Host Cell Engineering for the Production of Methacrylate Esters

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Abstract

Microbial bioprocess serves as an alternative route for the sustainable production of a variety of chemicals. Recent bioprocess development efforts has allowed its application for the commercial production of certain industrially relevant chemicals. However, most are still in the exploratory or pre-commercialization stage due to a variety of bottlenecks that needs to be addressed prior to commercialization. This includes the bioprocess route being developed by Lucite International for the production of butyl methacrylate, which could be part of an integrated process for the production of methacrylate esters. In this bioprocess, commercial viability is attainable with a butyl methacrylate titre of 10-20% v/v. One of the bottlenecks in this proposed bioprocess is the toxicity of the bioproduct towards the production strain, which could limit the attainable product titre. A previous study on its toxicity led to the isolation of *E. coli* strains that can grow vial cultures with BMA at 20% v/v. However, these strains were unable to demonstrate tolerance in a well-mixed environment. Thus, there is still a need to develop a robust host strain that can tolerate butyl methacrylate at the desired product titre.

E. coli BW25113 was explored as the potential host strain. Adaptive evolution *via* sequential batch and chemostat cultures were used to generate *E. coli* strains with tolerance for butyl methacrylate at 20% v/v. Genome shuffling was also used to further improve growth of *E. coli* with butyl methacrylate at 20% v/v. The possible mechanisms of tolerance for butyl methacrylate were determined with the use of genomic DNA and RNA sequencing of the evolved strains. The ability of the evolved strains to produce BMA was also tested by introduction of the heterologous pathway.

Adaptive evolution, through sequential batch and chemostat cultures, was successful in generating various *E. coli* strains with improved growth in the presence of BMA up to 20% v/v. Each of the evolved strains acquired various mutations that include an *acrR* mutation along with either a *marR*, *soxR*, and *rob*. The mutations acquired allowed increased expression in *acrAB*, which suggests that the AcrAB-TolC efflux pump might play an important part in the

tolerance for butyl methacrylate. Exposure of the evolved strain to butyl methacrylate stimulated the activation of genes that belong to the oxidative stress, heat shock, phage shock, and acid stress response systems and membrane modifying, energy generating, and essential building block synthesizing enzymes. It also resulted in the repression of the genes related to DNA replication and protein synthesis. The use of the evolved strains as host cell for production did not show an improvement in butyl methacrylate titre in comparison to the parental strain. However, butyl methacrylate production seems to be limited by factors other than toxicity. Thus, there is a need for further investigation and improvement of the production pathway.

Acknowledgements

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List of Abbreviations

BMA	N-butyl methacrylate
$CaCl_2$	Calcium chloride
$CoCl_2$	Cobalt (II) chloride
$CuSO_4$	Copper (II) sulfate
DI H_2O	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
$FeCl_3$	Iron (III) chloride
HCl	Hydrochloric acid
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
KOH	Potassium hydroxide
LB	Luria Bertani
NH_4Cl	Ammonium chloride
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
$MnSO_4$	Manganese (II) sulfate
Na_2HPO_4	Disodium hydrogen phosphate
$NaH_2PO_4 \cdot 2H_2O$	Sodium phosphate monobasic dehydrate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PEG	Poly (ethylene glycol)
Tris/HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TAE	Tris-acetate-EDTA
TE	Trace elements
$ZnSO_4$	Zinc sulfate

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Introduction

1.1 General introduction

Microbial fermentation provides a promising alternative route for the sustainable production of industrially relevant chemicals (Erickson et al., 2012; Singh, 2011). It exploits the ability of microorganisms to produce a diverse range of compounds that be can be further modified or directly used as a fuel, solvent, food ingredient, fragrance, bioactive compounds, building blocks for polymeric materials, and etc. from renewable feedstocks (Lee et al., 2019; Rabinovitch-Deere et al., 2013; Tsuge et al., 2016). Recently, a number of chemicals are commercially produced with the aid of microorganisms that includes 1,3 propanediol by DuPont Tate & Lyle, 1,4 BDO by Genomatica, 1-butanol by Geen Biologics, isobutanol by Gevo, succinic acid by Bioamber, Myriant, REverdia, and Succinity, atemisinin and ß-Farnesene by Amyris, polylactic acid (PLA) by NatureWorks LLC, polyhydroxyalkanoate (PHA) by Metabolix and SyntheZyme, polyethylene by Braskem, and polyols by BiOH (Benjamin et al., 2016; Burgard et al., 2016; Davies, 2013; Erickson et al., 2012; Komesu et al., 2017; Nghiem et al., 2017; Tao et al., 2014). Production of other chemicals *via* microbial bioprocesses are also being considered by various research and development efforts (Lamsen & Atsumi, 2012; Lu et al., 2019; Meadows et al., 2018), including methyl methacrylate (MMA) by Lucite International and Mitsubishi Chemicals (Eastham et al., 2017; O'malley et al., 2018; Sato et al., 2017).

Methacrylate esters are a versatile group of monomer used in plastics, coatings, adhesives, textile, paper, cosmetics, electronics, lubricants, fuel additives, and oil-field or construction chemicals (Darabi Mahboub et al., 2018; Fleischhaker et al., 2014; Nagai, 2001; Slone, 2010). Demands for methyl methacrylate (MMA), the methacrylate ester with largest use and production, is expected to surpass 4.8 million metric tonnes by 2020 (Darabi Mahboub et al., 2018). They can be produced from esterification of methacrylic acid (MAA) or trans-esterification of other methacrylate esters (Heeres et al., 2019; Nagai, 2001). Technologies that are under development or currently employed for

commercial production of MAA or MMA utilizes acetone-cyanohydrin (ACH) and light hydrocarbons as feedstocks and heterogeneous metals as catalysts (Darabi Mahboub et al., 2018; Nagai, 2001). The earliest commercial production process for MMA was the ACH process. In this process, acetone, hydrogen cyanide (HCN), methanol, and sulfuric acid (H_2SO_4) are used as starting materials to produce MMA with cyanohydrin and methacrylamide as intermediates, and ammonium bisulfate (NH_4HSO_4) as waste by-product (Fig. 1.1) (Ballarini et al., 2007; Darabi Mahboub et al., 2018; Nagai, 2001; Zheng et al., 2016). An improved version of the ACH process, developed by Mitsubishi Gas Chemical, eliminates the use of H_2SO_4 and waste by-product NH_4HSO_4 by reacting cyanohydrin with water in the presence MnO_2 to generate the intermediate 2-hydroxyisobutylamide. The second intermediate is further converted to methyl 2-hydroxyisobutylate and formamide *via* transesterification with methyl formate in the presence of CaO. MMA is finally obtained from the dehydration of 2-hydroxyisobutylate, while HCN can be obtained and recycled into the process *via* dehydration of formamide (Fig. 1.2) (Abe, 1999; Darabi Mahboub et al., 2018; Nagai, 2001). Another iteration of the ACH process, which also eliminates the use of H_2SO_4, is the AVENEER process developed by Evonik (Darabi Mahboub et al., 2018). In the AVENEER process, ammonia, methane, acetone, and methanol are used as feedstocks to produce MMA.

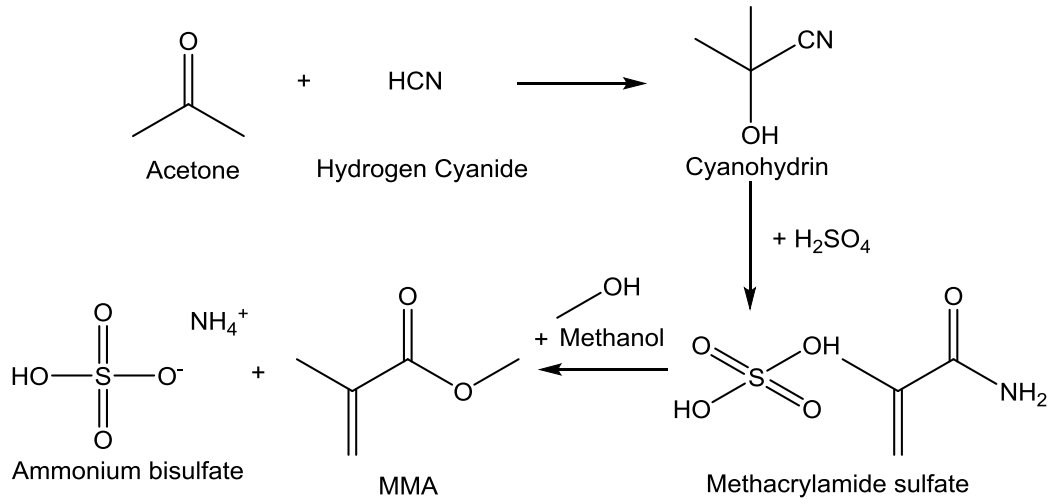

Figure 1.1 ACH Process for MMA production (Darabi Mahboub et al., 2018; Nagai, 2001).

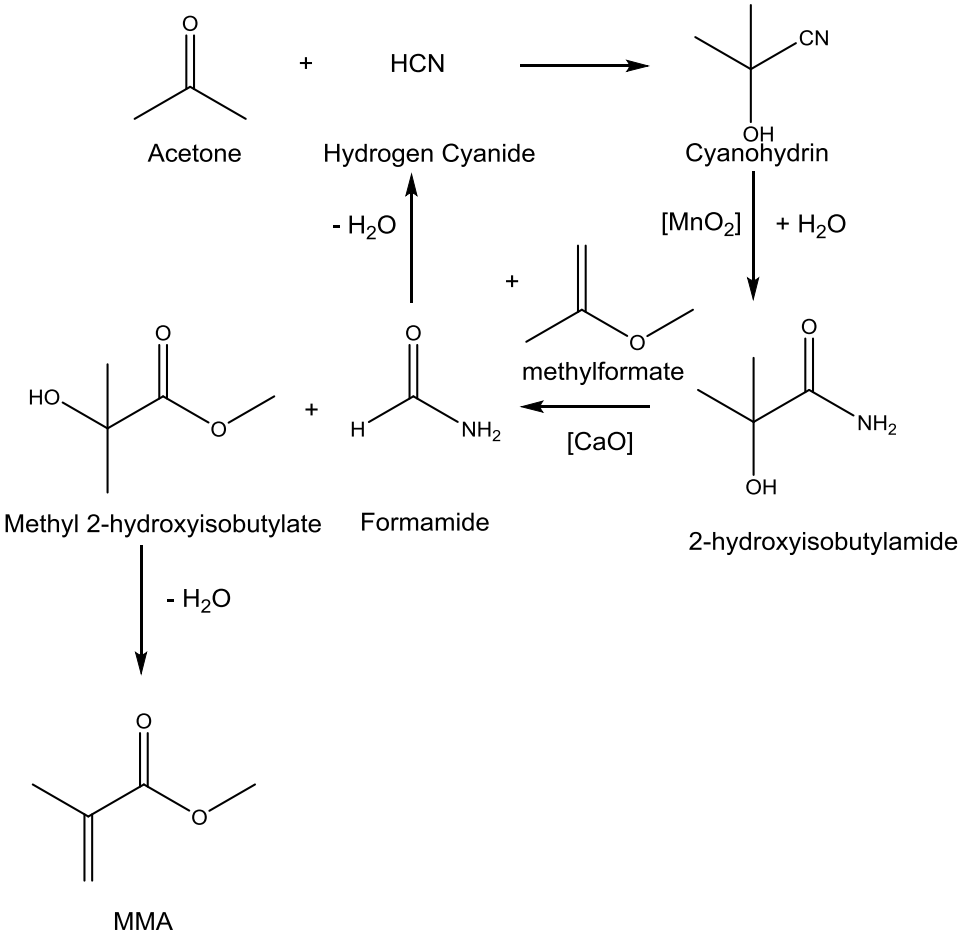

Figure 1.2 New ACH Process for MMA production (Darabi Mahboub et al., 2018; Nagai, 2001).

MMA is also produced commercially with the use of ethylene as the main feedstock *via* the 4-stage BASF process and 2-stage alpha process developed by Lucite International (Darabi Mahboub et al., 2018). In the BASF process (Fig. 1.3), ethylene is first reacted with carbon monoxide (CO) and hydrogen (H_2) to form propionaldehyde, which is then subjected to a condensation reaction with formaldehyde to form methacrolein (MAC). Oxidation of MAC leads to the formation of MAA, which is subsequently esterified with methanol to produce MMA (Darabi Mahboub et al., 2018; Duembengen et al., 1985; Merger & Foerster, 1983; Nagai, 2001). On the other hand, the alpha process (Fig. 1.4) generates methyl propionate from the reaction of ethylene with CO and methanol over a Pd catalyst in the first stage. MMA is produced upon reaction of methyl propionate with formaldehyde over the Cs-doped SBA-15 catalyst (Ai, 2005; Darabi Mahboub et al., 2018; Li et al., 2014a).

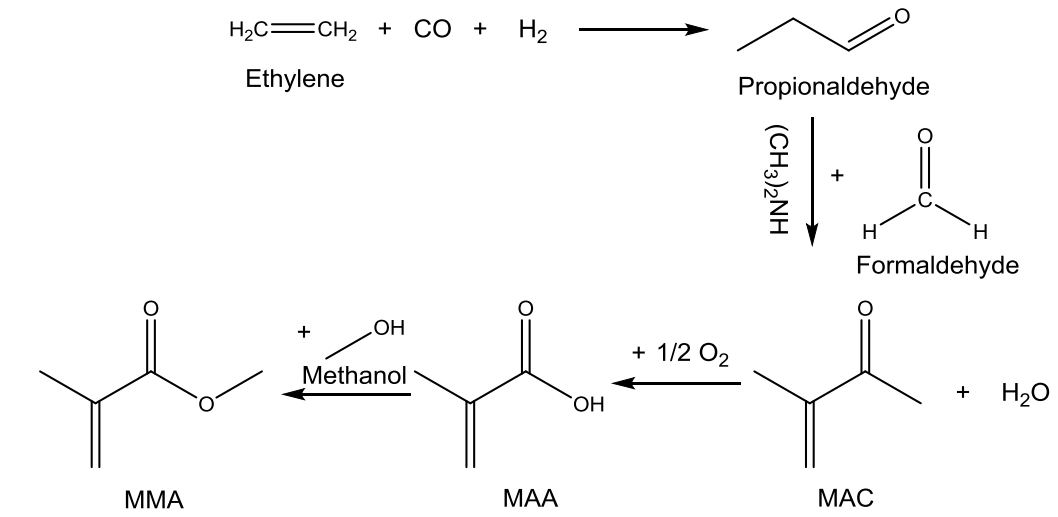

Figure 1.3 BASF Process for MMA production (Darabi Mahboub et al., 2018; Nagai, 2001).

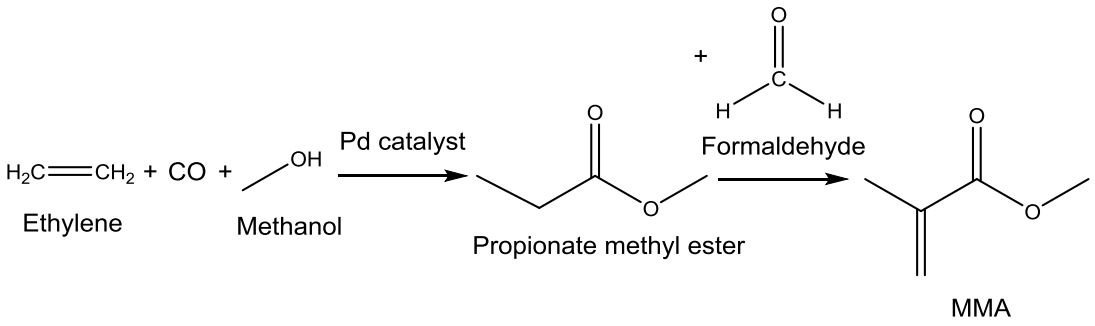

Figure 1.4 Alpha Process for MMA production (Darabi Mahboub et al., 2018; Nagai, 2001).

The other processes that were applied in commercial scale by Nihon Methacrylate Monomer and Mitsubishi Rayon involved the usage of isobutylene as feedstock to produce MMA via oxidation of isobutylene to MAC, then MAA, followed by esterification with methanol (Fig. 1.5) (Darabi Mahboub et al., 2018; Guan et al., 2008). MMA can also be produced from isobutylene *via* its ammoxidation to methacrylonitrile (MAN) (Fig. 1.6), which was commercialized by Asahi Chemical Company (Nagai, 2001; Onsan & Trimm, 1975).

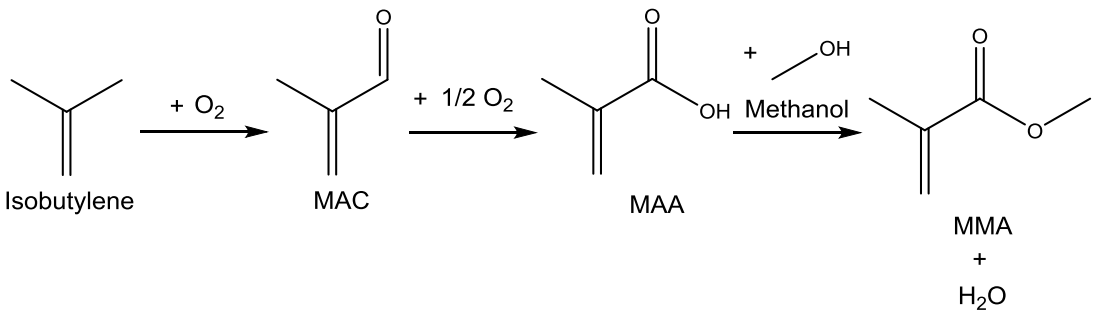

Figure 1.5 Isobutylene Oxidation Process for MMA production (Darabi Mahboub et al., 2018; Guan et al., 2008)

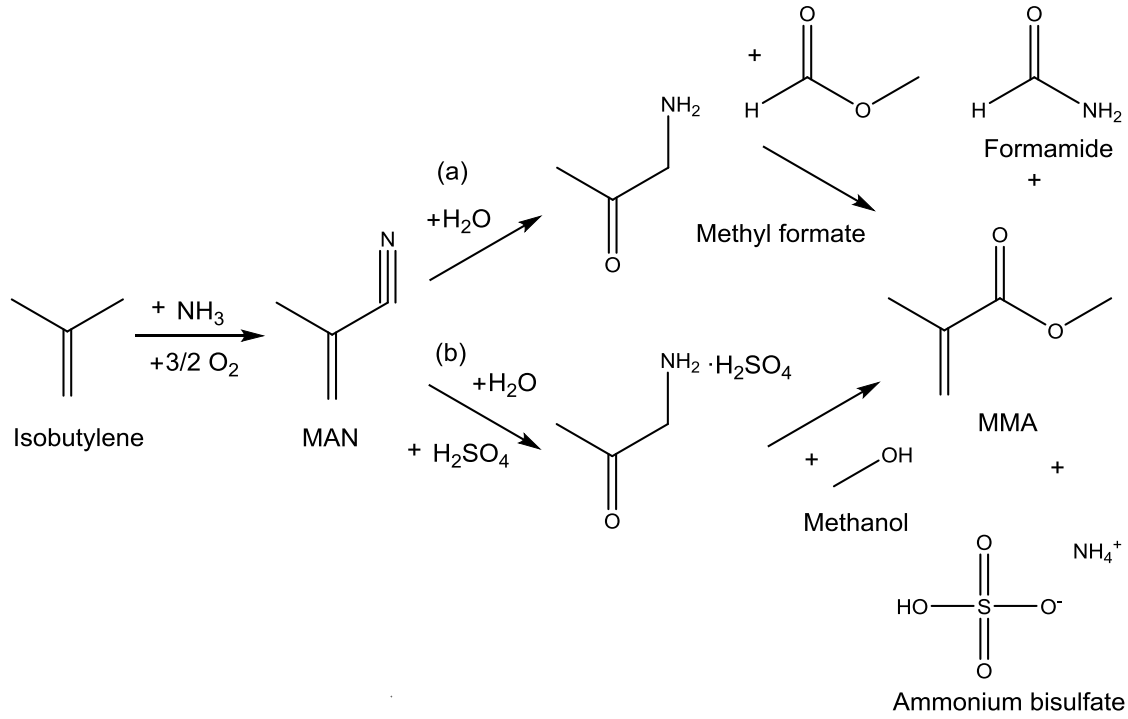

Figure 1.6 Isobutylene Ammoxidation Process for MMA production (a) Mitsubishi Gas Chemicals Process (b) Asahi Chemical Co Process (Nagai, 2001).

Other notable technologies that are still in the development pipeline go through similar routes making use of various C2 (ethylene), C3 (propane, propylene, and propyne), and C4 (isobutane, isobutene) compounds (Darabi Mahboub et al., 2018; Guan et al., 2009). A process developed from the research triangle institute (RTI) (Eastman-Bechtel route; Fig. 1.7) starts with ethylene, CO, and H_2O to generate the intermediate propionic acid through the hydroxycarbonylation reaction using metal carbonyl catalyst. A condensation reaction of propionic acid and formaldehyde leads to the formation of MAA, which can be converted to MMA *via* esterification with methanol (Darabi

Mahboub et al., 2018; Nagai, 2001; Xu, 2002). In a similar process, called LiMA (Leading in Methacrylates) that was developed and commercialized by Evonik, ethylene, CO, and H_2 are converted to propionaldehyde. Further conversion of propionaldehyde to MAC is achieved through an Aldol condensation reaction with formaldehyde. Finally, an oxyesterification reaction converts MAC and methanol to produce MMA (Darabi Mahboub et al., 2018).

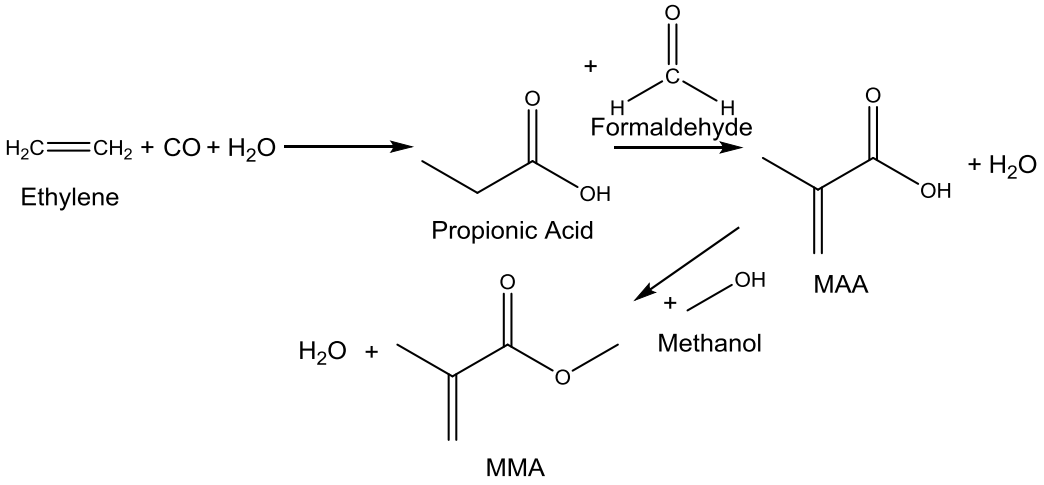

Figure 1.7 Reaction steps for the Eastman-Bechtel route (Darabi Mahboub et al., 2018).

The use of propylene as starting material goes through hydroxy carbonylation reaction with H_2O and CO to form the intermediate isobutyric acid, which is then further converted to MAA via oxidative dehydrogenation and MMA after esterification with methanol (Fig. 1.8) (Darabi Mahboub et al., 2018).

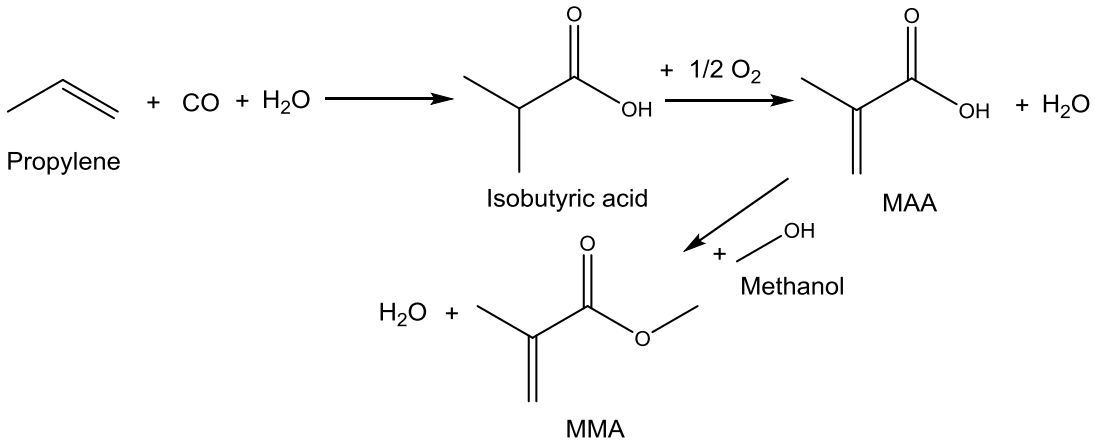

Figure 1.8 Reaction steps for the propylene route (Darabi Mahboub et al., 2018; Nagai, 2001).

In an alternative C3 route developed by shell, propyne is used as the feedstock and directly converted to MMA *via* a methoxy carbonylation reaction with CO and methanol over a Pd catalyst (Fig. 1.9) (Darabi Mahboub et al., 2018; Drent, 1988; Mizuno et al., 2008).

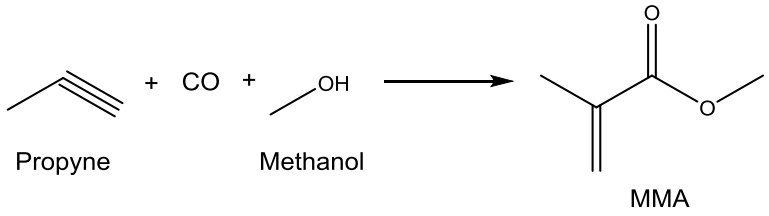

Figure 1.9 Reaction steps for the propyne route towards MMA (Mizuno et al., 2008).

The current commercial processes and most processes in the development pipeline for production of MAA and MMA relies heavily on petroleum based or sourced feedstocks and expensive metal based catalysts with short lifetimes (Darabi Mahboub et al., 2018; Nagai, 2001). As fossil reserves are limited along with tremendous global concerns for climate change (Gopalakrishnan et al., 2019; Jakob & Hilaire, 2015; Rogelj et al., 2018), there is an urgent need to switch from petroleum based feedstocks to the more sustainable biobased feedstocks (Bennich & Belyazid, 2017; Bennich et al., 2018; Werpy & Petersen, 2004). Lucite International's bioprocess route to MMA production utilizes renewable biobased feedstocks and microbial biocatalysts that can be regenerated through the renewable biobased feedstocks as well (Uppada et al., 2014). The bioprocess route would be part of the integrated process, wherein n-butyl methacrylate (BMA) will be produced as an intermediate for MMA production (Fig. 1.10).

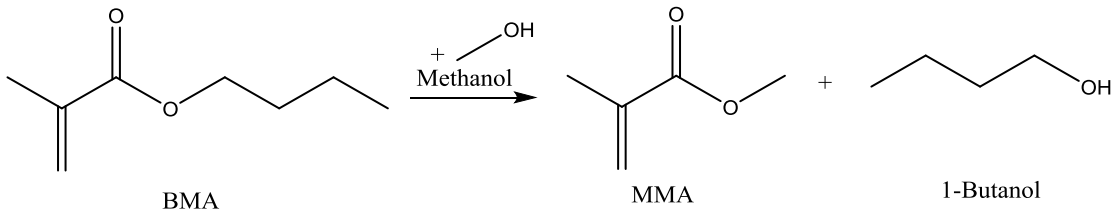

Figure 1.10 Transesterification of BMA to MMA.

BMA can be produced from pyruvate (Eastham et al., 2017; Eiji et al., 2013), which can be produced from various carbon sources (Li et al., 2001), and 1-butanol *via* bioconversion (Fig. 1.11).

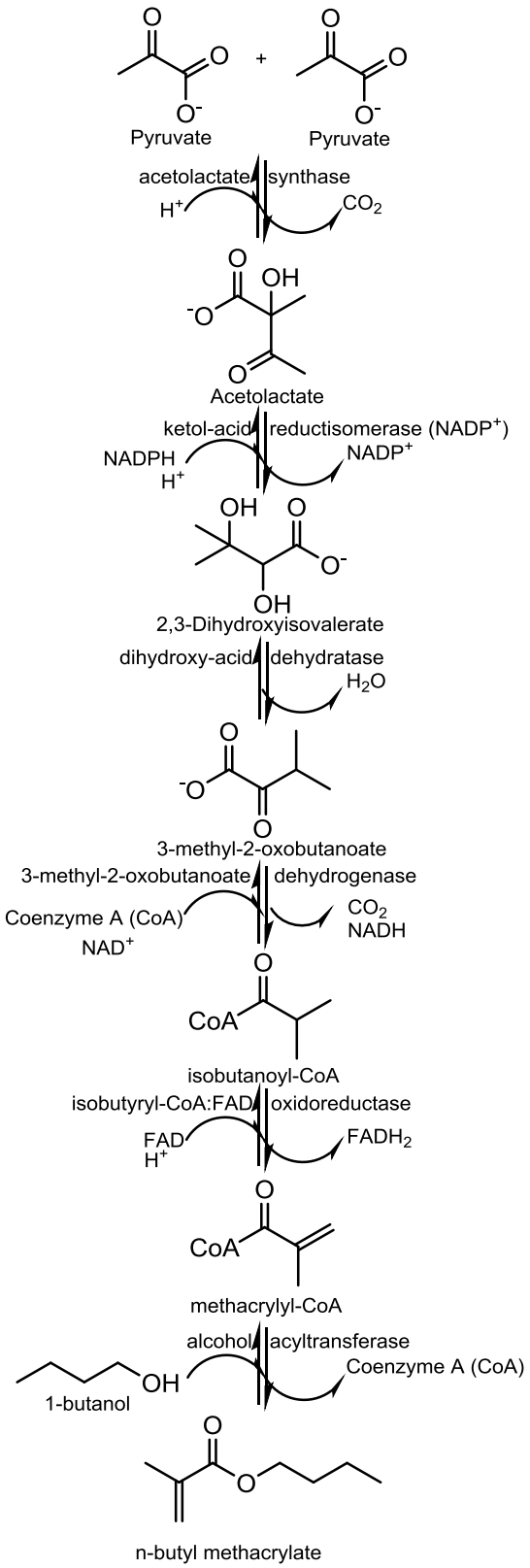

Figure 1.11 Bioconversion of pyruvate to BMA.

Two pyruvate molecules are converted to the intermediates acetolactate, 2,3 dihydroxyisovalerate, 3-methyl-2-oxobutanoate, isobutanoyl-COA, and methacrylyl-COA with the aid of the enzymes acetolactate synthase, ketol-acid

reductoisomerase, dihydroxy-acid dehydratase, 3-methyl-2-oxobutanoate dehydrogenase, and isobutyryl-CoA:FAD oxidoreductase, respectively. BMA can then be formed from methacrylyl-CoA and 1-butanol through an alcohol acyltransferase.

In Lucite International's process design, MMA production is economically favourable if BMA can be produced at 10-20% v/v (Fig. 1.12). The high BMA titres will cause phase separation of BMA from the aqueous phase and enable its recovery *via* decantation. One key constraint in achieving the target BMA concentration (10-20% v/v) is the ability of the production host cell to tolerate and be metabolically active in the presence 10-20% v/v BMA (Mukhopadhyay, 2015). This is because BMA, like other organic chemicals produced by microorganisms, can exhibit toxic effects towards the production host cell (Mukhopadhyay, 2015). Both vital cellular functions and product formation are inhibited with increasing bioproduct titres, which eventually leads to cell death and cessation of product formation and limits attainable BMA titres (Foo et al., 2014; Huffer et al., 2011; Lian et al., 2016; McKenna & Nielsen, 2011; Menchavez & Ha, 2019; Mukhopadhyay, 2015). *Escherichia coli*, which is one of the production host strains being developed by Lucite International is not known to be tolerant towards BMA at 10-20% v/v (Disley, 2018). *E. coli* strains with improved tolerance for BMA were isolated from a preceding study (Disley, 2018). However, the isolated strains were unable to display tolerance for BMA (10-20% v/v) in a well mixed system (Personal communication from Ingenza Ltd. And Lucite International). Thus, it will be necessary to engineer a more robust potential host strain to be capable of withstanding and performing at target BMA titres.

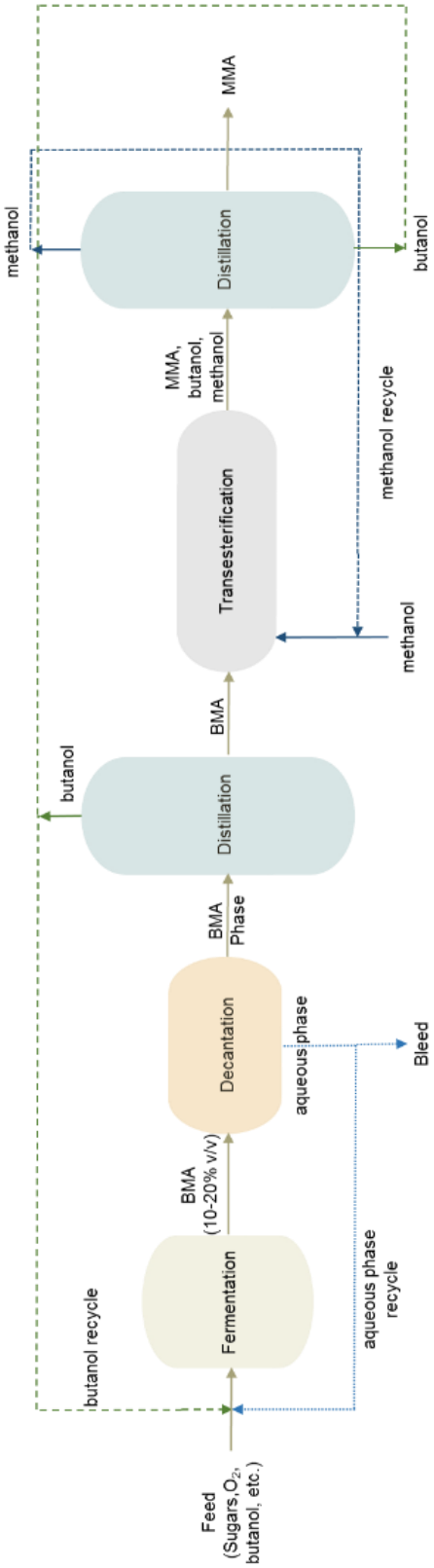

Figure 1.12 Simplified schematic diagram of the integrated process for MMA production as developed by Lucite International.
Legend: In and out of various unit operations (solid lines and arrows), aqueous phase bleed and recycle (blue dotted line), methanol streams (dark blue dashed-line), and butanol streams (green dashed-line).

2

Literature Review

2.1 Introduction

Microbial fermentation may offer a green and sustainable alternative to established chemicals manufacturing processes, to enable the production of commercially important chemicals that can be utilized as platform chemicals, fuels, therapeutics, fine chemicals, food additives, fragrances, bioactive compounds, and building blocks for various materials (Erickson et al., 2012; Hatti-Kaul et al., 2007; Lee et al., 2019; Soetaert & Vandamme, 2006; Tang & Zhao, 2009; Tsuge et al., 2016). The use of microbial processing (Pickens et al., 2011; Rabinovitch-Deere et al., 2013; Sun et al., 2015) has been greatly aided by advances in metabolic engineering (Kogure & Inui, 2018; Lian et al., 2018; Peña et al., 2018; Pontrelli et al., 2018), enzyme engineering (Li & Cirino, 2014; Newton et al., 2018), synthethic biology (Lee et al., 2018; Tan & Prather, 2017), and systems biology (Chae et al., 2017; Chubukov et al., 2016; Hansen et al., 2017). However, despite these technical breakthroughs in production *via* microbial bioprocesses, only a limited number of chemicals have reached commercial manufacturing (Benjamin et al., 2016; Burgard et al., 2016; Chubukov et al., 2016; Davies, 2013; Gallage & Møller, 2015; Komesu et al., 2017; Nghiem et al., 2017; Tao et al., 2014; Zhang et al., 2017). The vast majority of potential bioproducts are still languishing in the development pipeline, since typical product titres are frequently much too low (mg/L to a few g/L) (Lamsen & Atsumi, 2012; Lu et al., 2019; Meadows et al., 2018). Such dilute product streams result in excessive costs for dewatering and product recovery, due to high energy usage, capital intensive recovery processes (Efe et al., 2013; Mariano & Filho, 2012; Salemme et al., 2017) and significant costs for treating process waste and water (Mariano & Filho, 2012). Consequently, product titre, along with product yield and productivity, is among the key parameters that affect production cost and process economics. Therefore, product titre is a key determinant of commercial viability (Chubukov et al., 2016) and environmental impact (Mariano & Filho, 2012) of chemical production *via* microbial processes.

Until now, the main emphasis for R&D has been to relieve limitations in the metabolic pathway, enzymes, regulatory networks, spatial organization of the metabolites and enzymes, and cellular machineries to enable the bioconversion of sustainable feedstocks to chemical products (Chubukov et al., 2016; Lechner et al.; Lee et al., 2018; Meadows et al., 2018). Although there have been numerous successes, many chemical products are inherently toxic to microorganisms, and this toxicity towards the production host cell has long been recognised as a critical constraint in the product titres that can be obtained either from natural or metabolically engineered production strains (Brennan et al., 2012; Jarboe et al., 2013; Lee et al., 2008; McKenna et al., 2015; Meadows et al., 2018; Ng & Kuek, 2013). Thus, cellular activities and product formation are progressively inhibited with increasing bioproduct concentration until the product reaches a lethal concentration and/or maximum attainable titre (Chong et al., 2014; Dunlop et al., 2011; Foo et al., 2014; Foo & Leong, 2013; Huffer et al., 2011; Lian et al., 2016; Ma & Yu, 2012; McKenna & Nielsen, 2011; Menchavez & Ha, 2019; Mingardon et al., 2015; Tan et al., 2016a; Tan et al., 2017b; Tan et al., 2016b).

Although some microorganisms have mechanisms in place to combat the deleterious effects of toxic bioproducts, the concentrations at which the cells are able to function before bioproduct formation stalls is still well below target concentrations for commercial production (Chong et al., 2014; Dunlop et al., 2011; Foo et al., 2014; Foo & Leong, 2013; Huffer et al., 2011; Lian et al., 2016; Ma & Yu, 2012; McKenna & Nielsen, 2011; Menchavez & Ha, 2019; Mingardon et al., 2015; Tan et al., 2016a; Tan et al., 2017b; Tan et al., 2016b). This limitation can be resolved by engineering the host strain until it is able to tolerate and produce at the desired product titre (Dunlop et al., 2011), attaching/encapsulating the host strain with a protective material (Hinks et al., 2015; Menchavez & Ha, 2019), applying in-situ product recovery options (McKenna et al., 2015; Outram et al., 2017), or combinations thereof. Engineering of the host strain for product tolerance makes use of classical and modern biotechnological techniques to surpass the maximum product titre it can naturally endure before vital cellular and metabolic processes breakdown (Mukhopadhyay, 2015). However, more often than not, the improvements in

product tolerance do not reach product titres that are required for commercial production (Mariano & Filho, 2012; Mukhopadhyay, 2015).

Protective materials can also be used to enhance product tolerance of the host cell *via* encapsulation in a biocompatible carrier (Menchavez & Ha, 2019) or addition of membrane insertion molecules for cell membrane stabilization (Hinks et al., 2015). In-situ product recovery incorporates conventional or novel separation processes into the fermentation process for partial removal of the bioproduct and maintain its concentration below inhibitory levels to allow continued production (McKenna et al., 2015; Outram et al., 2017). Both of the latter options would entail additional capital costs with the maximum attainable titers still dependent on the product tolerance of the host strain. Thus, engineering the product tolerance of the host strain will still be an important component for these approaches. In some cases, combinations of these approaches might be necessary to for use in an integrated bioprocess to circumvent the production limitations resulting from product toxicity (Menchavez & Ha, 2019; Outram et al., 2017; Qureshi & Blaschek, 2000). In this review, recent strategies and efforts in the engineering of a more tolerant bacterial host strain for a range of bioproducts as an approach to break bioproduct toxicity titer limitations are discussed.

2.2 Mechanisms of chemical toxicity

One key step in solving any problem is understanding the problem itself inorder to formulate a sound approach. Thus, understanding how chemicals affect the host cell and its components to manisfest its toxic nature is an important step for the engineering of the host cell with improved product tolerance. Cell inhibition and death may result from the bioproduct's direct interaction with vital cellular components (proteins, lipids, and nucleic acids) (Asakura et al., 1978; Banerjee et al., 1981; Chen & Rand, 1998; Chu et al., 2013; Jarboe et al., 2013; Ly & Longo, 2004; Modig et al., 2002; Murínová & Dercová, 2014; Osman et al., 1988; Sardessai & Bhosle, 2002; Sikkema et al., 1995; Spears & Fascione, 2016; Tittensor & Walker, 1968; Yuan et al., 2010), alteration of the cellular environment (Ingram, 1981; Jarboe et al., 2013), inherent stress response mechanisms (Ezraty et al., 2017; Jarboe et al., 2013), or a combination of the

direct and indirect action of the bioproduct (Ingram, 1981; Jarboe et al., 2013; Martins et al., 2019).

In particular, many chemical products partition from the aqueous phase into the cell membrane and can interact with its lipid and protein components, which are critical components for the maintenance of the membrane's integrity, fluidity, and overall physical properties (Dombek & Ingram, 1984; Los & Murata, 2004; Sikkema et al., 1995; Silhavy et al., 2010; Weber & de Bont, 1996). As the bioproduct concentration increases, the amount of product within the lipid bilayer increases and starts to alter the membrane integrity and fluidity (Los & Murata, 2004; Sikkema et al., 1995; Silhavy et al., 2010; Weber & de Bont, 1996). The changes in membrane properties can disrupt various membrane-associated systems crucial to cellular functions, such as transport, energy conservation mechanisms, signaling, and cell division processes (Ingram, 1981; Lennen et al., 2011; Russell, 1992; Segura et al., 2012; Sikkema et al., 1994; Watson, 2015). Eventually, the bioproduct concentration reaches the threshold limit that results in leakage of essential macromolecules and cofactors, as well as dissipation of the proton motive force that leads to cell death (Cartwright et al., 1986; Hyldgaard et al., 2012; Ingram, 1981; Jarboe et al., 2013; Lennen et al., 2011; Royce et al., 2013b).

Some bioproducts interact directly with cellular components (lipids, protein, DNA, RNA) and inflict damage through denaturation (Asakura et al., 1978; Murínová & Dercová, 2014; Segura et al., 2012) and chemical reaction with the cellular macromolecules (Chu et al., 2013; Osman et al., 1988; Spears & Fascione, 2016; Tittensor & Walker, 1968; Yuan et al., 2010). Their accumulation may also lead to inhibition of enzymes essential for overall cell metabolism (Banerjee et al., 1981; Modig et al., 2002) or enzymes involved in the production pathway (Primak et al., 2011; Tian et al., 2019). Damage of macromolecules can also result from reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $\cdot OH$, $ROO\cdot$) generated from cell processes, which are exacerbated in the presence of potential bioproducts at elevated concentrations (Pérez-Gallardo et al., 2013). In the case of organic acid production, pH homeostasis can be significantly altered and cause acidification in the cytoplasm (Russell, 1992), which may result in the collapse of the transmembrane pH gradient , decrease

in proton motive force, inhibition of essential cell processes, increase in turgor pressure and osmolarity, and damage to DNA and RNA (Baronofsky et al., 1984; Beales, 2004; Herrero et al., 1985; Huesemann & Papoutsakis, 1986; McLaggan et al., 1994; Raja et al., 1991; Sinha, 1986).

2.3 Chemical tolerance mechanisms

In response to the deleterious effects caused by contact with a variety of toxic compounds, microorganisms may alter their cell envelope structure to maintain membrane integrity and fluidity, expel or prevent entry of toxic compounds, and activate various stress response and damage repair mechanisms (Brynildsen & Liao, 2009; Ezraty et al., 2017; Jarboe et al., 2013; Joly et al., 2010; Karschau et al., 2011; Martins et al., 2019; Petersohn et al., 2001; Sardessai & Bhosle, 2002; Sawant et al., 2016; Segura et al., 2012; Sikkema et al., 1995; Weber & de Bont, 1996; Yung et al., 2016). Microorganisms can maintain membrane integrity and fluidity by adjusting the saturated-to-unsaturated fatty acid ratio, the abundance of branched (iso and anteiso), hydroxy, and cyclopropane fatty acids, the degree of cis-trans isomerization of unsaturated fatty acids, abundance of specific phospholipid head groups, and the type and amount of membrane proteins (Carey & Ingram, 1983; Clark & Beard, 1979; Dombek & Ingram, 1984; Heipieper et al., 2003; Mrozik et al., 2005; Mrozik et al., 2004; Murínová & Dercová, 2014; Segura et al., 2012; Sikkema et al., 1995; Silveira et al., 2004; Sol Cuenca et al., 2015; Weber & de Bont, 1996; Yung et al., 2016; Zu et al., 2014). The changes in membrane composition (Murínová & Dercová, 2014; Sikkema et al., 1995), along with reduced expression of certain porins (Brynildsen & Liao, 2009; Roma-Rodrigues et al., 2010; Royce et al., 2014), also contribute to prevent or reduce the rate of permeation and diffusion of toxic compounds.

On the other hand, expulsion of toxic compounds from the cell is achieved *via* membrane vesicles (Baumgarten et al., 2012; Kobayashi et al., 2000), porins (Zhou et al., 2015), and efflux pumps (Li et al., 1998; Martins et al., 2019; Rojas et al., 2001). As toxic compounds interact with cellular components in a variety of ways, microorganisms can also respond to counteract the combined deleterious effects by recruiting complementary elements with diverse functions

from various stress response and damage repair systems (Martins et al., 2019; Molina-Santiago et al., 2017; Nicolaou et al., 2010; Rau et al., 2016; Shimizu, 2013b; Yung et al., 2016). The notable stress response and damage repair systems that respond to toxic compounds include the phage shock response (Chiou et al., 2004; Joly et al., 2010), acid stress response (Jarboe et al., 2013; Shimizu, 2013b), oxidative stress response (Cao et al., 2017; Ezraty et al., 2017; Rau et al., 2016), envelope stress response (Cao et al., 2017; Grabowicz & Silhavy, 2017), osmotic stress response (Cao et al., 2017; Krämer, 2010), heat and cold shock response (Barria et al., 2013; Brynildsen & Liao, 2009; Cao et al., 2017; Guisbert et al., 2004; Yung et al., 2016), and multidrug resistance (Duval & Lister, 2013), which integrate various functions that allow the cell to maintain membrane stability, expel or prevent entry of toxic compounds, adjust biosynthesis and energy metabolism, and repair or degrade damaged membrane components, protein, DNA, and RNA (Martins et al., 2019).

Even though microorganisms have mechanisms in place to combat deleterious effects of certain toxic chemical products, their action is limited to product concentrations well below the requirements for commercial bioprocess (Lamsen & Atsumi, 2012; Lu et al., 2019; Meadows et al., 2018; Mukhopadhyay, 2015). Thus, it will still be necessary to further improve their product tolerance. Nonetheless, these mechanisms serve as a crucial starting point to further improve the chemical product tolerance of the chosen host strain.

2.4 Host strain engineering for bioproduct tolerance

Host strain engineering for improved bioproduct tolerance has been acknowledged as a key component for successful bioprocesses (Lee & Kim, 2015) and various approaches have been reported for the generation of a host strain with improved bioproduct tolerance (Dunlop et al., 2011). Engineering of the host strain can be achieved either by a random or rational/targeted approach. The random approach makes use of the classical random mutagenesis tools, while the rational or targeted approach builds on knowledge of known tolerance mechanisms from native or engineered strains with the aid of ‘omics analysis and systems biology tools.

2.4.1 Random approach

Acquiring spontaneous mutations in a host cell is a rare phenomenon with an approximate rate of 10^{-7} mutations/gene/generation (Csörgő et al., 2012), but this can still be exploited to evolve new strains with desirable properties by imposing appropriate selection pressures. Adaptive evolution for product tolerance (Dragosits & Mattanovich, 2013) involves the continued propagation cells in a batch or continuous culture for many generations (100-1000) of the selected host microorganism in a culture medium with the toxic chemical product at the concentration desired for tolerance. This process exploits the random mutations that occur naturally, and any fitter mutants that arise will proliferate due their faster growth rates under the selection conditions. Therefore, the frequency of the mutants within the selected population is in proportion to their fitness (Dragosits & Mattanovich, 2013; Winkler & Kao, 2014).

Adaptive evolution through sequential batch cultures has been successfully used to increase *E. coli* tolerance towards ethanol (Horinouchi et al., 2010; Wang et al., 2011), isopropanol (Horinouchi et al., 2017), isobutanol (Minty et al., 2011), and 1-butanol (Menchavez et al., 2018) with improvements ranging from 7.8-100% (Table 2.1). Evolution of *E. coli* for enhanced 1-butanol tolerance was also successfully achieved in a continuous culture resulting in a 62.5% improvement (Reyes et al., 2012). Aside from *E. coli*, the 1-butanol tolerance of *Synechocystis. sp.* PCC 6803 (Wang et al., 2014) and *Clostridium acetobutylicum* D64 (Liu et al., 2013) were also improved *via* adaptive evolution in serial batch transfers. *Synechocystis. sp.* and *C. acetobutylicum* gained 150% and 57.7% improvements, respectively. The evolution process for *Synechocystis. sp.* was accomplished by growing the cultures in a broth with increasing levels of 1-butanol, while *C. acetobutylicum* was evolved and selected in both broth and plate containing 1-butanol. In the case of *C. acetobutylicum*, after each increment the cells were plated on YPS agar containing the same 1-butanol concentration. The largest colonies were then transferred to broth containing the same or a higher 1-butanol concentration, and the selection cycle repeated. The final *C. acetobutylicum* strain was tolerant to 31.2 g/L 1-butanol, which is about 5 g/L less than the minimum target product

concentration for 1-butanol production *via* fermentation (Mariano & Filho, 2012; Vane, 2008).

Table 2.1 Summary of studies with adaptive evolution as method to generate bioproduct tolerant strains.

Method	Conditions	Chemical (Max C observed from the parental strain for T; P in g/L)	Microorganism	Improvement (%)					Ref
				C	CD	CV	GR	P	
Adaptive evolution (batch/ serial transfers)	LB medium + xylose, anaerobic, ~350 generations	Ethanol (20 ; 15.9)	*E. coli* KC01	100	NT	NT	NT	48.8	(Wang et al., 2011)
	M9 medium + glucose, microaerobic ; 1000 generations	Ethanol (44.7; NT)	*E. coli* W3110	7.8	NT	NT	100	NT	(Horinouchi et al., 2010)
	M9 medium +glucose, microaerobic ; 210 generations	Iso-propanol (24; NT)	*E. coli* MDS42	12.3	NT	NT	NT	NT	(Horinouchi et al., 2017)
	M9 medium + xylose / glucose, microaerobic ,425–500 generations	Isobutanol (7.5; NT) / 10; NT)	*E. coli* EcNR1	130 / 100	NT	NT	200	NT	(Mintyet al., 2011)
	LB medium, aerobic, ~ 300 generations	Isobutanol (6; 19)	*E. coli* JCL260	25	500	NT	NT	NI	(Atsumi et al., 2010)
	M9 medium	1-Butanol (6; NT)	*E. coli* BW25113	75	NT	NT	NT	NT	(Menchavez et al., 2018)
	BG11 medium, ~ 700 generations	1-Butanol (1.6; NT)	*Synechocystis. sp*. PCC 6803	150	NT	NT	NT	NT	(Wang et al., 2014)
Artificial simulation of bio-evolution (batch)	YPS medium, anaeorobic, ~ 1 year total evolution	1-Butanol (19.8; 12.2)	*C. acetobutylicum* D64	57.7	NT	NT	NT	25.4	(Liu et al., 2013)
Adaptive evolution (conti-nuous)	M9 medium, aerobic, Dilution Rate 0.23 h^{-1}, 144 generations	1-Butanol (6.4; NT)	*E. coli* BW25113	62.5	NT	NT	NT	NT	(Reyes et al., 2012)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; NI, no improvement.

An evolved *E. coli* strain with improved tolerance for ethanol also showed 48.8% improvement in ethanol production (Wang et al., 2011). Likewise, the evolved *C. acetobutylicum* was able to produce 25.4% more 1-butanol than the

parental strain (Liu et al., 2013). Although the number of generations required to evolve the desired tolerance usually involves lengthy experiments (Atsumi et al., 2010; Horinouchi et al., 2010; Liu et al., 2013; Minty et al., 2011; Reyes et al., 2012; Wang et al., 2011; Wang et al., 2014), adaptive evolution presents a very effective method to generate host strains with improved product tolerance and production titres strains.

It is also possible to increase the mutation rate by exposure of the host strain to mutagens. A variety of mutagenic chemicals are known, including base analogues, 2-aminopurine deaminating agents, hydroxylamine, nitrous acid, alkylating agents, ethyl methanesulfonate, mustards, and intercalating agents (Parekh et al., 2000). These mutagens can be used to achieve random mutations, including deletion, addition, substitution (transversions), or breakage of DNA strands. Mutants of *Clostridium beijerinckii* NCIMB 8052 with enhanced 1-butanol tolerance and production were generated by chemical mutagenesis using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) (Table 2.2) (Annous & Blaschek, 1991; Qureshi & Blaschek, 2000). One strain gained improvements in 1-butanol tolerance and production by 45.8% and 82.4%, respectively. NTG mutagenesis was also used to generate *C. beijerinckii* strains with enhanced tolerance for isopropanol (Gérando et al., 2016). One strain acquired 11.1% improvement in tolerance for isopropanol, but was unable to produce more isopropanol than the parental strain.

Exposure of microorganisms to UV irradiation (<280-400 nm) serves as an alternative option to accelerate mutant generation, since UV irradiation damages the DNA by causing the formation of cyclobutane pyrimidine dimers, pyrimidine (6-4) pyrimidone photoproducts at dipyrimidine sites, and oxidation through generation of reactive oxygen species (Ikehata & Ono, 2011; Rastogi et al., 2010). The DNA damage caused by UV irradiation results in mutations (tranversions, deletions, frameshifts, and transitions) due to errors in DNA repair and replication (Ikehata & Ono, 2011; Parekh et al., 2000; Rastogi et al., 2010). UV mutagenesis of *C. thermocellum* generated strains with 160% higher tolerance for in ethanol (Table 2.2). This was achieved by subjecting the microorganism to several rounds of UV irradiation and selection with ethanol in liquid cultures until cells were tolerant to 40.0 g/L of ethanol. An isolated mutant

strain produced 250% more than the parental strain, using cellulose as substrate (Tailliez et al., 1989).

Transposon mutagenesis has also been instrumental in improving *C. beijerinkii* NCIMB 8052 tolerance for 1-butanol (Table 2.2). Transposons, or transposable elements, are discrete DNA segments with the ability to relocate between genomic sites, causing a loss-of-function frame shift mutation (Hayes, 2003; Lennen & Herrgård, 2014). Transposon mutagenesis of *C. beijerinckii* NCIMB 8052 generated mutant strains with 75-83% improvements in 1-butanol concentration tolerated (Liyanage et al., 2000). The transposon was found in close proximity to *gldA*, which affected the level of expression of the glycerol dehydrogenase (*gldA*) and consequently reduced its total activity. However, it is uncertain how this contributed to the improved 1-butanol tolerance. Although this method is effective, its use may be limited, since the only expected outcome is reduced or loss of function of the affected gene.

Mutator strains can also be employed to generate mutants, since these strains carry defects in the DNA repair or replication system that increases the rate of spontaneous random mutations (Luan et al., 2013; Selifonova et al., 2001; Shiwa et al., 2012; Zhu et al., 2015). When coupled with a selective pressure for product tolerance, tolerant strains can be obtained and then isolated (Table 2.2). 1-Butanol tolerant *E. coli* strains were generated through introduction of a two plasmid module system based on stress induced mutagenesis (SIM) into a *mutL* deficient *E. coli* strain (Zhu et al., 2015). Genes encoding stress response regulators (RecA, RpoS and RpoE), error prone polymerases (Pol IV and Pol V), and transcription antitermination (NusA) were tested for SIM, and were expressed from one of the plasmids, and controlled by IPTG induction, whilst the second plasmid contained a gene encoding MutL, required for DNA mismatch repair, under the control of the Tet promoter. The plasmid used in this system was designed such that acceleration of SIM-based mutagenesis could be induced by addition of IPTG, while deceleration could be achieved by addition of anhydrotetracycline (aTc), which binds to TetR without inhibiting translation, and allows transcription of *mutL*. Thus, SIM results from the up regulation of the SIM-inducing genes, and down regulation of the mismatch repair system, resulting in increased mutation rates (Foster, 2007; Galhardo et

al., 2007; Rosche & Foster, 1999; Zhu et al., 2015). The final SIM module comprised a first plasmid containing SIM accelerator genes *dinB* (PolIV), *recA* (RecA), and *rpoS* (RpoS), while a second plasmid harboured the decelerator gene *mutL* (MutlL). Each cycle of the SIM included selection, mutagenesis, isolation, and screening steps. The best performing strain for each cycle was used as the starting strain for the next cycle of evolution (Zhu et al., 2014; Zhu et al., 2015). The mutant strain obtained after 10 rounds of mutagenesis was tolerant to 1-butanol concentration of 12.0 g/L, an increase of 71.4% compared with the parental strain (Zhu et al., 2015).

1-Butanol tolerant *E. coli* strains were also generated using a method called "Genome Replication Engineering Assisted Continuous Evolution (GREACE) (Table 2.2) (Luan et al., 2013). GREACE uses mutants of *dnaQ* to generate errors in DNA replication, thus introducing mutations. The gene *dnaQ* encodes the ε subunit of DNA polymerase III, which is the proof-reading element of DNA polymerase. The *dnaQ* mutants were transformed into the parental strain, then selected for 1-butanol tolerance through adaptive evolution by serial transfers in LB broth containing 1-butanol. An isolated strain from GREACE showed 20% higher tolerance for 1-butanol. However, both SIM and GREACE generated 1-butanol tolerant strains were not tested to check whether or not they can produce more 1-butanol than the parental strain.

Both SIM and GREACE boost the mutation rate and lessens the time necessary for evolution and generation of product tolerant strains. However, high mutation rates could also be deleterious for the host strain as they can also accumulate non-beneficial mutations (Sprouffske et al., 2018). Accumulation of too many mutations would also make it difficult to get a better understanding of the tolerance mechanism for the chemical product of choice (Atsumi et al., 2010). Thus, a delicate balance of mutation rate and length of the evolution process coupled with the proper selection scheme to obtain robust product tolerant strains will be necessary for these techniques.

Table 2.2 - Summary of bioproduct tolerant strains generated from various random mutagenesis approaches.

Method	Conditions	Chemical (Max C observed from the parental strain for T; P in g/L)	Micro-organism	Improvement (%)					Ref
				C	CD	CV	GR	P	
NTG mutagenesis	NTG at 50 µg/ml, 15 minute incubation	1-Butanol (12; 10.8)	*C. beijerinckii* NCIMB 8052	45.8	NT	NT	NT	82.4	(Qureshi & Blaschek, 2000)
	NTG at 50 µg/ml, 1 hour incubation	Isopropanol (45; 1.5)	*C. beijerinckii* DSM 6423	11.1	NT	NT	NT	NI	(Gérando et al., 2016)
UV mutagenesis	UV fluence of 5300 J/m2 at 8 W/m2	Ethanol (15; 3.6)	*C. thermocellum* NCIB 10682	160	NT	NT	<100	250	(Tailliez et al., 1989)
Transposon mutagenesis	Tn1545	1-Butanol (6; NT)	*C. beijerinckii* DSM 6423	83	NT	NT	NT	NT	(Liyanage et al., 2000)
Stress induced mutagenesis	LB broth/agar, 10 rounds of evolution	1-Butanol (8; NT)	*E. coli* SMB07	71.4	NT	NT	NT	NT	(Zhu et al., 2015)
Genome replication engineering assisted continuous evolution	LB broth, 3 transfers per concentration and 36 days in total	1-Butanol (8; NT)	*E. coli* DH5α	20	NT	NT	NT	NT	(Luan et al., 2013)
Genome Shuffling	NTG mutants as starting strains, 2 rounds of shuffling	Isopropanol (50; 1.5)	*C. beijerinckii* DSM 6423	N.I.	NT	+	NT	23.4	(Gérando et al., 2016)
	hybridization with *Lb. brevis,* 1 round of shuffling	1-Butanol (8.0; NT)	*E. coli* BW25113	98.8	NT	NT	NT	NT	(Winkler et al., 2010)
	adaptive evolution mutants as starting culture, 1 round of shuffling	1-Butanol (10.4; NT)	*E. coli* BW25113	7.7	NT	NT	NT	NT	(Reyes et al., 2012)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; NI, no improvement; +, growth observed but % improvement not calculated.

Another way to generate chimeras is through genome shuffling. Genome shuffling makes use of the entire genome of a mixed cell population to generate new combinations of mutations by recombination (Biot-Pelletier & Martin, 2014). The genome shuffling process is usually carried out by generating a

genetically diverse starting population, followed by recombination, selection, and screening, which can be repeated a number of times until the desired/acceptable product tolerance is achieved (Biot-Pelletier & Martin, 2014; Gong et al., 2009; Winkler et al., 2010). The starting population can be taken from natural sources or DNA fragment libraries, or generated *via* adaptive evolution, chemical mutagenesis, UV mutagenesis, transposon mutagenesis, or use of mutator strains or unmutated heterologous strains (Atsumi et al., 2010; Gérando et al., 2016; Horinouchi et al., 2010; Liyanage et al., 2000; Luan et al., 2013; Minty et al., 2011; Tailliez et al., 1989; Wang et al., 2011; Wang et al., 2014; Winkler et al., 2010; Zhu et al., 2015). The method of choice to introduce the DNA for recombination is highly dependent on the host microorganism and the source of diversity, which includes protoplast fusion, conjugation, phage-mediated transduction, direct transformation, liposomal delivery, or, in the case of yeast, sexual recombination (Gong et al., 2009).

Genome shuffling has been exploited to improve the tolerance of *C. beijerinckii* DSM 6423 for various compounds, by protoplast fusion of variants generated by NTG mutagenesis (Table 2.2). The resulting mutant strains exhibited improved tolerance for isopropanol, bromobutyrate, ethylbromobutyrate, and 5-methyl bromobutyrate. After 2 rounds of genome shuffling, mutant strains with enhanced tolerance for isopropanol at 50 g/L were generated, while the NTG mutants could barely grow at the same concentration in agar plates (Gérando et al., 2016). Although tolerance was barely improved, isopropanol production of the mutant increased by about 23.0% compared with the parental strain and the mutant produced by NTG mutagenesis. In an attempt to improve 1-butanol tolerance of *E. coli*, hybrid strains of *E. coli* BW25113 and *Lactobacillus brevis* 367 were generated via protoplast fusion of individual cultures without prior mutagenesis (Table 2.2) (Winkler et al., 2010). Two of the mutant hybrid strains exhibited 1-butanol tolerance that was twice the starting tolerance of the *E. coli* strain (Winkler et al., 2010). The 1-butanol tolerance of *E. coli* was also improved by 7.7% through genome shuffling of a population taken from a the chemostat culture grown up to 10.4 g/L 1-butanol (Table 2.2) (Reyes et al., 2012).

The different methods of generating bioproduct tolerant strains all seem to be effective to some extent. It will be difficult to identify which are the most effective methods unless the methods are compared systematically, by using the same parental strain and the same compound throughout, and then extending systematically to other organisms and compounds. Nonetheless, adaptive evolution appears to be the method of choice for generation of product tolerant strains *via* the random approach due to its effective and simple nature. Genome shuffling shows considerable promise to further enhance the level of tolerance achieved after adaptive evolution or other random mutagenesis approaches (Gérando et al., 2016; Reyes et al., 2012). However, further work is needed to prove that the resulting strains are sufficiently productive for industrial manufacturing of the chosen bioproduct.

2.4.2 Reverse/Inverse metabolic engineering

Reverse/inverse metabolic engineering (Bailey et al., 2002; Oud et al., 2012) depends on determining the genetic basis of the enhanced chemical product tolerance by comparing the genome sequence of the product tolerant strain with the parental strain, to identify the beneficial mutation/s (Fig. 2.1) (Atsumi et al., 2010; Bailey et al., 2002; Hong et al., 2010; Minty et al., 2011; Oud et al., 2012).

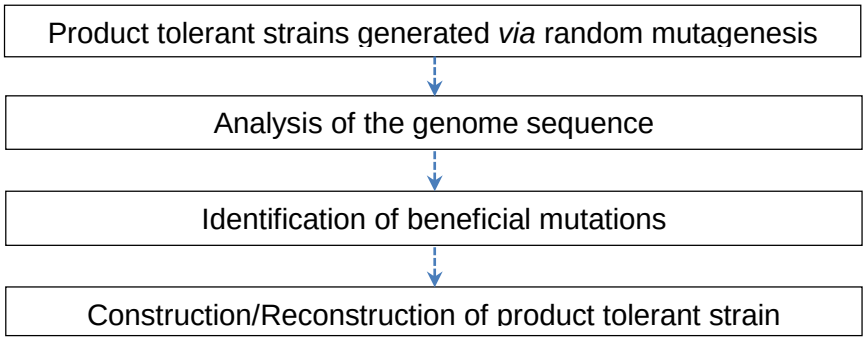

Fig. 2.1 Flow diagram of reverse/inverse engineering of a product tolerant strain.

The mutated gene or set of genes are then introduced into the parental strain, to confirm the role of the gene(s) in the resistance phenotype (Bailey et al., 2002; Hong et al., 2010; Oud et al., 2012). Further confirmation is obtained by reverting the mutation back to the wild type and confirming loss of tolerance.

Once the beneficial mutated genes are identified, they can be used or combined with other beneficial mutated genes to construct a superior product tolerant strain (Atsumi et al., 2010; Bailey et al., 2002; Hong et al., 2010; Minty et al., 2011; Oud et al., 2012).

Genomic sequence analysis and insertion or deletion of mutated genes revealed that mutations in *acrA*, *gatY*, *tnaA*, *yhbJ*, *hipA-flxA*, *marC*, *hfq*, *mdh*, *acrAB*, *gatYZABCD* and *rph* genes were beneficial for *E. coli* in increasing tolerance towards isobutanol from 6-7.5 g/L to 20 g/L (Atsumi et al., 2010; Minty et al., 2011). *gatY* and *acrA* were the most important of the mutant genes in conferring product tolerance, since the repair of both mutations in the isobutanol tolerant mutant reduced its cell density by 2-3 fold after growth in LB containing 8 g/L isobutanol for 24 hours as compared to the original isobutanol tolerant strain (Atsumi et al., 2010). Reconstruction of isobutanol tolerance was accomplished by deletion of the genes, *acrA*, *gatY*, *tnaA*, *yhbj*, and *marCRAB*, in the parental strain (Atsumi et al., 2010). However, there was no improvement in isobutanol production by the reconstructed strain. Other than this study, rather little work has been done to demonstrate the benefits of reverse engineering for improving chemical tolerance, and more work is necessary to determine whether or not this approach can yield further improvements. However, it serves as a very useful experimental tool to study the significance of each mutation in conferring the observed phenotype of the bioproduct tolerant mutants (Horinouchi et al., 2017).

2.4.3 Rational/Targetted approach

2.4.3.1 Cell membrane engineering

Alteration in fatty acid composition is an important mechanism to improve survival when microorganisms are exposed to toxic organic compounds (Heipieper & de Bont, 1994; Heipieper et al., 1992; Heipieper et al., 2003; Mrozik et al., 2005; Mrozik et al., 2004; Pinkart et al., 1996; Weber et al., 1994). For this reason, membrane engineering has been exploited to engineer improved tolerance of microorganisms for bioproducts (Oh et al., 2012; Si et al., 2016; Tan et al., 2017b; Tan et al., 2016b). Membrane composition can be modifed by altering the expression of the genes relevant to regulation of fatty

acid synthesis (Fujita et al., 2007; Oku et al., 2003), introduction of key enzymes to produce the relevant membrane components (Allakhverdiev et al., 2001; Alterman & Hanzlik, 2002; Beck, 2005; Chazarreta Cifré et al., 2013; Courtois et al., 2004; Cronan et al., 1979; Cybulski et al., 2002; Fujita et al., 2007; Grogan & Cronan, 1984; Grogan & Cronan, 1997; Heipieper et al., 2010; Heipieper et al., 2003; Holtwick et al., 1999; Junker & Ramos, 1999; Kaneda, 1977; Kaneda, 1991; Kim & Oh, 2013; Kolattukudy & Walton, 1972; Oku et al., 2003; Oku & Kaneda, 1988; Uttaro, 2006; von Wallbrunn et al., 2003; Wada et al., 1989; Yu et al., 2014; Zhang & Rock, 2009), or supplementation with fatty acids that are not produced naturally by the host strain (Beck, 2005; Kaneda, 1977; Oku et al., 2003; Oku & Kaneda, 1988). Key enzymes for the modification of the fatty acid structure include desaturases for introduction of a double bond (Allakhverdiev et al., 2001; Chazarreta Cifré et al., 2013; Cybulski et al., 2002; Uttaro, 2006; Wada et al., 1989), and *cis-trans* isomerase (Cti) for isomerization of *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids (Heipieper et al., 2010; Holtwick et al., 1999; Junker & Ramos, 1999; von Wallbrunn et al., 2003). In addition, cytochrome P450 monooxygenases, hydratases, 12-hydroxylases, lipoxygenases, and diol synthases can be used for addition of hydroxyl groups (Alterman & Hanzlik, 2002; Kim & Oh, 2013; Kolattukudy & Walton, 1972), whilst branched chain fatty acid synthetase (Beck, 2005; Kaneda, 1977; Kaneda, 1991; Oku et al., 2003; Oku & Kaneda, 1988), branched-chain α-keto acid decarboxylase (Oku & Kaneda, 1988), branched chain amino acid transferase (Beck, 2005), and malonyl-CoA:ACP transacylase (Oku et al., 2003) can be used for extension with a branched chain. Cyclopropane fatty acid synthase can be utilized for cyclopropane ring formation (Courtois et al., 2004; Cronan et al., 1979; Grogan & Cronan, 1984; Grogan & Cronan, 1997). However, rational optimization of the fatty acid composition could be difficult, as maintenance of membrane fluidity might not depend on changing a single component but rather a delicate balance of changes in amounts of multiple membrane components (Courtois et al., 2004; Eze, 1991; Heipieper & de Bont, 1994; Heipieper et al., 1992; Heipieper et al., 2003; Löbbecke & Cevc, 1995; Mrozik et al., 2005; Mrozik et al., 2004; Murínová & Dercová, 2014; Perly et al., 1985; Pinkart et al., 1996; Segura et al., 1999; Sikkema et al., 1995; Sol Cuenca et al., 2015; Weber & de Bont, 1996; Weber et al., 1994).

Cell membrane engineering approaches such as *cis-trans* isomerisation of unsaturated fatty acids (Tan et al., 2016b), alteration of membrane fatty acid profiles (Sherkhanov et al., 2014), adjustment of the saturated to unsaturated fatty acids ratio (Oh et al., 2012; Si et al., 2016), and changes in the phospholipid head group abundance (Tan et al., 2017b) have been successful in improving tolerance for various chemicals. *Cis-trans* isomerisation of unsaturated fatty acids is catalysed by the enzyme, *cis-trans* isomerase (Cti) (Heipieper et al., 2003). Cti is expressed naturally in various *Pseudomonas* and *Vibrio* species (Heipieper et al., 2003), but not in *E. coli* (Tan et al., 2016b). Heterologous expression of a Cti and its native signal peptide from *P. aeruginosa* in *E. coli* led to the isomerisation of *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids, which reduced membrane fluidity, and increased membrane rigidity (Tan et al., 2016b). The strain expressing Cti had a *trans/cis* unsaturated fatty acid ratio of 0.078, and showed a 12.0% increase of growth rate with exogenous octanoic acid (1.4 g/L) and 41.0% improvement in octanoic acid production (Tan et al., 2016b). This strain also exhibited an improved growth rate in the presence of exogenous hexanoic acid (1.2 g/L), decanoic acid (1.7 g/L), 1-butanol (4.8 g/L), hexanol (0.8 g/L), acetate (1.8 g/L), succinate (23.6 g/L), phenol (1.0 g/L), styrene (0.2 g/L), or toluene (0.9 g/L). However, the strain grew less rapidly in the presence of ethanol (15.5 g/L) or isobutanol (4.8 g/L).

E. coli (Δ*fadD* BTE) is used to produce medium chain fatty acids (Sherkhanov et al., 2014) but the native 2-acyl-glycerophosphoethanolamine acyltransferase/acyl-ACP synthetase (Aas) catalyses incorporation of medium chain free fatty acids (FFAs) into membrane phospholipids (Hsu et al., 1991; Jackowski et al., 1994; Sherkhanov et al., 2014) and makes the host cell more susceptible to medium chain fatty acid toxicity (Sherkhanov et al., 2014). Although the underlying mechanism of the increase in susceptibility of the cells to the fatty acid was not identified, it was possible to decrease the incorporation of medium chain fatty acids into the membrane lipids by deleting the *aas* gene (Sherkhanov et al., 2014). This led to partial restoration of the normal membrane fatty acid profile by reduction in the medium chain (C12-14) to long chain (C16) fatty acid ratio in comparison with the control strain. The strain with

the *aas* deletion grew faster, attained higher cell density, and retained at least twice the viable count after 48 h compared to the control strain grown in the presence of exogenous C12:0 (1.0 g/L) or C14:0 (1.0 g/L). It also produced about 20% more FFA (Sherkhanov et al., 2014).

Adjustment of the saturated to unsaturated fatty acid ratio has also been beneficial for the improvement of alkane (Oh et al., 2012) and alcohol tolerance (Oh et al., 2012; Si et al., 2016). FadR represses fatty acid degradation (Feng & Cronan, 2009) and activates expression of two key enzymes (FabA and FabB) for unsaturated fatty acid synthesis (Campbell & Cronan, 2001). Deletion of the *fadR* gene resulted in an increase of the saturated to unsaturated fatty acid ratio in *E. coli*. This improved growth in LBGMg media containing cyclohexane-hexane (14.1 g/L) mixture (1:1 ratio), with a 3-fold increase in cell concentration after 9 hours of growth compared with the wild type (Oh et al., 2012). By contrast, the saturated to unsaturated fatty acid ratio could be decreased by deleting the uncharacterized proteins, YibT or YghW, and this improved the growth of *E. coli* in agar plates containing 1-butanol (6.4 g/L) (Si et al., 2016). However, the growth in liquid culture was not assessed.

The alteration of phospholipid head groups allows cells to adapt and tolerate toxic organic compounds (Clark & Beard, 1979; Weber & de Bont, 1996), complementing alterations of the fatty acid composition. Therefore, manipulation of the amount and head-group composition of phospholipids has been explored as a means to engineer tolerance of microorganisms to bioproducts (Cronan, 2003; Geiger & Sohlenkamp, 2015; Heath et al., 2002; Pieringer, 1968; Smith, 1969; Tan et al., 2017b). The effect of changing the abundance of different phospholipid head groups was tested as a means of improving the tolerance of *E. coli* towards octanoic acid. The *pssA*, *pgsA*, and *clsA* genes encode the enzymes necessary for the synthesis of PE, PG, and CL, respectively (Tan et al., 2017b). Over-expression of *pssA*, *pgsA*, or *clsA* resulted in a 7%, 38%, or 12% increase in PE, PG, or CL, respectively. An increase in expression of *pssA* led to a 29% improvement in growth rate and about 2-fold increase in cell concentration when *E. coli* was grown with octanoic acid (2.9 g/L) as compared to the control strain. It also led to 1.4 fold improvement in octanoic acid to 0.22 g/L and total carboxylic acid to 0.27 g/L

production. Increased expression of *pssA* also improved resistance to inhibitory compounds from lignocellulosic feedstocks and various other bioproducts (Tan et al., 2017b). However, the level of fatty acid unsaturation increased and the level of cyclic fatty acids decreased, indicating that the membrane composition also changed when the head group composition is altered. This leaves room for discussion whether any effects on chemicals tolerance and production were due solely to the increased PE production or a result of the overall change in membrane composition. By contrast, increased expression of *clsA* did not improve octanoic acid tolerance, while overexpression of *pgsA* caused a 50% decline a growth rate (Tan et al., 2017b).

Table 2.3 Effect of membrane engineering on microbial tolerance towards bioproducts.

Method	**Chemical** (Max C observed from the parental strain for T; P in g/L)	**Microorganism**	**Improvement (%)**					**Ref**
			C	CD	CV	GR	P	
Overexpression of Cti	Octanoic acid (1.4; 0.31)	*E. coli* MG1655	NT	NT	NT	12	41	(Tan et al., 2016b)
Deletion of *fadR*	1:1 v/v Cyclohexane-hexane mixture (14.1)	*E. coli* BW25113	NT	200	NT	NT	NT	(Oh et al., 2012)
Deletion of *yibT* or *yghW*	1-Butanol (6.4)	*E. coli* JM109	NT	NT	< 500	NT	NT	(Si et al., 2016)
Overexpression of PssA	Octanoic acid (2.9; 0.22)	*E. coli* MG1655	NT	NT	NT	29	40	(Tan et al., 2017b)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested.

Although changes in membrane composition of microorganisms has been widely reported as an adaptation mechanism for chemical tolerance (Murínová & Dercová, 2014; Sardessai & Bhosle, 2002; Segura et al., 2012; Sherkhanov et al., 2014; Sol Cuenca et al., 2015), it is surprising that only very few studies have exploited this mechanism as a target for cell engineering to improve bioproduct tolerance. Also, only a few studies (Sherkhanov et al., 2014; Tan et al., 2017b; Tan et al., 2016b) have demonstrated that such membrane engineering results in improved product titres. These studies were restricted to systems for production of fatty acids, and further studies are needed to test the

impact of membrane engineering on the manufacture of other type of compounds. In addition, the results from the membrane engineering efforts suggest that membrane composition must be tailored specifically for the target bioproduct (Si et al., 2016; Tan et al., 2016b). It should be noted that the key principle for membrane engineering for solvent or bioproduct tolerance is membrane homeostasis (Zhang & Rock, 2008) or restoration of the cell membrane fluidity to the state prior to the perturbation caused by the presence of the toxic compound (Murínová & Dercová, 2014; Peabody et al., 2014; Sardessai & Bhosle, 2002; Segura et al., 2012; Sol Cuenca et al., 2015). As manipulation of metabolic pathways (Sherkhanov et al., 2014; Si et al., 2016), regulatory networks (Aono, 1998; Duval & Lister, 2013; Grkovic et al., 2002; Grkovic et al., 2001; Shimizu, 2013a; Shimizu, 2013b) or altered expression of membrane components (*e.g*. transporters) (Lennen et al., 2013; Mukhopadhyay, 2015; Tan et al., 2017a; Turner & Dunlop, 2015) could also alter membrane fluidity, membrane engineering should be attempted only after incorporation of the metabolic pathway for the desired bioproduct and other interventions to improve bioproduct tolerance and production titres.

2.4.3.2 Overexpression of heat shock proteins (HSPs)

Based on the observation that expression of certain HSPs were induced during exposure to various alcohols and other toxic chemicals (Anfelt et al., 2013; Blom et al., 1992; Bormann et al., 2014; Brynildsen & Liao, 2009; Cao et al., 2017; Desmond et al., 2004; Fiocco et al., 2007; Foo et al., 2014; Kang et al., 2007; Mann et al., 2012; Rau et al., 2016; Yung et al., 2016), numerous attempts have been made to increase cellular tolerance towards short chain alcohols by overexpressing specific HSPs (Anfelt et al., 2013; Desmond et al., 2004; Fiocco et al., 2007; Foo et al., 2014; Kang et al., 2007; Mann et al., 2012; Tomas et al., 2003; Zingaro & Papoutsakis, 2012; Zingaro & Terry Papoutsakis, 2013) or a combination of HSPs (Zingaro & Papoutsakis, 2012) (Table 2.4). Heat shock proteins (HSPs) protect against protein aggregation, help in refolding/unfolding of damaged proteins, repair damaged proteins, reactivate inactivated proteins, or help degrade irreparably denatured proteins (Guzzo, 2012; Murínová & Dercová, 2014; Richter et al., 2010; Segura et al., 2012; Verghese et al., 2012; Weber & de Bont, 1996; Whitley et al., 1999; Yura et al.,

1993). These proteins are also involved in RNA and DNA repair, metabolism, regulation, cell structure maintenance, and restoration of membrane stability (Guisbert et al., 2004; Richter et al., 2010; Straus et al., 1987).

One of the most studied HSPs is the ATP-dependent GroESL chaperonin system, which aids in the folding/refolding of unfolded/misfolded proteins (Masters et al., 2009; Xu & Sigler, 1998). The overexpression of the native GroESL in *C. acetobutylicum* increased the cell density in liquid cultures exposed to 1-butanol (6.0 g/L) by 50% as compared with the control strain (Tomas et al., 2003). It also allowed survival of 45% of the cells after exposure to 1-butanol at 15.9 g/L for 2 h, while the control strain failed to survive (Mann et al., 2012). A 30% increase in 1-butanol titres was obtained after overexpression of GroESL in *C. acetobutylicum* (Mann et al., 2012; Tomas et al., 2003). Similarly, overexpression of *Lactobacillus paracasei* GroESL in *Lb. paracasei* and *Lactococcus lactis* NZ9800 allowed growth in liquid cultures containing 1-butanol (4.0 g/L), while the parental strain failed to grow (Desmond et al., 2004). Homologous overexpression of GroESL in *E. coli* enhanced cell growth in the presence of ethanol (30.4 g/L), 1-butanol (6.0 g/L), 2-butanol (10.0 g/L), isobutanol (7.9 g/L) and 1,2,4-butanetriol (198.3 g/L) by 12, 2.8, 3, 1.8, and 4-fold higher cell density in liquid cultures as compared to the control strain, respectively (Zingaro & Terry Papoutsakis, 2013). It also improved cell viability after exposure to ethanol (51.6 g/L) for 24 hours by 38% with respect to the control strain (Zingaro & Papoutsakis, 2012).

Aside from GroESL, other HSPs were also tested for their ability to improve alcohol tolerance. In *C. acetobutylicum*, GrpE and HtpG were individually overexpressed, leading to 25% and 56% improvements in cell viability (Mann et al., 2012). However, overexpression of GrpE or HtpG did not improve solvent production. Individual overexpression of native GrpE (a co-chaperone in the DnaK chaperone system) (Brehmer et al., 2004; Rüngeling et al., 1999; Wu et al., 1996) and ClpB (a chaperone protein that aids in disaggregation of proteins) (Barnett et al., 2000; Kedzierska et al., 2003; Mogk et al., 2003) in *E. coli* improved its cell viability by 27% and 15%, respectively. (Zingaro & Papoutsakis, 2012). Hsp33, a chaperone protein that helps prevent protein folding and aggregation (Cremers et al., 2010; Jakob et al., 2000), from *Bacillus*

psychrosaccharolyticus was also overexpressed in *E. coli*. This resulted in a higher viability upon exposure to isopropyl alcohol (22.9 g/L) and 1-butanol (15.9-19.8 g/L) (Kang et al., 2007).

The benefit of overexpressing small HSPs to improve alcohol tolerance was also assessed (Anfelt et al., 2013; Fiocco et al., 2007; Foo et al., 2014). Small HSPs are proteins with molecular masses in the range of 15-42 kDa, which have the ability to bind to non-native proteins and prevent their aggregation and irreversible folding (Caspers et al., 1995; Kitagawa et al., 2002; Kuczynska-Wisnik et al., 2002; Narberhaus, 2002; Roy et al., 1999; Török et al., 2001). For example, IbpA and IbpB (Kitagawa et al., 2002; Kuczynska-Wisnik et al., 2002) were overexpressed in *E. coli* (Foo et al., 2014). IbpA overexpression improved the growth rate and cell density in the presence of isopentenol (1.5 g/L) and also improved isopentenol production by 16%, while overexpression of IbpB did not enhance *E. coli* tolerance towards isopentenol (Foo et al., 2014). Since it helps stabilize membranes and prevents protein aggregation, the effect of HspA overexpression was also investigated (Török et al., 2001). Overexpression of native HspA in *Synechocystis* sp. at approximately 10 times the normal level improved cell growth rate and cell density (after 6 days) in the presence of exogenous 1-butanol (4.0 g/L) by around 60% and 50%, respectively (Anfelt et al., 2013). In *Lactobacillus plantarum*, the over expression of native small HSPs, Hsp18.55 and Hsp19.3 resulted in higher viability exposure to ethanol (84.6 g/L) and 1-butanol (8.0 g/L). On the contrary, the over expression of the small HSP, Hsp18.5, did not change the susceptibility of *Lb. plantarum* towards ethanol and 1-butanol under the same conditions (Fiocco et al., 2007).

Effects of the co-overexpression of various HSPs on alcohol tolerance were also evaluated. The overexpression of GroESL-GrpE and GroESL-ClpB combination in *E. coli* led to a 100% and 1,130% increase in viability after exposure to ethanol (37.6 g/L), respectively (Zingaro & Papoutsakis, 2012). The co-overexpression of GroESL and ClpB also enhanced cell viability of *E. coli* when exposed to 1-butanol (8.0 g/L) and isobutanol (7.9 g/L) by 78% and 25%, respectively (Zingaro & Papoutsakis, 2012). Likewise, co-overexpression of GrpE, GroESL, and ClpB in *E. coli* led to improvements in viability after exposure for to ethanol (51.6 g/L), 1-butanol (8.0 g/L), and 1,2,4-butanetriol

(238 g/L) by 200%, 390%, and 78%, respectively (Zingaro & Papoutsakis, 2012). However, overexpression of other HSPs such as DnaK, DnaJ, IbpA, and IbpB individually or in combinations did not help improve *E. coli* tolerance for alcohols (Zingaro & Papoutsakis, 2012).

Table 2.4 Effect of HSPs overexpression on microbial tolerance towards bioproducts.

Method	**Chemical** (Max C observed from the parental strain for T; P in g/L)	**Microorganism**	**Improvement (%)**					**Ref**
			C	CD	CV	GR	P	
Overexpression of GroESL	1-Butanol (6; 13.0)	*C. acetobutylicum* ATCC 824	NT	50	NT	NT	30	(Tomas et al., 2003)
	1-Butanol (4; NT)	*Lb. paracasei* NFBC 338; *L. lactis* NZ9800	NT	+; +	NT	NT	NT	(Desmond et al., 2004)
	Ethanol (51.6; NT); 1-Butanol (6 ; NT); 2-Butanol (10; NT); Isobutanol (7.9; NT); 1,2,4-Butanetriol (198.3; NT)	*E. coli* 10-ß	NT	1100; 180; 200; 80; 300	NT	NT	NT	(Zingaro & Terry Papoutsakis, 2013)
Overexpression of GroESL/ GrpE/ HtpG	1-Butanol (15.9; 6.7)	*C. acetobutylicum* ATCC 824	NT	NT	+/ +/ +	NT	30/ -49/ -32	(Mann et al., 2012)
Overexpression of GroESL/ GrpE/ ClpB	Ethanol (51.6; NT)	*E. coli* MG1655	NT	38/ 27/ 15	NT	NT	NT	(Zingaro & Papoutsakis, 2012)
Overexpression of Hsp33	Isopropanol (22.9; NT), 1-butanol (19.8; NT)	*E. coli* JW176 / *E. coli* JW49	NT	NT	+,+ / +,+	NT	NT	(Kang et al., 2007)
Overexpression of IbpA	Isopentenol (1.5; 0.83)	*E. coli* DH1	NT	+	NT	+	16	(Foo et al., 2014)
Overexpression of HspA	1-Butanol (4 g/L)	*Synechocystis sp.*PCC 6803	NT	50	NT	60	NT	(Anfelt et al., 2013)
Overexpression of Hsp 18.55 ; Hsp 19.3	Ethanol (84.6 g/L), 1-Butanol (8 g/L)	*Lb. plantarum*	NT	NT	+,+ ; +,+	NT	NT	(Fiocco et al., 2007)
Overexpression of GrpE, GroESL and ClpB	Ethanol (51.6; NT), 1-Butanol (8 ; NT), 1,2,4 Butanetriol (238 g/L)	*E. coli* MG1655	NT	200 , 390, 78	NT	NT	NT	(Zingaro & Papoutsakis, 2012)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; NI, no improvement; +, growth observed but % improvement not calculated.

Although the use of some HSPs provided promising improvements in tolerance for short chain alcohols (especially the combinations of several HSPs), the use of HSPs to improve tolerance towards other classes of bioproducts has not been reported. Furthermore, the majority of studies have only shown that overexpression of HSPs improve cell growth or survival (Anfelt et al., 2013; Desmond et al., 2004; Fiocco et al., 2007; Kang et al., 2007; Zingaro & Papoutsakis, 2012; Zingaro & Terry Papoutsakis, 2013), and demonstrations of improved product titres have been limited (Foo et al., 2014; Mann et al., 2012; Tomas et al., 2003). Therefore, it will be interesting to verify if the benefit of the overexpression of HSPs can be extended to improvements in tolerance and titre for bioproducts of different functional groups.

2.4.3.3 Modulation of transport protein expression

Given the involvement of transport proteins in stress responses to toxic chemicals, a number of studies have tested the effects of modulating their expression in attempts to improve bioproduct titres (Table 2.5). The first group of transport proteins that were heavily implicated for their role in toxic compound tolerance are the efflux pumps (Martins et al., 2019; Mukhopadhyay, 2015; Murínová & Dercová, 2014; Sardessai & Bhosle, 2002; Segura et al., 1999; Segura et al., 2012; Sol Cuenca et al., 2015). Efflux pumps are membrane proteins with the ability to actively export compounds through energy-dependent transport processes. They are divided into six superfamilies: namely, the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound exporters (MATE) family, the resistance-nodulation-division (RND) family (Saier et al., 2006) and the proteobacterial antimicrobial compound efflux (PACE) family (Chitsaz & Brown, 2017; Hassan et al., 2015; Hassan et al., 2018). The ABC pumps utilize ATP as the energy source, whereas the MFS, RND and SMR pumps are H^+-dependent antiporters, while the MATE pumps are Na^+ or H^+-dependent antiporters (Du et al., 2015; Hassan et al., 2018; Wong et al., 2014). The energy source for PACE pumps is not yet known, but they are suspected to operate using electrochemical potential (Hassan et al., 2015). ABC, MFS, SMR, PACE and MATE transporters reside in the inner membrane proteins and are found in both Gram negative and Gram

positive bacteria (Du et al., 2015). On the other hand, RND transport systems are associated with Gram negative bacteria only. RND pumps form a tripartite complex across the cell envelope, including an inner membrane protein (RND type pump), a periplasmic protein (belonging to the membrane fusion proteins family), and an outer membrane porin (belonging to the outer membrane proteins family) (Du et al., 2015; Wandersman, 1992).

In one investigation, a pool of 43 efflux pumps from various microorganisms were selected for their homology with the substrate binding regions of the toluene efflux pump, TtgB, and expressed in *E. coli* (Dunlop et al., 2011). The best pumps were selected using a growth competition assay in the presence of 1-butanol (0-8.0 g/L), iso-pentenol (0-4.2 g/L), geranyl acetate (0-43.6 g/L), geraniol (0-1.3 g/L), α-pinene (0-40.9 g/L), limonene (0.3 g/L), or farnesyl hexanoate (26.2 g/L) (Dunlop et al., 2011). The *E. coli* strain overexpressing the native efflux pump, AcrAB, exhibited the best tolerance for a number of bioproducts, whilst an uncharacterized pump from *Alcanivorax borkumensis* provided the best tolerance for limonene, improving limonene tolerance by 50%. The strain overexpressing the efflux pump from *A. borkumensis* produced about 0.055 g/L of limonene, which is approximately 50% more limonene than the parental *E. coli* strain. However, this low yield is likely to be below the toxic threshold, so further work is needed to prove that this intervention can improve titres to industrially relevant concentrations. Similarly, overexpression of the ABC transporter, MdlB, improved the growth rate of *E. coli* in the presence of isopentenol (1.5 g/L), and improved isopentenol production by 12-60% in comparison with the control strain (0.3-0.8 g/L) (Foo et al., 2014).

Knockout studies revealed the importance of AcrAB-TolC efflux system for 1-hexene tolerance in *E. coli* (Mingardon et al., 2015). Overexpression of TolC alone did not improve growth of *E. coli*, while overexpression of AcrAB improved growth of *E. coli* in the presence of 1-hexene by approximately 10-fold as compared to the wild-type strain with native expression levels of AcrAB. The co-overexpression of AcrAB-TolC further improved growth of *E. coli* in the presence of 1-hexene by approximately 10-fold as compared to the wild-type strain (Mingardon et al., 2015). The inability of TolC overexpression to improve 1-hexene tolerance of *E. coli* was attributed to the possibility that AcrAB rather

than TolC is the limiting protein in the wild-type *E. coli* strain for the AcrAB-TolC efflux system (Mingardon et al., 2015).

Aside from efflux systems, the modulation in expression of outer membrane proteins/porins can be an effective strategy for improvement of bioproduct tolerance (Doukyu et al., 2012; Tan et al., 2017a; Zhou et al., 2015). Porins are water-filled channels found in the outer membranes of Gram negative bacteria or in the outer layer of mycobacteria andinvolved in the size-selective diffusion of hydrophilic compounds across the outer membrane (Fernández & Hancock, 2012). They are formed with a monomeric or trimeric transmembrane β-barrels and can be classified as specific or non-specific/general porins, depending on the pore size and the amino acid composition (Vollan et al., 2016). The pore size may vary slightly depending on the environmental conditions, including pH, salinity, temperature and the presence of flexible or constriction loops (Sleator & Hill, 2002; Vollan et al., 2016) . Such constriction loops are located inside the channel and, together with the hydrophilic amino acids of the channel, generate an electrostatic field that determines the selectivity of the pore (Fernández & Hancock, 2012; Vollan et al., 2016).

In *E. coli*, deletion of *ompF* (encoding the outer membrane porin F, OmpF; participates in the transport of sugars, ions, protein, and antibiotics) (Tan et al., 2017a), and/or increase in expression *fadL* (encoding the long chain fatty acid outer membrane porin, FadL) improved octanoic acid (1.4 g/L) tolerance, membrane integrity, and fatty acid production (Tan et al., 2017a). Deletion of *ompF* alone led to a 7%, 15%, 18%, and 10% improvement in growth rate, cell concentration, membrane integrity and fatty acid production, respectively. On the other hand, increased expression of *fadL* improved the growth rate, cell concentration, membrane integrity, and fatty acid production by 8%, 20%, 25%, and 34% respectively. The *E. coli* strain with a combination of *ompF* deletion and increased expression of *fadL* gained higher improvements in growth rate, cell concentration, membrane integrity, and fatty acid production at 18%, 50%, 37%, and 53%, respectively. The total fatty acid titre reached 2.33 g/L, which was 53% more than the control. These improvements were thought to arise from reduced uptake of short chain fatty acids *via* OmpF and increased uptake of long chain fatty acids *via* FadL for use in membrane damage repair (Tan et

al., 2017a). Similarly, deletion of the gene for any of the three components of the ProU (ProVWX) system for compatible solutes uptake (Wood, 1999) resulted in a 1000 fold improvement in colony forming efficiency of *E. coli* in agar plates overlaid with n-hexane-cyclohexane mixture (1:1 v/v) as compared to the control strain *E. coli* BW25113 or *E. coli* JA300 (Doukyu et al., 2012). About 24% reduction of intracellular n-hexane after 60 min exposure was observed in *E. coli* JA300 with a *proV* deletion compared to the wild type.

Proteomics analysis revealed differential expression of some transport related proteins with growth of *E. coli* in the presence of phenylpropanoids (1.0 g/L), resveratol, naringenin, or rutin (Zhou et al., 2015). Significant increase of OmpW, OmpF, FadL, OppD, and PotG and decrease of LamB, MalK, MalE, ManX, TolC, OppA protein levels were observed. Overexpression of the genes encoding OmpW, OmpF, FadL, OppD, and PotG resulted in a higher growth rate, while silencing of the genes encoding LamB, MalK, MalE, ManX, TolC, and OppA resulted in a lower growth rate as compared to the control strain in the presence of resveratrol (1.0 g/L), naringenin (1.0 g/L), or rutin (1.0 g/L). LamB (maltose outer membrane porin), MalK (maltose/maltodextrin ABC transporter, ATP binding protein), MalE (maltose ABC transporter; maltose transporter subunit), and ManX (fused mannose specific phosphotransferase system enzyme) are part of the maltose transport system for transport of small molecules such as carbohydrates, organic acids, and alcohols (Boos & Shuman, 1998; Davidson et al., 1992; Joly et al., 2004; Okochi et al., 2006).

It is possible that the reduced expression of these proteins may have decreased the influx of the phenylpropanoids into *E. coli*, lessening their toxic effects (Zhou et al., 2015), but further work is needed to confirm this hypothesis. OppA is an ABC transporter with broad specificity for uptake of oligopeptides (Baev et al., 2006), while OppD is another ABC transport system for efflux of oligopeptides that is linked to increased antibiotic resistance (Higgins et al., 1985; Ito et al., 2009). It is also possible that silencing OppA may have decreased the uptake of phenypropanoids and upregulating OppD may have increased their export system for the phenypropanoids (Zhou et al., 2015).

Table 2.5 Effect of modulation of transport protein expression on microbial tolerance towards bioproducts.

Method	Chemical (Max C observed from the parental strain for T; P in g/L)	Microorganism	Improvement (%)					Ref
			C	CD	CV	GR	P	
Overexpression of an uncha-racterized pump *A. borkumensis*	Limonene (0.3; 0.035)	*E. coli* DH1 ΔacrAB	60	NT	NT	NT	50	(Dunlop et al., 2011)
Overexpression of MdlB	Isopentenol (1.5; 0.35)	*E. coli* DH1	NT	NI	NT	+	60	(Foo et al., 2014)
Over expression of AcrAB; AcrAB-TolC	1-Hexene (saturated vapor)	*E. coli* K-12	NT	NT	10^3; 10^4	NT	NT	(Mingard on et al., 2015)
Δ***ompF***; **Overexpression of fadL** ; Δ***ompF* + overexpression of FadL**	Octanoic acid (1.4; 1.50)	*E. coli* MG1655	NT; NT; NT	15; 20; 50	NT; NT; NT	7; 8; 18;	10; 34; 53	(Tan et al., 2017a)
Δ***proV***, Δ***proW***, Δ***proX***	1:1 v/v n-Hexane-cyclohexan e mixture (over laid on top of agar plates)	*E. coli* BW25113; *E. coli* JA300	NT; NT	NT; NT;	10^5; 10^5	NT; NT	NT; NT	(Doukyu et al., 2012)
Overexpression of OmpW, OmpF, FadL, OppD, and PotG or silencing of LamB, MalK, MalE, ManX, and TolC	Resveratrol (1; NT), Naringenin (1; NT), Rutin (1; NT)	*E. coli* BL21	NT, NT, NT	NT, NT, NT	NT, NT, NT	+, +, +	NT, NT, NT	(Zhou et al., 2015)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; NI, no improvement; +, growth observed but % improvement not calculated.

Similarly, overexpression of OmpW may have enhanced the efflux of the phenylpropanoids, since it belongs to a family of small outer membrane proteins, linked to export of small hydrophobic molecules (Beketskaia et al., 2014; Hong et al., 2006). PotG is a subunit of PotFGHI which serves as an uptake system for putescrine (Kurihara et al., 2009; Terui et al., 2014), while OmpF and FadL are porins involved in uptake of small molecules and long chain fatty acids (Black, 1988; Call et al., 2016; Jaktaji & Heidari, 2013; Tan et al., 2017a; Ziervogel & Roux, 2013). On the other hand, TolC is an outer membrane protein that is a key part of various efflux systems in *E. coli* (*e.g.* AcrAB-TolC) (Deininger et al., 2011; Zgurskaya et al., 2011). The

improvements in phenylpropanoid tolerance with the increased expression of PotG, OmpF, and FadL or silencing of the gene encoding TolC seems to imply that they function in an opposite manner with phenylpropanoids as compared to their known function as part of uptake or efflux systems; however, it is also possible that the changes in their expression simply altered membrane integrity (Zhou et al., 2015). Thus, further work is needed to establish the mechanisms for improved tolerance to phenylpropanoids.

The modulation of suitable efflux and import systems has shown to be effective in improving both bioproduct tolerance and production titres (Dunlop et al., 2011; Foo et al., 2014; Tan et al., 2017a). A major challenge is the identification of the suitable efflux or import system/s, especially for bio-products not naturally produced by the chosen production host (Doukyu et al., 2012; Dunlop et al., 2011; Foo et al., 2014; Mingardon et al., 2015; Tan et al., 2017a; Zhou et al., 2015).

2.4.3.4 Overexpression of regulatory proteins of the multidrug resistance response system (MarA, SoxS, and Rob)

Aside from functional proteins, the overexpression of regulatory proteins such as MarA, SoxS, and Rob has shown to be effective in conferring tolerance towards toxic compounds (Table 2.6) (Asako et al., 1997; Nakajima et al., 1995; Shah et al., 2013; White et al., 1997). MarA, SoxS, and Rob are responsible for the regulation of the multidrug resistance in *E. coli* (Gambino et al., 1993; Grkovic et al., 2002; Grkovic et al., 2001). In addition to the presence of toxic organic compounds, MarA, SoxS, and Rob responds to the other stresses, including presence of antibiotics, oxidative agents, and changes in environment (*i.e.* pH) (Alekshun & Levy, 1997; Duval & Lister, 2013; Jain & Saini, 2016), which makes them a valuable stress regulatory network for bioproduct tolerance. MarA, SoxS, and Rob are closely related, homologues that are part of the AraC/XylS family of positive transcriptional regulators (Gallegos et al., 1997). Together they form a system known as the Mar (multiple antibiotic resistance)-Sox (superoxide response)-Rob (right oriC-binding protein) regulon (Grkovic et al., 2002; Grkovic et al., 2001). The Mar-Sox-Rob regulon includes efflux pumps (*e.g*. AcrAB-TolC) and other stress related proteins, and porins

(*e.g.* OmpF) (Alekshun & Levy, 1999; Duval & Lister, 2013; Griffith et al., 2009). Many of the genes within the regulon have a similar sequence of 20 bp within the promoter region, known as the marbox (Martin et al., 1999), which is a common binding site for MarA, SoxS, and Rob (Duval & Lister, 2013; Grkovic et al., 2002; Grkovic et al., 2001; Jain & Saini, 2016). For this reason, each of these regulators can activate the same multidrug resistance response, either singly or in combination. Nevertheless, the manner and extent of their activation may vary between particular genes (Martin et al., 2008). Similar systems also exist in different microorganisms, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, and *P. aeruginosa* (Grkovic et al., 2002; Grkovic et al., 2001).

Table 2.6 Effect of MarA, SoxS, or Rob overexpression on microbial tolerance towards chemicals.

Method	**Chemical** (Max C observed from the parental strain for T; P in g/L)	**Microorganism**	**Improvement (%)**					**Ref**
			C	CD	CV	GR	P	
Overexpression of MarA, SoxS, or Rob	Cyclo-hexane (overlaid on agar plates)	*E. coli* AG100; *E. coli* AG100K; *E. coli* GC4468; *E. coli* RA4468; *E. coli* DJ901	+; +; +; +; +	NT	NT	NT	NT	(White et al., 1997)
Overexpression of MarA	Cyclo-hexane (overlaid on agar plates)	*E. coli* W3110; *E.coli* JA300; *E.coli* MC1061; *E.coli* FS1576	+; +; +; +	NT	NT	NT	NT	(Asako et al., 1997)
	geraniol (8.8; NT)	*E.coli* DH5α	NT	NT	+	NT	NT	(Shah et al., 2013)
Overexpression of SoxS	Cyclo-hexane (overlaid on agar plates)	*E.coli* JA300; *E.coli* OST4251; *E.coli* MC1061	+; +; +	NT	NT	NT	NT	(Nakajima et al., 1995)
Overexpression of Rob	Cyclo-hexane (overlaid on agar plates)	*E.coli* JA300; *E.coli* OST4251; *E.coli* MC1061; *E.coli* MV1184; *E.coli* DH1; *E.coli* FS1576	+; +; +; +; +; +	NT	NT	NT	NT	(Nakajima et al., 1995)
***marR* mutation → overexpression of MarA**	pine oil (8.0; NT)	*E.coli* AG100	343	NT	NT	NT	NT	(Moken et al., 1997)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; +, growth observed but % improvement not calculated.

The overexpression of either MarA, SoxS, or Rob conferred tolerance towards cyclohexane in *E. coli* (Aono, 1998; Asako et al., 1997; Nakajima et al., 1995; White et al., 1997). The AcrAB-TolC efflux pump was shown to play a key role

in the acquired trait, as deletion of the *acrAB* locus resulted in loss of tolerance towards cyclohexane (Aono, 1998; White et al., 1997). *E. coli* strains that acquired mutations in *marR*, (at amino acid residue 73 Arg→Ser) (Asako et al., 1997) or *soxR* (10-amino acid residue truncation at the C-terminus) (Nakajima et al., 1995) also obtained tolerance towards cyclohexane. Pine oil tolerance (35.4 g/L) was acquired by *E. coli* with mutations in *marR*, since this led to overexpression of MarA (Moken et al., 1997).

The overexpression of *MarA* was also successful in improving *E. coli* tolerance for geraniol (8.8 g/L) (Shah et al., 2013). Again, the AcrAB-TolC efflux pump was suggested to play a key role, as the deletion of the *acrAB tolC* genes made *E. coli* more susceptible to geraniol toxicity (Shah et al., 2013). On the contrary, an *E. coli* strain with a *marR* mutation (Ankarloo et al., 2010; Oethinger et al., 2000) that allowed overexpression of MarA did not lead to tolerance towards short chain alcohols (Ankarloo et al., 2010). Deletion of the *mar* or the *acrAB* locus did not increase susceptibility towards these alcohols, suggesting that tolerance to these alcohols is not dependent on the MarA regulatory network nor on the AcrAB-TolC efflux system (Ankarloo et al., 2010). Therefore, the MarA, SoxS, Rob regulatory system serves as a promising target for manipulation in order to obtain tolerance for hydrophobic compounds but may not be a suitable system for short chain alcohols.

2.4.3.5 Global transcription machinery engineering (GTME)

Other transcription factors have also been utilized to generate bioproduct tolerant strains not by overexpression, but *via* directed evolution (Table 2.7) (Alper & Stephanopoulos, 2007; Basak et al., 2012; Chen et al., 2011; Chong et al., 2014; Chong et al., 2013a; Chong et al., 2013b; Klein-Marcuschamer et al., 2009; Klein-Marcuschamer & Stephanopoulos, 2008; Lee et al., 2011; Ma & Yu, 2012; Si et al., 2016; Tan et al., 2016a; Zhang et al., 2015; Zhang et al., 2012a). This approach, which has been termed as global transcription machinery engineering (GTME), targets key proteins responsible for the regulation of transcription at a global level, resulting in altered gene expression levels of tens to hundreds of genes (Alper & Stephanopoulos, 2007; Zhang et al., 2015).

In GTME, the directed evolution of the target transcription factor is usually achieved *via* an *in vitro* random mutagenesis approach (*e.g.* error prone PCR) to generate a mutant library, which is then cloned into a suitable vector and transformed into the host of choice, prior to selection for tolerance towards the desired bioproduct (Alper & Stephanopoulos, 2007; Zhang et al., 2015). Global transcriptional regulators that have been altered to improve bioproduct tolerance include σ^{70} (RpoD) in *E. coli* (Alper & Stephanopoulos, 2007; Si et al., 2016; Zhang et al., 2015), *Zymomonas mobilis* (Tan et al., 2016a), and *Lactobacillus plantarum* (Klein-Marcuschamer & Stephanopoulos, 2008); σ^{A} (SigA) in *Rhodococcus ruber (Ma & Yu, 2012)*; and cAMP receptor protein (CRP) in *E. coli* (Chong et al., 2014; Chong et al., 2013a; Chong et al., 2013b; Zhang et al., 2012a). This approach has also been used to cause perturbations in global transcription with the aid of an external global regulator, IrrE, from *Deinococcus radiodurans* (Chen et al., 2011), an artificial transcription factor (ATF) DNA-binding zinc finger protein fused to *E. coli* CRP (Lee et al., 2011) and mutations in the *E. coli* RNA polymerase subunit α (Klein-Marcuschamer et al., 2009) expressed in *E. coli* (Table 2.7).

Sigma factors regulate the specificity of RNA polymerase for promoter recognition, and are essential for the regulation and initiation of transcription (Paget, 2015; Paget & Helmann, 2003; Tripathi et al., 2014). In bacteria, the main sigma factor for transcription during normal growth belongs to the σ^{70} family, frequently annotated as RpoD in Gram negative bacteria (Alper & Stephanopoulos, 2007; Klein-Marcuschamer & Stephanopoulos, 2008; Paget, 2015; Paget & Helmann, 2003; Tan et al., 2016a; Tripathi et al., 2014; Zhang et al., 2015) or σ^{A} (SigA) in Gram positive bacteria (Hu & Coates, 1999; Ma & Yu, 2012; Pátek & Nešvera, 2011). Mutations in σ^{70} (RpoD) of *E. coli* improved the tolerance for ethanol (Alper & Stephanopoulos, 2007), 1-butanol (Si et al., 2016; Zhang et al., 2015), and cyclohexane (Zhang et al., 2015) by 40%, 65.6%, and <80%, respectively. RpoD mutants of *Z. mobilis* grew in the presence of 71.8 g/L ethanol, which is a 22.7% increase in the level of ethanol tolerance as compared to the control strain (Tan et al., 2016a). However, the strains with improved ethanol tolerance were not able to produce more ethanol (16 g/L) under normal conditions. Increased ethanol production was only

observed after external addition of ethanol (65.2 g/L). Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are key enzymes in the ethanol-producing pathway, which had their specific activities increased significantly in the RpoD mutant when the ethanol concentration was increased to 65.2 g/L (Tan et al., 2016a). Nonetheless, the full significance of this observation is unclear. The best σ^{70} (annotated as RpoD) mutants in *Lb. plantarum* were able to grow at least twice as fast, with 3 times more biomass, and produced 8% more lactate as compared to the wild-type (Klein-Marcuschamer & Stephanopoulos, 2008). Under low pH conditions (pH = 3.85), the strain carrying the mutant RpoD produced 25% more lactate than the control strain (Klein-Marcuschamer & Stephanopoulos, 2008). Effects on gene expression resulting from such RpoD mutations in both *Z. mobilis* (Tan et al., 2016a) and *Lb. Plantarum* (Klein-Marcuschamer & Stephanopoulos, 2008) were not further investigated.

R. ruber was used as a whole cell biocatalyst to produce acrylamide with acrylonitrile as substrate and its native σ^{A} (SigA) was subjected to directed evolution for improved tolerance for both the substrate and bioproduct. In comparison to the wild-type, the best *R. ruber* σ^{A} (SigA) mutant achieved a cell density of at least 3 and 2 times higher in the presence of acrylonitrile (0.8 g/L) and acrylamide (6.7 g/L), respectively (Ma & Yu, 2012). The *R. ruber* strain carrying a σ^{A} mutation also produced 10-37% more acrylamide than the control strain, while being used as a whole cell biocatalyst (Ma & Yu, 2012). However, the underlying mechanism of the improvements observed was not investigated further.

The α-subunit of the RNA polymerase, which is the core enzyme and plays a key role in RNA polymerase assembly (*via* its N-terminal region), interaction with class I transcription factors (e.g. CRP, MarA, Rob, SoxS, and etc.) and the upstream element of the promoter (*via* its C-terminal region) (Murakami et al., 1997), has also been evolved to confer 1-butanol tolerance. A mutant with an altered α-subunit of RNA polymerase achieved a 129% higher cell density in comparison to the wild-type when grown with 1-butanol (7.2 g/L). This mutant also exhibited better tolerance towards 2-butanol (12.7 g/L), 1-pentanol (2.0 g/L), and 3-pentanol(4.9 g/L) (Klein-Marcuschamer et al., 2009). However,

alcohol production was not attempted (Klein-Marcuschamer et al., 2009), and the effects on gene expression resulting from the mutations were not further investigated.

CRP, which is present in enteric bacteria (Soberón-Chávez et al., 2017), is a transcriptional regulator that affects ~ 400 genes and plays a lead role in the activation of key genes for the utilization of non-glucose carbon sources (Basak et al., 2012; Chong et al., 2013a; Gunasekara et al., 2015; Shimada et al., 2011; Zhang et al., 2012a). It has been evolved and tested for effectiveness in delivering 1-butanol (Zhang et al., 2012a; Zhang et al., 2012b), isobutanol (Chong et al., 2014), ethanol (Chong et al., 2013a), acetate (Chong et al., 2013b), and toluene (Basak et al., 2012) tolerance in *E. coli*. The CRP mutant selected for the best 1-butanol (Zhang et al., 2012a) and isobutanol (Chong et al., 2014) tolerance achieved at least 2 times higher growth rate and cell density as compared to the wild type with 1-butanol (9.7 g/L) (Zhang et al., 2012a) or isobutanol (9.5 g/L) (Chong et al., 2014). Ethanol (62.0 g/L) selection gave rise to a different CRP mutant able to grow as fast as the wild type but with at least 1.5 times the cell density, which also exhibited higher tolerance for propanol (24.2 g/L), 1-butanol (10.4 g/L), and 1-pentanol (3.6 g/L) (Chong et al., 2013a). Selection for acetate tolerance led to the isolation of a CRP mutant that grows about 5 times faster and 3 times higher cell density with sodium acetate (15.0 g/L) (Chong et al., 2013b). The *E. coli* strain with the mutant CRP also exhibited higher tolerance for sodium formate (5.0 g/L) and sodium propionate (18.0 g/L) as compared to the wild-type (Chong et al., 2013b). Furthermore, it improved acetate production by about 70% more as compared to the control strain (Chong et al., 2013b). CRP mutants were also selected with toluene (1.7-2.0 g/L) at concentrations that prevented growth of the wild type control strain (Basak et al., 2012). The CRP mutations resulted in 1-4 amino acid substitutions located at the N-terminal cAMP binding domain (residues 1-134) (Basak et al., 2012; Chong et al., 2013a; Zhang et al., 2012a), C-terminal DNA binding domain (residues 140-209) (Basak et al., 2012; Chong et al., 2014; Chong et al., 2013a; Zhang et al., 2012a), and/or the hinge (residues 135-139) (Basak et al., 2012; Chong et al., 2013a; Zhang et al., 2012a). These changes in amino acid composition were shown to significantly alter gene expression of

tens to hundreds of genes including stress related genes both in the presence or the absence of the compound selected for (Basak et al., 2012; Chong et al., 2014; Chong et al., 2013a; Zhang et al., 2012a).

A number of DNA-binding artificial transcription factors-fused zinc finger (ATF-ZF) protein mutants with *E. coli* CRP as an effector domain were tested for their ability to confer 1-butanol tolerance in *E. coli* (Lee et al., 2011). Zinc finger (ZF) proteins are a large group of proteins that require zinc for folding and interact with other proteins, lipids, RNA, or DNA (Jantz et al., 2004), with a wide range of cellular functions, including protein folding and assembly, lipid binding, RNA packaging, DNA recognition, and transcriptional regulation (Laity et al., 2001). Their ability to recognize and bind to specific DNA sequences and alter transcriptional regulation have been exploited to modulate gene expression through generation of artificial transcription factors (ATF) ZF proteins (Jantz et al., 2004; Sera, 2009) that are fused with effector domains (Frietze & Farnham, 2011). The best ATF-ZF mutant grew about 9 times faster and with 3 times higher cell density in the presence of 1-butanol (12.0 g/L) as compared to the wild type *E. coli* (Lee et al., 2011). A total of 284 genes were differentially expressed in the strain carrying the mutant ATF-ZF in comparison with strain not carrying any ATF (Lee et al., 2011).

IrrE (a metalloprotease that cleaves the repressor protein DrdO) from *D. radiodurans,* which has been found to be a crucial switch for radiation tolerance (Bauermeister et al., 2009; Chen et al., 2011; Hua et al., 2003; Ludanyi et al., 2014), was used to generate a mutant library and assessed for its ability to confer tolerance towards bioproducts (Chen et al., 2011). Although IrrE was not previously linked to chemicals tolerance, some mutations in IrrE resulted in improvements of ethanol, 1-butanol, or acetate tolerance (Chen et al., 2011). The best mutant IrrE selected for ethanol (37.6 g/L) tolerance was able to grow by at least 48 fold higher cell density as compared to the host cell with or without the wild type IrrE, while the best 1-butanol (7.0 g/L) tolerant mutant achieved at least 3 times cell density as compared to the *E. coli* strains with or without the wild type IrrE. In the case of the acetate (5.0 g/L) tolerant IrrE mutant, at least 50% improvement in cell density compared to the *E. coli* strains with or without the wild type IrrE was observed. The mutations in IrrE caused 1-4 amino acid

residue changes at the N-terminal domain (protease function), HTH domain (DNA binding), and/or C-terminal domain (small molecule sensor function) (Chen et al., 2011). However, the effect of these mutations on global transcription were not investigated further. Although the rationale for using the ATF-ZF fused with *E. coli* CRP (Lee et al., 2011) and IrrE from *D. Radiodurans* (Chen et al., 2011) as targets for GTME were unclear, this study demonstrates that GTME can be expanded with the use of artificial transcription factors and exogenous global regulators to improve bioproduct tolerance of a chosen production host (Chen et al., 2011).

As chemical tolerance is complex and would likely require the alteration of expression of a number of genes (Horinouchi et al., 2017; Molina-Santiago et al., 2017; Murínová & Dercová, 2014; Nicolaou et al., 2010; Peabody et al., 2014; Rau et al., 2016; Rutherford et al., 2010; Sardessai & Bhosle, 2002; Segura et al., 2012; Shimizu, 2013b; Sol Cuenca et al., 2015; Yang et al., 2013; Yung et al., 2016; Zhang et al., 2015), GTME presents a very promising targeted approach that allows simultaneous perturbations in expression of multiple genes to confer bioproduct tolerance (Zhang et al., 2015). However, its application has been limited to a few global regulators and potential chemical products (Alper & Stephanopoulos, 2007; Si et al., 2016; Tan et al., 2016a; Zhang et al., 2015) with only a few studies demonstrating an improvement in titres for the desired bioproduct (Chong et al., 2013a; Klein-Marcuschamer & Stephanopoulos, 2008; Ma & Yu, 2012; Tan et al., 2016a). Thus, succeeding works on GTME should focus on enhancing production titres. Nonetheless, the improvement in titres under stress conditions demonstrates some promise for this approach.

Table 2.7 - Effect of GTME on microbial tolerance towards bioproducts.

Method	Chemical (Max C observed from the parental strain for T; P in g/L)	Microorganism	Improvement (%)					Ref
			C	CD	CV	GR	P	
Directed evolution of RpoD	Ethanol (50; NT)	*E. coli* DH5α	40	NT	NT	NT	NT	(Alper & Stephanopoulos, 2007)
	1-Butanol (9.6; NT)	*E. coli* JM109	65.8	NT	NT	NT	NT	(Si et al., 2016)
	Cyclo-hexane (217.2; NT)	*E. coli* JM109	< 48.3	NT	NT	NT	NT	(Zhang et al., 2015)
	Ethanol (58.5; 16.0)	*Z. mobilis* ZM4	22.7	NT	NT	NT	NI	(Tan et al., 2016a)
	L-Lactate (5.5; 8.6)	*Lb. plantarum*	NT	< 200	NT	< 100	~7	(Klein-Marcuschamer & Stephanopoulos, 2008)
	Acrylonitrile (0.8; NT), Acrylamide (6.7; 300)	*R. ruber*	NT	200, 100	NT	NT	10-20	(Ma & Yu, 2012)
Directed evolution of CRP	1-Butanol (9.7; NT)	*E. coli* DH5α (Δcrp)	NT	NT	NT	100	NT	(Zhang et al., 2012a)
	Isobutanol (9.5; NT)	*E. coli* DH5α (Δcrp)	NT	NT	NT	260	NT	(Chong et al., 2014)
	Ethanol (62; NT)	*E. coli* JW5702 (Δcrp)	NT	<50	NT	25	NT	(Chong et al., 2013a)
	Acetate (15; 0.16)	*E. coli* DH5α (Δcrp)	NT	~200	NT	419	70	(Chong et al., 2013b)
	Toluene (0; NT)	*E. coli* DH5α (Δcrp)	+	NT	NT	NT	NT	(Basak et al., 2012)
Directed evolution of an artificial transcription factor fused with CRP	1-Butanol (12.0; NT)	*E. coli* MG1655	NI	~800	NT	~20	NT	(Lee et al., 2011)
Directed evolution of IrrE	Ethanol (37.6; NT), 1-Butanol (7; NT), Acetate (5; NT)	*E. coli* DH5α	NI, NI, NI	< 4700, 200, <50	NT, NT, NT	NT, NT, NT	NT, NT, NT	(Chen et al., 2011)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C, CD, CV, or GR); NT, not tested; NI, no improvement; +, growth observed but % improvement not calculated

2.5 Summary of highest bioproduct tolerance enhancement

Although no comprehensive comparison of methods for the enhancement of bioproduct tolerance has been reported, the studies presented in this review

showed potential approaches as options for the generation of a bioproduct tolerant strain. The approaches that allowed increase in chemical tolerance (in terms of chemical concentration) by at least 50% are summarized in Table 2.6. Among them adaptive evolution related approaches have been reported the most to give great enhancement (<50 %) in chemical tolerance (Table 2.8), which suggests that adaptive evolution serves as the best option as preliminary approach to enhance bioproduct tolerance of a chosen bacterial host cell.

Table 2.8 - List of reports for enhanced bioproduct tolerance with improvements of at least 50%

Method	Chemical (Max C observed from the parental strain for T; P in g/L)	Microorganism	Improvement (%)		Ref
			C	P	
Adaptive evolution (batch/ serial transfers)	Ethanol (20 ; 15.9)	*E. coli* KC01	100	48.8	(Wang et al., 2011)
Adaptive evolution (batch/ serial transfers)	Isobutanol +xylose (7.5; NT) / + glucose (10; NT)	*E. coli* EcNR1	130/ 100	NT	(Minty et al., 2011)
Adaptive evolution (batch/ serial transfers)	1-Butanol (6; NT)	*E. coli* BW25113	75	NT	(Menchavez et al., 2018)
Adaptive evolution (batch/ serial transfers)	1-Butanol (1.6; NT)	*Synechocystis. sp*. PCC 6803	150	NT	(Wang et al., 2014)
Artificial simulation of bio-evolution (batch)	1-Butanol (19.8; 12.2)	*C. acetobutylicum* D64	57.7	25.4	(Liu et al., 2013)
Adaptive evolution (continuous)	1-Butanol (6.4; NT)	*E. coli* BW25113	62.5	NT	(Reyes et al., 2012)
UV mutagenesis	Ethanol (15; 3.6)	*C. thermocellum* NCIB 10682	160	250	(Tailliez et al., 1989)
Transposon mutagenesis	1-Butanol (6; NT)	*C. beijerinckii* DSM 6423	83	NT	(Liyanage et al., 2000)
Stress induced mutagenesis	1-Butanol (8; NT)	*E. coli* SMB07	71.4	NT	(Zhu et al., 2015)
Genome Shuffling	1-Butanol (8.0; NT)	*E. coli* BW25113	98.8	NT	(Winkler et al., 2010)
Overexpression of an uncharacterized pump from *A. borkumensis*	Limonene (0.3; 0.035)	*E. coli* DH1 ΔacrAB	60	50	(Dunlop et al., 2011)
marR mutation → overexpression of MarA	pine oil (8.0; NT)	*E.coli* AG100	343	NT	(Moken et al., 1997)
Directed evolution of RpoD	1-Butanol (9.6; NT)	*E. coli* JM109	65.8	NT	(Si et al., 2016)

C, concentration; CD, cell density; CV, cell viability; GR, growth rate; P, production; T, tolerance (in terms of C); NT, not tested; NI, no improvement.

2.6 Concluding remarks

Various classical and modern biotechnology tools are available for the engineering of the production host cell for improved bioproduct tolerance. Both the classical random mutagenesis and the modern rational approaches have proven to be effective in generating a host strain with improved bioproduct tolerance. Among the approaches, adaptive evolution has shown to be the most promising approach in generating bioproduct tolerant strains. However, the enhancement in bioproduct tolerance does not necessarily translate to higher product titres. In addition, none of the reported studies has improved bioproduct tolerance at industrial production scale relevant titres (which is the subject of this study). Nevertheless, reports of enhanced production under stress conditions by exogenously adding the bioproduct at near inhibitory concentrations shows a glimpse of the potential of bioproduct tolerant strains to break bioproduct toxicity titre limitations.

3

Aim and Objectives

3.1 Aim

The overall aim of this study was to develop a robust host cell suitable for the commercial production of methacrylate esters based on Lucite International's butyl methacrylate (BMA) bioprocess route. In line with the overall aim, this study intended to address four objectives.

3.2 Objectives

1. To generate strains with tolerance towards BMA (10-20% v/v)

It is essential for the host strain to have tolerance for BMA at 10-20% in order to realize the bioprocess. The potential host strain explored in this study was E. coli. Based on literature review, preceding study that isolated BMA tolerant E. coli , and preliminary growth studies in the presence of BMA, adaptive evolution was applied to generate BMA tolerant E. coli strains.

2. To understand the cause of BMA tolerance

Understanding the underlying mechanisms of BMA tolerance may help in identifying genes or gene networks that are vital for BMA tolerance, which could be used for further engineering of the host cell. In this regard, Genomics and Transcriptomics were used on a few select BMA tolerant strains to identify the mutations acquired and changes in transcription profile due to the mutations acquired as well as exposure to BMA.

3. To further enhance desirable traits of the BMA tolerant strains

BMA tolerant strains experience different degrees of inhibition as reflected with their growth in the presence of BMA (20% v/v). Tolerant strains either grew at high rate or high cell density. Cell density correlates with the efficiency of resource utilization and allocation. Thus, it would be beneficial to have a host strain that is resource efficient to maximize utilization of resources for growth and product formation. In this regard, genome shuffling was utilized to exploit the diverse mutations found in BMA tolerant strains followed by selection to

identify strains with superior cell density grown in the presence of BMA (20% v/v).

4. To test the BMA tolerant strains for actual BMA production

The purpose of engineering the potential host strain to be more tolerant to BMA was to allow production at desired titres that are beyond the previous toxicity limit. In this regard, the BMA tolerant strains were tested for their ability to produce BMA.

4

Materials and Methods

4.1 Materials

4.1.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, Mo, USA): ampicillin, $CaCl_2$ (≥96.0 %), DMSO (≥99.9 %), EDTA disodium salt (>99.0 %), $FeCl_3$ (98.0%), KH_2PO_4 (≥98 %), K_2HPO_4 (≥98.0 %), KOH (≥85 %), $MgCl_2$ (≥98.0 %), $MnSO_4 \cdot H_2O$ (≥99 %), NaCl (≥99.0 %), Na_2HPO_4 (>99.5 %), $NaH_2PO_4 \cdot 2H_2O$ (≥99.0 %), NaOH (≥97.0 %), NH_4Cl (≥99.5 %), $(NH_4)_2SO_4$ (≥99.0 %), ammonium citrate dibasic (98 %), sodium 3-methyl-2-oxobutyrate (95.0 %), sodium maleate dibasic (≥98 %), spectinomycin dihydrochloride pentahydrate, Tris/HCl (>99%), agarose, arabinose (≥99.0 %), glucose (>99.0 %), glycerol (>98.0 %), sucrose (≥99.0 %), BMA (99.0 %), and inhibitor remover (prepacked column). The chemicals $MgCl_2$ and $CuSO_4 \cdot 5H_2O$ (99.5%) were purchased from BDH Chemicals Limited, whilst $ZnSO_4 \cdot 7H_2O$ and $CoCl_2 \cdot 6H_2O$ were purchased from Acros Chemicals. PEG6000 was purchased from Fluka. Granulated LB broth was purchased from Melford Biolaboratories Ltd. (Ipswich, UK), whilst the bacteriological agar was from Oxoid Ltd. (Altrincham, UK).

4.1.2 Reagents

TAE buffer (50X) for routine gel electrophoresis analysis was purchased from Bio-Rad (Hercules, California). The Quick-Load Purple 1 kb Plus DNA ladder from New England Biolabs (Hitchin, UK) was used to estimate the size of DNA fragments.

4.1.3 Enzymes

The DNase I and Q5® High-Fidelity DNA polymerase were purchased from New England Biolabs (Hitchin, UK), whilst the lysozyme used was purchased from Melford Laboratories (Ipswich, UK).

4.1.4 Kits

GenElute™ bacterial genomic DNA kit, plasmid miniprep kit, and PCR clean-up kit were used for extraction and purification of genomic DNA, plasmid DNA, and PCR products and purchased from Sigma-Aldrich (St. Louis, Mo, USA). QIAquick Gel Extraction Kit was purchased from Qiagen (Hilden, Germany).

4.1.5 DNA oligonucleotides

The oligonucleotides, which include the primers used in this study, were ordered from Integrated DNA Technologies (Leuven, Belgium).

4.1.6 Plasmids

The plasmids pKIV_ara (Appendix Fig. 11.1) and pBAD-MMA050_mACX4_ corrected (3) (Appendix Fig. 11.2) used for testing the production of BMA in *Escherichia coli* strains were kindly provided by Ingenza Ltd. (Roslin, Edinburgh).

Table 4.1 List of plasmids used in this study

Plasmid	Features	Reference/Source
pKIV_ara	2-Ketoisovalerate production vector: 11, 242 bp with a p15 ori; $Spec^R$; *araC; ilvC*, *ilvD*, and *katE* from *E. coli*; *alsS* from *Bacillus subtilis*; under the pBAD promoter system	Ingenza Ltd.
pBAD-MMA050_mACX4_ corrected (3)	BMA from 2-ketoisovalerate production vector: 13, 394 bp with a pUC ori; Amp^R; *araC*; *bkdA1, bkdA2, bkDB,* and *lpdV* from *Pseudomonas aeruginosa*; *acx4* from *Arabidopsis thaliana*; *aat* from *Malus domestica*; under the araBAD promoter system	Ingenza Ltd.

Plasmids and their sources are shown. ori - origin of replication, $Spec^R$ – spectinomycin resistance marker, Amp^R - ampicillin resistance marker, *araC* – arabinose operon regulatory protein, *ilvC* – ketol-acid reductoisomerase, *ilvD* – dihydroxy-acid dehydratase, *katE* – catalase HPII, *alsS* – acetolactate synthase, *bkdA1*– 2-oxoisovalerate dehydrogenase subunit alpha, *bkdA2* - 2-oxoisovalerate dehydrogenase subunit beta, *bkdB* – lipoamide acyltransferase, *lpdV* - dihydrolipoyl dehydrogenase, *acx4* – acyl-coenzyme A oxidase 4, *aat* – alcohol acyl transferase

4.1.7 Strains

The parental strain *E. coli* BW25113 was purchased from the American Type Culture Collection (ATCC), while the best isolate from the previous study (strain LM-2) (Disley, 2018) was kindly provided by Laura Martins. The other strains

used in the study were generated from *E. coli* BW25113 with their characteristics listed in Table 4.2.

Table 4.2 List of bacterial strains used in this study

Strain	Features	Source / Reference
E. coli BW25113	From *E. coli* MG1655: Δ*(araD-araB)567* Δ*(rhaD-rhaB)568* Δ*lacZ4787 (::rrnB-3) hsdR514 rph*-1	ATCC (Grenier et al., 2014)
LM-2	From *E. coli* MG1655: *soxR*(R20H) *acrR*(V29G)	(Disley, 2018)
RNM-2	From *E. coli* BW25113: *acrR*(E91fs) *rob*(R156H) *rpoC*(L361R) *ilvN*(C41Y) *ygbK*(A294E) *lpxM*(267_272 del) *ompT*(indels)	This study
RNM-3	From *E. coli* BW25113: *acrR*(E91fs) *rob*(R156H) *ilvN*(C41Y) *phoP*(L11F) *acrB*(V448L) *icd*(398Dfs) *ΔymfD ΔymfE Δlit ΔintE ΔxisE Δymf IΔymfJ ΔcohE ΔcroE ΔymfL ΔymfM ΔoweE ΔaaaE ΔymfR ΔbeeE ΔjayE ΔymfQ ΔstfP ΔtfaP ΔtfaE ΔstfE ΔpinE ΔmcrA* yhDE(indels)	This study
RNM-5	From *E. coli* BW25113: *acrR*(N214fs) *soxR*(Leu139X) *mscK*(indels) 580116(G>T)	This study
RNM-6	From *E. coli* BW25113: *acrR*(Y77fs) *soxR*(A146del)	This study
RNM-7	From *E. coli* BW25113: *acrR*(E91fs) *rob*(A70V) *stfP*(indels)	This study
RNM-18	From *E. coli* BW25113: *acrR*(A191fs) *rob*(A70T) *creA*(V85V) *yohJ*(L109R) *dnaK*(V377G) 927777(C>T) *cra*(I270fs) *clsA*(A448fs) *rpoC*(K215fs) *opgH*(R95P) *cpxA*(P177Q) *ompX*(indels) *atpI*(indels) Δ*psuT ΔpsuG ΔpsuK ΔfruA ΔfruK ΔfruB ΔsetB*	This study
RNM-8	From *E. coli* BW25113: *acrR*(K53fs) *rob*(R156H) *rpoB*(T1037P) *torY*(A87T)	This study
RNM-19	From *E. coli* BW25113: *acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *ompR*(R15S) *acrB*(T379I) *yieL*(indels) *mioC*(indels)	This study
RNM-20	From *E. coli* BW25113: *acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *ompR*(R15S) *yhiN*(indels) *pitA*(indels)	This study
RNM-21	From *E. coli* BW25113: *acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *rpoC*(A787V) *ompR*(R15S) *acrB*(V901I) *ΔyhhJ ΔrbbA ΔyhiI ΔyhiJ ΔyhiL ΔyhiM ΔyhiN ΔpitA*	This study
RNM-22	From *E. coli* BW25113: *acrR*(K53Yfs) *rob*(R156H) *rpoB*(T1037P) *groL*(P279L) *torY*(A87T) 1197659(C>A)	This study
RNM-23	From *E. coli* BW25113: *acrR*(E74fs) *soxR*(R20L) *rpoC*(r1075C) *torY*(A87T) 2133236(T>A) 3915915(T>G)	This study

4.2 Methods

4.2.1 Growth media, agar, and buffer preparations

4.2.1.1 Growth media

M9 minimal medium

Stock solutions of M9 salts (5X concentrate), $MgSO_4$ (1M), $CaCl_2$ (1M), glucose (40% w/v) were prepared and autoclaved separately. M9 salts (5X concentrate) stock solution contained Na_2HPO_4 (33.89 g), KH_2PO_4 (15 g), NaCl (2.5 g), and NH_4Cl (5.0 g) in deionized (DI) water (1L). M9 minimal medium (amount from stock solution; final concentration) was prepared by adding the autoclaved M9 salts aseptically (200 mL; 1X), $MgSO_4$ (2.0 mL; 2 mM), $CaCl_2$ (0.1 mL; 0.1 mM), and glucose (25.0 mL; 10 g/L) to DI H_2O (773 mL).

MSX minimal medium

Stock solutions of MSA, MSB, Vishniac trace elements, and glucose (40% w/v) were prepared and autoclaved separately. Vishniac trace elements solution (1L) was prepared by combining EDTA disodium salt (50 g) with DI H_2O (800 mL) and dissolved by addition of KOH pellets (1 at a time). The salts were added in the following order: $ZnSO_4$ (2.2 g), $CaCl_2$ (5.54 g), $MnCl_2{\cdot}4H_2O$ (5.06 g), $FeSO_4{\cdot}7H_2O$ (5 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1.1 g), $CuSO_4{\cdot}5H_2O$ (1.57 g) and $CoCl_2{\cdot}6H_2O$ (1.61 g). The pH of the salt solution with EDTA was adjusted to 6 using KOH (1M) and DI H_2O was added to make up for the desired final volume (1L). MSA was prepared by dissolving KH_2PO_4 (6.0 g) and Vishniac trace elements (2.0 mL) in DI H_2O (660 mL). The pH of the solution was adjusted to 7 with addition of KOH (1M) and DI H_2O was added to make up for the desired final volume (760 mL). MSB was prepared by dissolving NH_4Cl (3.0 g) and $MgSO_4{\cdot}7H_2O$ (0.4 g) in DI H_2O (200 mL). The final MSX solution was prepared by aseptically adding the autoclaved MSA (760 mL), MSB (200 mL), 40% w/v glucose solution (25 mL), and DI H_2O (15 mL).

Lund medium

Stock solutions of 5X salt solution, glycerol solution (40% w/v), $MgSO_4$ (1M), yeast extract (50 g/L), trace elements (TE) solution, and arabinose (20% w/v) were prepared and autoclaved separately. Stock solutions of Ampicillin (0.1 g/L) and Spectinomycin (0.05 g/L) were prepared and filter sterilized separately. The 5X salt solution was prepared by dissolving $(NH_4)_2SO_4$ (10.0 g), K_2HPO_4 (73.0 g), $NaHPO_4 \cdot 2H_2O$ (18.0 g), and ammonium citrate (2.5 g) in DI H_2O (800 mL). The pH of the 5X salt solution was adjusted to pH 7.0 and DI H_2O was added to reach the desired final volume (1L). The TE solution was prepared by dissolving EDTA disodium salt dihydrate (22.3 g), $CaCl_2 \cdot 2H_2O$ (0.5 g), $FeCl_3$ (10.03 g), $ZnSO_4 \cdot 7H_2O$ (0.18 g), $CuSO_4 \cdot 5H_2O$ (0.16 g), $MnSO_4 \cdot H_2O$ (0.15 g), and $CoCl_2 \cdot 6H_2O$ (0.18 g) in DI H_2O (800 mL). The pH of the solution was adjusted to pH 7.0 and DI H_2O was further added to reach the desired volume (1L). The Lund medium was prepared by aseptically adding the autoclaved 5X salt solution (200 mL), yeast extract (15.4 mL), $MgSO_4$ solution (2.0 mL), TE solution (2.0 mL), glycerol solution (25.0 mL), Antibiotic solution (1.0 mL) and DI H_2O (755 mL).

4.2.1.2 Agars

LB agar

LB agar was prepared by adding LB (25.0 g) and bacteriological agar (15.0 g) with DI H_2O (1 L). The mixture was sterilized via autoclave and allowed to cool down (55-60 °C) prior to addition of antibiotic for selection (if necessary) and poured onto sterile disposable Petri dish (~25 mL). Once the agar has solidified, the agar was allowed to dry (45-60 min) inside the sterile microbiological safety cabinet prior to use or storage at 4°C for later use.

M9 agar

M9 agar was prepared in a similar manner as preparing M9. In M9 agar, the bacteriological agar (1.5 g) was dissolved in DI H_2O (773 mL) then sterilized by autoclave and allowed to cool down to 55-60 °C. The stock solutions of M9 salts (200 mL), $MgSO_4$ (2.0 mL), $CaCl_2$ (0.1 mL), and glucose (25.0 mL) were preheated to 55-60°C prior to aseptic addition into the sterile agar solution (773

mL). The M9 agar solution was poured onto sterile disposable Petri dish (~25 mL). Once the agar has solidified, the agar was allowed to dry (45-60 min) inside the sterile microbiological safety cabinet prior to use or storage at 4°C for later use.

MSX agar

MSX agar was prepared in a similar manner as preparing M9. In MSX agar, the bacteriological agar (1.5 g) was dissolved in MSA (760 mL) then sterilized by autoclave and allowed to cool down to 55-60 °C. The stock solutions of MSB (200 mL), glucose (40% w/v; 25.0 mL), and DI H_2O (15 mL) were preheated to 55-60°C prior to aseptic addition into the sterile agar solution (773 mL). The MSX agar solution was poured onto sterile disposable Petri dish (~25 mL). Once the agar has solidified, the agar was allowed to dry (45-60 min) inside the sterile microbiological safety cabinet prior to use or storage at 4°C for later use.

Agarose gel

Agarose gel for DNA gel electrophoresis was prepared by dissolving agarose (10 g) in TAE buffer (1X; 1L).

4.2.1.3 Buffers

Tris/HCl buffer

A stock of Tris/HCl buffer (0.1 M) was prepared by dissolving TRIS hydrochloride (15.76 g) in DI H_2O (800 mL). The pH of the TRIS hydrochloride solution was adjusted to pH 8.0 with KOH (10 M) and DI H_2O was further added to reach the desired final volume (1L). A 0.01 M Tris/HCl buffer (pH = 8.0) was prepared by addition of the autoclaved Tris/HCl stock solution (100 mL) to DI H_2O (900 mL).

SMM Buffer

Stock solutions of sodium maleate (40 mM) with $MgCl_2$ (40 mM) and sucrose (1M) were prepared and autoclaved separately. Sodium maleate (40 mM) with $MgCl_2$ (40 mM) stock solution was prepared by dissolving sodium maleate dibasic salt (6.40 g) and $MgCl_2$ (3.81 g) in DI H_2O (800 mL). The pH of the salt

solution was adjusted to pH 6.5 by addition of HCl (1 M) and DI H_2O was added to reach the desired total volume (1 L). The sucrose solution (1M) was prepared by dissolving sucrose (342.3 g) in DI H_2O (800 mL) and DI H_2O was further added to attain the desired total volume (1 L). SMM buffer (amount from stock solution; final concentration) was prepared by aseptically combining the autoclaved solution of sodium maleate with $MgCl_2$ (500 mL; 20 mM of each) and solution (500 mL; 0.5 M) (Dai et al., 2005).

PEG buffer

Stock solutions PEG6000 (60% w/v) with the salts sodium maleate (30 mM), $MgCl_2$ (30 mM), and $CaCl_2$ (15 mM) and sucrose (2.5 M) were prepared and sterilized separately. PEG6000 (600 g), sodium maleate (4.80 g), $MgCl_2$ (2.86 g), and $CaCl_2 \cdot H_2O$ (2.21 g) were dissolved in DI H_2O (900 mL). The pH of the mixture was adjusted to 6.5 and DI H_2O was further added to reach the desired total volume (1 L) then filter sterilized. The sucrose solution (1M) was prepared by dissolving sucrose (855.75 g) in DI H_2O (800 mL) and DI H_2O was further added to attain the desired total volume (1 L) then autoclaved. The PEG buffer (amount from stock solution; final concentration) was prepared by aseptically combining the filter sterilized stock solution of PEG6000 (666 mL; 40% w/v) with the salts sodium maleate (15 mM), $MgCl_2$ (15 mM), and $CaCl_2$ (10 mM) and sucrose solution (200 mL; 0.5 M), filter sterilized DMSO (50 mL; 5% vol.), and autoclaved DI H_2O (84 mL) (Dai et al., 2005).

4.2.2 Growth studies

E. coli strains were grown in M9 minimal medium (50 mL) with glucose (10 g/L) contained in a conical flask (250 mL) fitted with a rubber Suba-seal using a shaker incubator (Innova®40, New Brunswick Scientific, NJ, USA) at 37°C and 200 rpm. BMA in appropriate amounts were added immediately after inoculation. The maximum growth rate at the exponential growth phase was estimated using the slope in the plot of ln OD600 vs time (h) (Hall et al., 2013).

4.2.3 Adaptive evolution

4.2.3.1 Sequential batch cultures

E. coli strains were grown in M9 minimal medium (10 mL) with glucose (10 g/L) contained in a Falcon tube (50 mL) at vertical position using a shaker incubator (Innova®40, New Brunswick Scientific, NJ, USA) at 37°C and 200 rpm. BMA in appropriate amounts was added immediately after inoculation.

4.2.3.2 Chemostat cultures

Cells were grown in M9 minimal medium with glucose (1 g/L) using a home-made jacketed mini-bioreactor (Fig. 4.1) with a working volume of 55 mL at 37°C and an aeration rate of 0.3 L/h. The bioreactor was fitted with a mini pH probe (Mini-pH probe; 180 mm, Cole-Parmer) attached to a pH controller (Fermac 200, Electrolab) for automatic pH control. The fresh media, base, and BMA were fed separately using peristaltic pumps (120U, Watson Marlow).

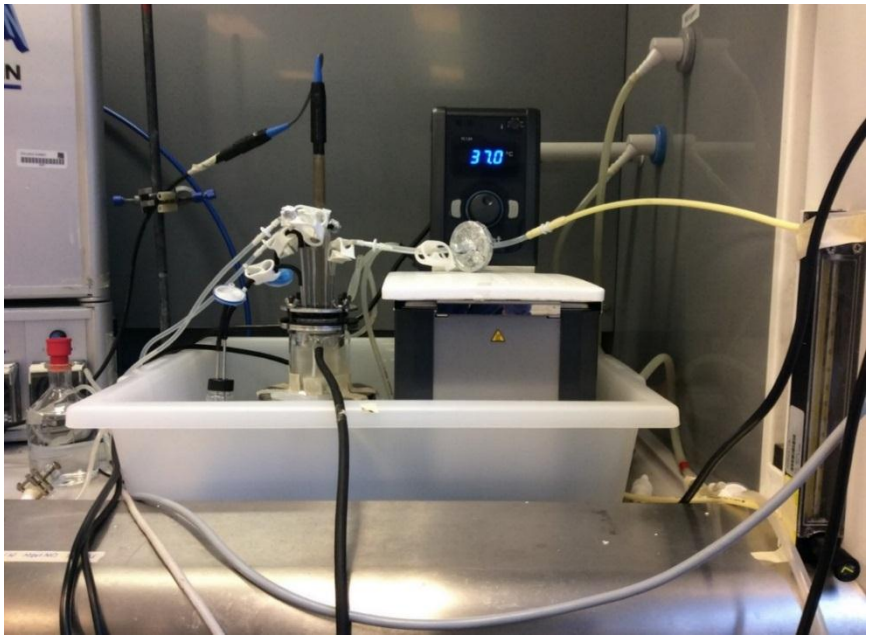

Fig. 4.1 Mini-bioreactor set-up for the chemostat cultures.

4.2.4 Genome shuffling

4.2.4.1 Protoplast formation

Protoplasts of the BMA tolerant *E. coli* strains were generated using a modified protocol of Dai et al. (2005) and Reyes et al. (2012). Cells were grown in LB medium (30 mL) in a 250 mL baffled flasks at 37°C and 200 RPM until an OD (600 nm) of about 1.0. Cells were harvested by centrifugation (5000g; 4°C; 10 min) and washed with ice cold Tris/HCl buffer (30mL; 0.01 M; pH 8.0) for three times. After the final wash, cells were pelleted *via* centrifugation (5000g; 4°C; 10 min) and re-suspended in Tris/HCl (27 mL; 0.01 M; pH 8.0) containing sucrose (0.5 M). EDTA disodium salt (0.1 M; pH 8.0) in Tris/HCl (0.01 M; pH 8.0) was added to the re-suspended cells as three separate aliquots (1 mL/aliquot) at 10 min intervals to a final concentration of 0.01 M. The re-suspended cells were incubated at 37°C and 200 RPM for an additional 20 min to initiate removal of the outer membrane. After the incubation, cells were again harvested by centrifugation (5000g; 4°C; 10 min) and washed with ice cold SMM buffer (30 mL) for two times. This was followed by the re-suspension of the cells in ice cold SMM buffer (30 mL) containing lysozyme (2 mg/mL) and incubation at 37°C at 200 RPM for 1 h to allow digestion of the peptidoglycan layer (Dai et al., 2005; Reyes et al., 2012).

4.2.4.2 Protoplast fusion

Protoplasts were fused by mixing protoplasts from each of the 12 unique BMA tolerant strains. The volume of the protoplasts were added in proportion to their OD (600 nm) such that the final cell density will be equal for each strain (i.e. 0.5 mL for OD 1.0). Once the protoplasts were mixed, DNaseI (1 U) was added to digest DNA released from lysed cells and prevent transformation. The mixed protoplasts was incubated at 25°C for 10 min, then harvested by centrifugation (3000g; 4 °C; 20 min) and re-suspended in PEG buffer (0.5 mL). The re-suspended protoplasts in PEG buffer was incubated for another 30 min at 25°C to allow the fusion of the protoplasts (Dai et al., 2005; Reyes et al., 2012).

4.2.4.3 *Protoplast regeneration*

SMM buffer (1.0 mL) was added to the fused protoplasts in PEG buffer prior to harvesting by centrifugation (3000g; 4 °C; 20 min) and re-suspended in LB medium (0.5 mL) containing sucrose (0.5 M). Serial dilutions of the re-suspended fused protoplasts were immediately spread on soft LB plates (8 g/L agar) containing sucrose (0.5 M) using a plastic spreader and incubated at 37°C for 48 h (Dai et al., 2005; Reyes et al., 2012). Regenerated cells were recovered from plates by addition of LB medium (2.0 mL) to the plates followed by careful scraping for cell suspension. The cells in suspension were transferred to conical flasks (500 mL) with LB medium (100 mL) and incubated overnight at 37°C and 200 RPM. The overnight cultures were used to generate cryogenic stocks for future use.

4.2.5 BMA production

4.2.5.1 *Biotransformation*

An overnight culture of an *E. coli* strain carrying the plasmid pBAD-MMA050-ARA was inoculated (initial OD600 = 0.1) in Lund medium (65 mL) with glycerol (10 g/L) using a conical flask (250 mL) and incubated with aeration (0.1 L/h) at 37°C and 250 rpm. After 12 h of incubation, an aliquot (15 mL) was taken as pre-induction sample. The cells were induced with arabinose (50 µL) from a stock solution (20% w/v). After induction, stock solutions (initial concentration; volume added; final concentration) of $(NH_4)_2SO_4$ (20% w/v; 250 µL; 1 g/L), glycerol (40% w/v; 375 µL; 3 g/L), sodium 3-methyl-2-oxobutyrate (500 mM; 2.0 mL; 20 mM), and 1-butanol (250 mM; 1.0 mL; 5 mM) were added. Two further additions of 1-butanol (1.0 mL) from a stock solution (250 mM) were added at 1 h intervals to reach the desired total 1-butanol added (15 mM). Aliquote samples (13-14 mL) were taken 6 and 24 h after induction and was used to prepare samples for GC-MS analysis. Samples for GC-MS analysis were prepared by centrifugation of the aliquote from the biotransformation mixture at 10, 000 RPM for 10 min. The supernatant (10 mL) was mixed with an equal volume of ethyl acetate and mixed vigorously for 1 min. Aliquotes (1 mL) of the ethyl acetate phase was used sample for analysis.

4.2.6 Molecular biology methods

4.2.6.1 Primer design

Designed primers were checked for its melting temperature (Tm) and potential secondary structures with the aid of the OligoAnalyzer tool from Integrated DNA Technologies (US).

4.2.6.2 DNA/RNA extractions

Genomic DNA extraction

The buffers and binding column used for genomic extractions were all taken from the GenElute™ Bacterial Genomic DNA kit. A single colony from a cryogenic stock of the desired strain plated overnight was picked and inoculated in LB (10 mL) using a Falcon tube (50 mL) and incubated at 37°C and 200-250 RPM for 12-16 h. An aliquot (1.5-2.0 mL) of the overnight culture was transferred to a microcentrifuge tube (2.0 mL) and pelleted using a microcentrifuge (MiniSpin™, Eppendorf, Hamburg, Germany) at ≥12,000 g for 2 min. After the supernatant liquid was discarded, the cell pellet was resuspended in Lysis Solution T (180 µL) and followed by addition of RNase A (20 µL). After incubation at room temperature for 2 min, Proteinase K (20 g/L; 20 µL) was added to the suspended cells and incubated at 55°C for 30 min. This was followed by addition of Lysis solution C (200 µL) and incubation at 55°C for an additional 10 min. Ethanol (200 µL) was added to the lysed cells and inverted for 3-5 times to allow gentle mixing. A Column Preparation Solution (500 µL) was added to the binding column in a collection tube (2.0 mL) and centrifuged at ≥6,500 g for 2 min. After the flow through liquid was discarded, the lysate with ethanol was loaded to the binding column and centrifuged at ≥6,500 g for 2 min. The flow through liquid lysate was discarded and the Wash Solution (500 µL) was loaded to the column and centrifuged at ≥12,000 g for 2 min. The flow through of the wash solution was discarded and the column was centrifuged at ≥12,000 g for 2 min to allow removal of excess ethanol. The binding column was transferred to a fresh collection tube. Genomic DNA was eluted by addition of molecular biology grade water (50-100 µL) to the binding column and centrifugation at ≥12,000 g for 3 min.

Plasmid DNA extraction

The buffers and binding column used for plasmid DNA extractions were all taken from the GenElute™ Plasmid Miniprep kit. A single colony from a cryogenic stock of the strain carrying the desired plasmid plated overnight was picked and inoculated in LB (10 mL) using a Falcon tube (50 mL) and incubated at 37°C and 200-250 rpm for 12-16 h. An aliquot (1.5-2.0 mL) of the overnight culture was transferred to a microcentrifuge tube (2.0 mL) and pelleted using a microcentrifuge at ≥12,000 g for 2 min. After the supernatant liquid was discarded, the cell pellet was resuspended using the Resuspension Solution (200 µL). The resuspended cells were lysed by addition of the Lysis Solution (200 µL) and incubated for 5 min. The lysed cell suspension was neutralized by addition of the Neutralization Solution (350 µL) and centrifugation at ≥12,000 g for 10 min. A Column Preparation Solution (500 µL) was added to the binding column in a collection tube (2.0 mL) and centrifuged at ≥12,000 g for 2 min. After the flow through of the Column Preparation Solution, the supernatant of the neutralized cells was added to the binding column in a collection tube and centrifuged at ≥12,000 g for 2 min. The flow through of the lysate was discarded prior to addition of the Wash Solution (750 µL) to the binding column and centrifugation at ≥12,000 g for 2 min. The flow through of the wash solution was discarded and the column was centrifuged at ≥12,000 g for 2 min to allow removal of excess ethanol. The binding column was transferred to a fresh collection tube. Plasmid DNA was eluted by addition of molecular biology grade water (50 µL) to the binding column and centrifugation at ≥12,000 g for 3 min.

DNA extraction from agarose gel electrophoresis

The buffers and binding column used for DNA extractions from agarose gel were all taken from the QIAquick Gel Extraction Kit. The desired DNA fragment was excised from the agarose gel using a scalpel. The excised gel was weighed and dissolved by addition of Buffer QG (300 µL/ 100 mg of gel) and incubation at 50°C for 15-30 min. Once the gel was visibly dissolved, isopropanol (100 µL/ 100 mg of gel) was added to the solution and mixed gently. The gel solution was applied to QIAquick column in a collection tube (2.0 mL) and centrifuged at ≥12,000 g for 3 min. After the flow through of the solution was discarded,

Buffer PE (750 µL) was added to the QIAquick column in the same collection tube and centrifuged at ≥12,000 g for 2 min. The flow through was again discarded with the QIAquick column in the collection tube centrifuged for an additional 2 min to remove residual ethanol. The QIAquick column was transferred to a fresh collection tube (1.5 mL). DNA was eluted by addition of molecular biology grade water (10-50 µL) to the binding column and centrifugation at ≥12,000 g for 3 min.

RNA extraction

The mRNA extraction was handled by Ingenza Ltd (Roslin Innovation Centre, Edinburgh, UK) as part of a collaborative effort for the project with Lucite International. All buffers and binding column used for RNA purification were taken from the RNeasy® Mini Kit (Qiagen), while RNase-Free DNase Set (Qiagen) was used for removal of DNA. A single colony from a cryogenic stock of the desired strain plated in MSX agar plate overnight was picked and inoculated in MSX (10 mL) using a Falcon tube (50 mL) and incubated at 37°C and 200-250 rpm for 12-16 h. An aliquot of the overnight culture was inoculated in MSX (120 mL) for an initial OD600 of 0.05 using conical flasks fitted with Suba seals and incubated at 37°C and 200 rpm. A sample (50 mL) was taken into a Falcon tube (50 mL) when the OD600 of the culture reached 0.3 and kept on ice prior to RNA extraction. Immediately after the first sample was taken BMA (13 mL) was added to the flask cultures. The cultures were incubated for an additional 1 h prior to taking another sample for RNA extraction. Samples were processed immediately for RNA extraction.

The cells from the samples were harvested by centrifugation at 5000 g for 5 min at 4 °C. After the supernatant was discarded, the cell pellet was resuspended by addition of Buffer RLT (350 µL) followed by vortexing for 5-10 s. The suspension was transferred into a Safe-Lock tube (2 mL) that contains acid washed beads (25-50 mg) and vortexed vigorously for 5 min. The ruptured cell mixture was centrifuged at ≥12,000 g for 10 s with the resulting supernatant transferred to an RNase free microcentrifuge tube (2.0 mL). Ethanol (70 vol. %; 350 µL) was added to the supernatant and the mixture was gently mixed by pipetting. The lysate was transferred to an RNeasy spin column in a collection

tube (2 mL) and centrifuged at ≥8000 x g for 15 s. After the lysate flow through was discarded, Buffer RW1 (350 µL) was added to the RNeasy spin column in a collection tube (2 mL) and centrifuged at ≥8000 x g for 15 s. The wash solution flow through was discarded. A mixture of DNase I stock solution (27.2 Kunitz/µL; 10 µL) and Buffer RDD (70 µL) from the RNase-Free DNase Set was loaded into the RNeasy spin column membrane and incubated at room temperature for 15 min. After incubation, Buffer RW1 (350 µL) was loaded into the RNeasy column and centrifuged at ≥8000 x g for 15 s. The wash solution flow through was discarded, which was followed by addition of Buffer RPE to the RNeasy spin column (500 µL) and centrifugation at ≥8000 x g for 15 s. The flow through was discarded, followed by addition of fresh Buffer RPE (500 µL) and centrifugation at ≥8000 x g for 2 min. The RNeasy spin column was transferred to a fresh collection tube (2 mL) and centrifuged at ≥12000 x g for 2 min. The RNeasy spin column was transferred to another fresh collection tube (1.5 mL) and RNase free water (30-50 µL) was loaded directly to the spin column membrane. RNA was eluted by centrifugation at ≥8000 x g for 1 min.

4.2.6.3 PCR

General PCR reactions were performed by mixing the Reaction Buffer (5X; 10 µL), dNTPs (10 mM; 1µL), forward primer (10 µM; 2.5 µL), reverse primer (10 µM; 2.5 µL), template DNA (1-1000 ng), Q5 High-Fidelity DNA polymerase (0.5 µL), and nuclease free water (to 50 µL) using a PCR tube (0.2 mL). The PCR mixture in the tube were incubated at 98°C for 30 s (initial denaturation) in a thermocycler (Mastercycler®-Personal, Eppendorf, Hamburg, Germany). This was followed by sequential incubation at 98°C for 10 s, 50-72°C for 30 s/kb of desired amplification, and 72 °C for 30 s, which was repeated for 25-35 cycles. A final extension step was implemented by further incubation 72 °C for 5-10 min. The reaction was stopped by storing the PCR tubes at 4-10 °C.

4.2.6.4 Chemical transformation

Chemical transformation was used for introduction of plasmid into the *E. coli* strains used.

Preparation of competent cells

A single colony from a cryogenic stock of the desired strain plated overnight in LB (with antibiotics if necessary) was picked and inoculated in LB (10 mL; with antibiotics if necessary) using a Falcon tube (50 mL) and incubated at 37 °C and 200-250 rpm for 12-16 h. An aliquot of the overnight culture was inoculated in a fresh LB medium (50 mL; with antibiotics if necessary) at a starting OD600 of 0.1 using a baffled flask (250 mL) and incubated at 30 or 37 °C at 250 rpm. The cells were transferred to a Faclon tube (50 mL) when the OD600 reached 0.6-0.7 and placed on ice for at least 5 min. The ice cold cell culture were centrifuged at 5000 g and 4 °C for 10 min. After the supernatant was discarded, the cell pellet was resuspended in an ice cold $CaCl_2$ solution (0.1 M; 10 mL). The resuspended cells were placed on ice for 10 min prior to centrifugation at 5000 g and 4 °C for 10 min. The supernatant was discarded and the cell pellet was resuspended with $CaCl_2$ solution (0.1 M; 2 mL) (Dagert & Ehrlich, 1979).

Transformation of competent cells

An aliquot (200 µL) of the chemical competent cell was transferred into a sterile microcentrifuge tube (1.5 mL) and placed on ice. The plasmid (1-50 ng) was added to the chemical competent cells and the mixture was placed on ice for 30 min. The transformation mixture was then incubated in a water bath at 42 °C for 30 s and immediately placed on ice for 2 min. LB broth (1 mL) was added to the ice cold mixture and incubated at 30/37 °C and 250 rpm for 1-2 h. The transformed cells were selected by plating in LB agar plates containing appropriate antibiotics.

4.2.7 Bioinformatics

4.2.7.1 DNA/Amino acid sequence

The National Center for Biotechnology Information (NCBI) gene data bank (Maglott et al., 2005) was used as the database of the reference DNA sequence, while the universal protein (UniProt) knowledgebase (UniProt Consortium, 2018) was used as a database for the reference protein/amino acid sequence. The Translate tool from ExPASy: SIB

bioinformatics resource portal (Artimo et al., 2012) was used to determine the amino acid sequence from a given DNA sequence.

4.2.7.2 DNA/Amino acid sequence alignments

Alignment of DNA sequences was implemented using the BLASTN tool from NCBI, while the EMBOSS Water tool from the European Bioinformatics Institute (EMBL-EBI) (Kanz et al., 2005) was used for amino acid sequence alignments.

4.2.7.3 Genome sequencing

The Genomic DNA sequencing and data processing were handled by Edinburgh Genomics (The University of Edinburgh, Edinburgh, UK) as part of a Genomic DNA sequencing package service. Genomic DNA sequence was analyzed using MiSeq v2 150PE (Illumina®) to yield at least 11M + 11M reads per run.

Trimming

Reads were trimmed to a minimum length of 36 for quality at the 3' end with a threshold of 30 and adapter sequences of the Nextera XT kit (CTGTCTCTTATA) using Cutadapt version 1.12 (Martin, 2011).

Alignment and variant calling

Genome alignment and variant calling were undertaken with the Snippy pipeline to identify the difference in genomic DNA sequence between the isolated strains and parental strain/reference genome (*Escherichia coli* strain BW25113, assembly ASM75055v1, annotation version 34 from Ensembl) (Grenier et al., 2014). Snippy version 3 was used with a minimum of 10 reads covering each position, and 0.9 as the minimum fraction of the reads that must differ from the reference. VCFtools was used for any manipulation of VCF files not done from within Snippy (Danecek et al., 2011).

4.2.7.4 RNA sequencing

RNA sequencing and differential analysis were conducted by Edinburgh Genomics (The University of Edinburgh, Edinburgh, UK) as part of an RNA sequencing package service.

Trimming

Cutadapt1 (version cutadapt-1.9.dev2) (Martin, 2011) was used to trim the reads for quality at the 3' end with a quality threshold of 30 for adapter sequences of the TruSeq Stranded Total RNA with RiboZero rRNA Removal kit kit (AGATCGGAAGAGC). Reads after trimming had a minimum length of 50.

Alignment

The genome of *E. coli* BW25113 was used as reference for mapping, while annotations for *E. coli* K-12 group strain was used for counting (Baba et al., 2006; Grenier et al., 2014). STAR (version 2.5.2b) (Dobin et al., 2013) was used to align the reads to the reference genome. Paired-end reads were specified with the options –outSAMtype BAM Unsorted, while all other parameters were set as default.

Read counting by feature

The program featureCounts (version 1.5.1) (Liao et al., 2013) was used to read counts. The reads were assigned to features of type 'exon' in the input annotation grouped by gene_id in the reference genome. A minimum alignment quality of 10 was specified with the strandedness set to 'reverse'.

Count preprocessing

Filtering

Filtering of the raw counts was accomplished by removal of genes consisting mostly of virtually zero counts, filtering on counts per million (CPM) to avoid artefacts due to library depth. A row of the expression matrix was required to obtain values of no less than 0.1 in at least 3 samples. This corresponds to the smallest sample group as defined by Group, once any samples were removed.

Normalisation

Normalisation of the reads was achieved using the weighted trimmed mean of M-values method (Robinson & Oshlack, 2010) with 'TMM' as the method to the calNormFactors method of edgeR (version 3.16.5) (Robinson et al., 2010).

Analysis

Differential analysis

Differential expression of genes were calculated with the aid of edgeR (version 3.16.5) (Robinson et al., 2010) with the contrasts shown in Table 4.3. The default settings of edgeR was used to estimate the fold changes. Prior to fitting a model, a small read count in proportion to the library sized is added to avoid infinite fold changes in genes with zero or close to zero counts. The statistical assessment of differential expression was accomplished with the quasi-likelihood (QL) F-test using the contrast shown in Table 4.3.

Table 4.3 Contrasts specified for differential analysis

Group 1	Group 2
A_BW25113	A_RNM_2
A_BW25113	A_RNM_5
A_BW25113	A_RNM_18
A_BW25113	A_RNM_21
A_BW25113	A_RNM_22
A_BW25113	A_RNM_23
A_RNM_2	B_RNM_2
A_RNM_5	B_RNM_5
A_RNM_18	B_RNM_18
A_RNM_21	B_RNM_21
A_RNM_22	B_RNM_22
A_RNM_23	B_RNM_23

Notes: A – Prior to BMA addition, B – After BMA addition.

Venn diagrams

From the differential analysis, differentially expressed genes were sorted according to their fold changes with respect to reference strain or state. Genes with log-fold change of 1 and -1, were considered as up and down regulated genes, respectively provided that they have a p-value of <0.05 (Yung et al., 2016).

Gene list comparison

Gene lists were compared using the Multiple List Comparator tool from molbiotools.com.

Enrichment analysis

In order to facilitate functional characterization and understanding of how the differentially expressed genes relate to cellular process, function, component, and regulatory networks enrichment analysis were performed. Enrichment analysis for the differentially expressed genes belonging to the Gene Ontology (GO) (Ashburner et al., 2000; The Gene Ontology Consortium, 2018) terms biological process, molecular function, and cellular component were achieved with the Cytoscape plug-in ClueGo (version 2.5.4) (Bindea et al., 2009) , while enrichment analysis for the differentially expressed genes belonging to regulatory networks were implemented in FunRich (version 2.1.1) (Pathan et al., 2015).

Heat maps

Heat maps were generated using the GENE-E (Broad Institute) to facilitate visualization of the changes in gene expression.

Genome sequence viewing

The complete genome sequencing results visualization was aided by the use of the Integrative Genomics Viewer from Broad Institute (Robinson et al., 2011).

4.2.8 Analytical Methods

4.2.8.1 UV-Vis Spectrophotometry

Growth of *E. coli* strains was monitored by measuring the absorbance of the culture at 600 nm (OD600) using a spectrophotometer (UV mini 1240, Shimadzu).

4.2.8.2 Gas Chromatography with Mass Spectrometry (GC-MS)

Butyl methacrylate, butyl acetate, butyl isobutyrate, and butyl isovalerate were detected and quantified using a gas chromatograph (Agilent 7890 A, Agilent Technologies, Santa Clara, CA, USA) equipped with an electron ionization source, an inert mass selective detector (MSD) , a quadrupole mass analyser (Agilent 5975C Agilent Technologies, Santa Clara, CA, USA), and an Agilent 19091s-433 column (30 m x 250 µm x 0.25 µm). Helium at flowrate of 1.1971

mL/min was used as a carrier gas. Samples (1µL) were injected using an automated liquid sampler with a split ratio of 10:1. The inlet temperature was set at 280°C. The temperature of the oven was initially set at 45 °C for 5 min and was increased to 300 °C at a rate of 20 °C/min. The final temperature was kept constant for 10 min. To improve the sensitivity for detection of the butyl esters, the most characteristic ions were monitored in selected ion monitoring (SIM) mode with a cutoff value of 50 m/z. Amount of the butyl esters was estimate from a standard calibration curve (Appendix Figures 11.12-11.15).

4.2.8.3 Agarose gel electrophoresis

The size and concentration of DNA fragments were estimated using the agarose gel electrophoresis with Quick-Load Purple 1 kb Plus DNA ladder as reference. A typical gel electrophoresis involved casting a melted agarose gel onto a cast with the desired well combs and the gel staining agent SYBR Safe (5-10 µL). Once the gel has fully solidified, the well combs were carefully removed and the gel was transferred to the electrophoresis cell containing enough TAE buffer to fully soak the gel (350-400 mL). The DNA ladder (1.5-2.0 µL) and samples (5-25 µL) were loaded to the wells. A typical agarose gel electrophoresis run was at 90 V for 45-50 min (Powerpac, Bio-Rad, California, USA).

4.2.8.4 DNA sequencing

All of the DNA sequence were analysed through Sanger method of DNA sequencing (Sanger et al., 1977) by Eurofins Genomics (Ebersberg, Germany). The purified DNA sample (15 µL) was combined with a primer (2 µL) in a microcentrifuge tube (1.5 mL). Samples were sent *via* the TubeSeq Service of Eurofins Genomics.

5

Generation of BMA tolerant *E. coli* strains *via* adaptive evolution

5.1 Introduction

A previous work investigated the toxicity of BMA in *E. coli* using glass vials containing BMA (Disley, 2018). In the first 24 h, no growth was observed in the vial containing 20% v/v BMA. Surprisingly, growth was observed after the culture was left in the incubator for an additional 48 h. This led to the isolation of strains with the ability to grow in a glass vial containing BMA (20% v/v). However, these strains were unable to grow in a well-mixed environment containing BMA (20% v/v) (Personal communication from Ingenza Ltd. and Lucite International). Thus, a more robust strain has to be developed. In order to generate more robust strains, adaptive evolution (ADE) through sequential batch cultures and continuous cultures was used as an initial approach.

5.2 Effect of BMA concentration of cell growth

In order to get a rough idea on the *E. coli* tolerance for BMA and BMA concentrations that can be used to initiate the adaptive evolution experiments, the effect of BMA at various concentrations on the growth of *E. coli* was investigated. This was done by adding exogenous BMA to the growth medium at increasing concentrations from 0.01% to 20% v/v (Fig. 5.1). Growth of *E. coli* with 0.01% and 0.05% v/v BMA was very similar to its growth in the absence of BMA, but with a slight decline in its maximum growth rate (Fig. 5.1 and Table 5.1). As the BMA concentration was increased further to 0.1% v/v a decline in cell density followed by growth after a long lag (18 h) was observed (Fig. 5.1 and Table 5.1), which suggests that BMA inhibits the growth of *E. coli* at 0.1% v/v or higher. As the exogenous BMA concentration was raised further to 0.5-20% v/v (Fig. 5.1), cell density declined and no growth was observed after 36 h of incubation. This suggests that *E. coli* growth was completely inhibited with BMA at 0.5-20% v/v.

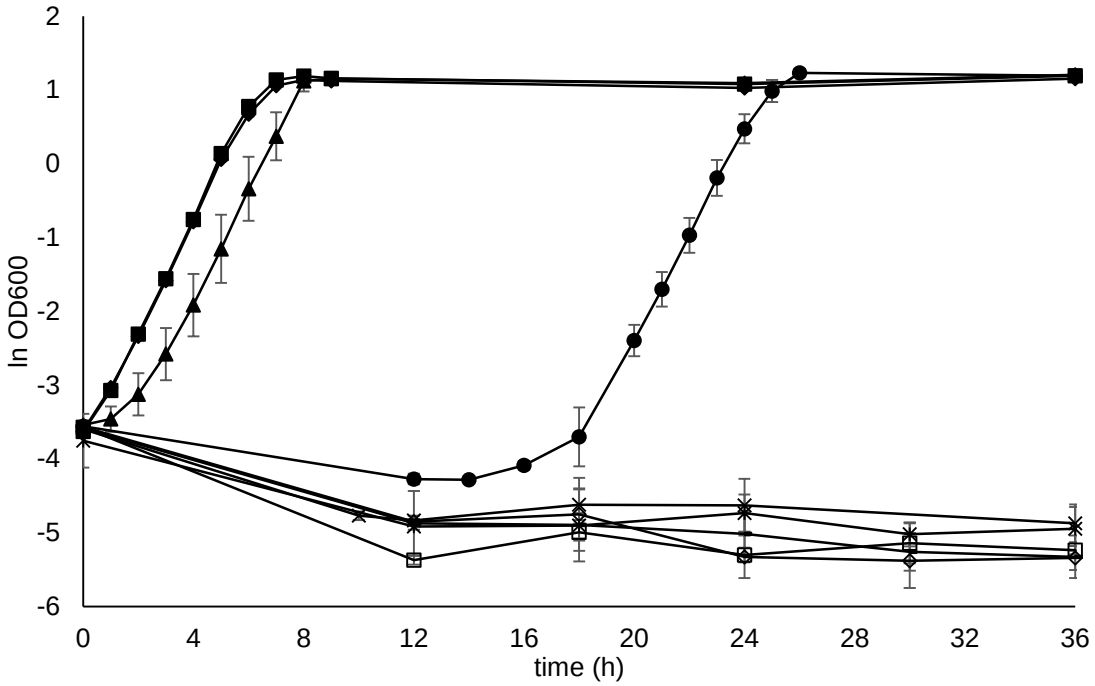

Figure 5.1 Effect of BMA concentration on the growth of *E. coli*. Growth of *E. coli* in M9 minimal medium (50 mL) containing BMA at 0% (■), 0.01% (♦), 0.05% (▲) 0.1% (●), 0.5% (x), 1.0% (+), 5.0% (-), 10% (□), and 20% (◊) v/v in conical flasks (250 mL) at 37°C and 200 RPM.

Table 5.1 Growth kinetic parameters of *E. coli* at various BMA concentrations

BMA content (% v/v)	Max cell density (OD at 600nm)	Growth rate (/h)	Lag phase (h)
0	3.303 ± 0.149	0.783 ± 0.016	1
0.01	3.173 ± 0.055	0.758 ± 0.001	1
0.05	3.377 ± 0.257	0.724 ± 0.009	1
0.1	3.441 ± 0.187	0.725 ± 0.003	18

5.3 Adaptive evolution

5.3.1 Adaptive evolution in sequential batch cultures

Two short ADE experiments (ADE-1 and ADE-2) were used as an initial attempt to generate *E. coli* strains with tolerance for BMA at 20% v/v. In ADE-1 (Fig. 5.2), three separate cultures were grown in parallel. The cultures were transferred sequentially in parallel, while the BMA concentration was increased after each sequential transfer. ADE-2 was completed in a similar manner with ADE-1, except that the culture with highest cell density was used as starting culture for the subsequent cultures (Fig. 5.3). In both cases (ADE-1 and ADE-

2), cell growth was observed for each of the sequential cultures at all BMA concentrations (Figs. 5.2 and 5.3).

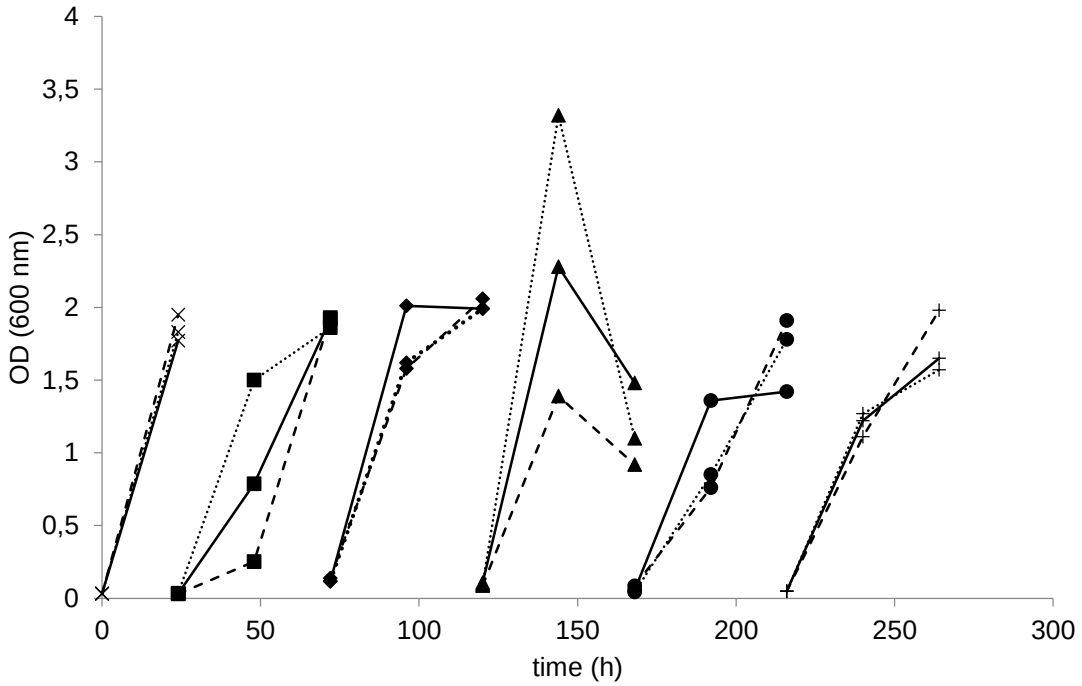

Figure 5.2 ADE-1. Adaptive evolution in serial batch cultures with sequential increases in BMA concentration. *E. coli* was grown in M9 minimal medium (10 mL) with BMA at 37°C and 200 RPM in 3 parallel FALCON® tubes (50 mL); tube 1 (Solid Line), tube 2 (dotted line), and tube 3 (dashed line). BMA concentration was increased in each sequential transfer at 0.1% BMA (x), 0.5% BMA (■), 1 % BMA (♦), 5 % (▲), 10% (●), and 20% (+) using each separate tube as seed culture for subsequent transfer.

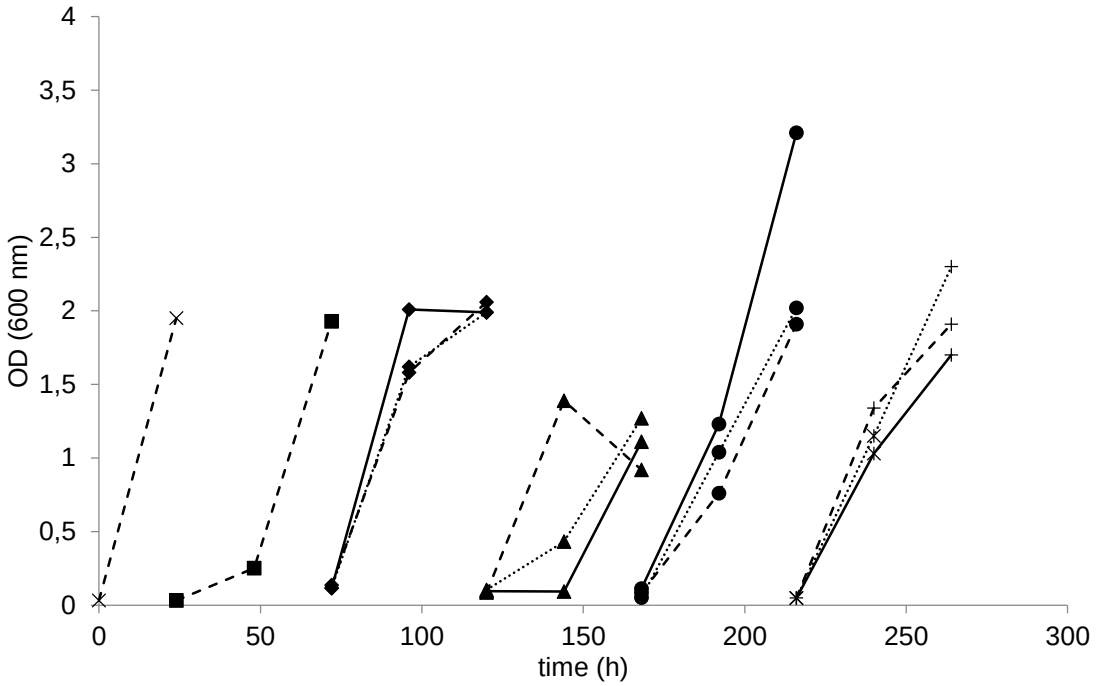

Figure 5.3 ADE-2. Adaptive evolution in serial batch cultures with sequential increases in BMA concentration. *E. coli* was grown in M9 minimal medium (10 mL) with BMA at 37°C and 200 RPM in 3 parallel FALCON® tubes (50 mL); tube 1 (Solid Line), tube 2 (dotted line), and tube 3 (dashed line). BMA concentration was increased in each sequential transfer at 0.1% BMA (x), 0.5% BMA (■), 1 % BMA (♦), 5 % (▲), 10% (●), and 20% (+) using culture with highest cell density as starting culture for each of the tubes.

In an attempt to generate BMA-tolerant *E. coli* strains with better fitness and more diversity, a longer evolution experiment (ADE-3) was performed (Dragosits & Mattanovich, 2013). ADE-3 (Fig. 5.4) was done in a similar way as ADE-1 (Fig. 5.2), but with 5 serial transfers at the same BMA concentration before subsequent culture to a medium with higher BMA content. The increase in the number of sequential transfers corresponds to a greater number of generations for evolution, which could facilitate selection and enrichment of fitter BMA tolerant strains (Atwood et al., 1951; Barrick et al., 2009; Dragosits & Mattanovich, 2013; Elena & Lenski, 2003). As with the short evolution experiments (ADE-1 and ADE-2), cell growth was observed in ADE-3 for every serial transfer at all BMA concentrations tested (Fig. 5.4).

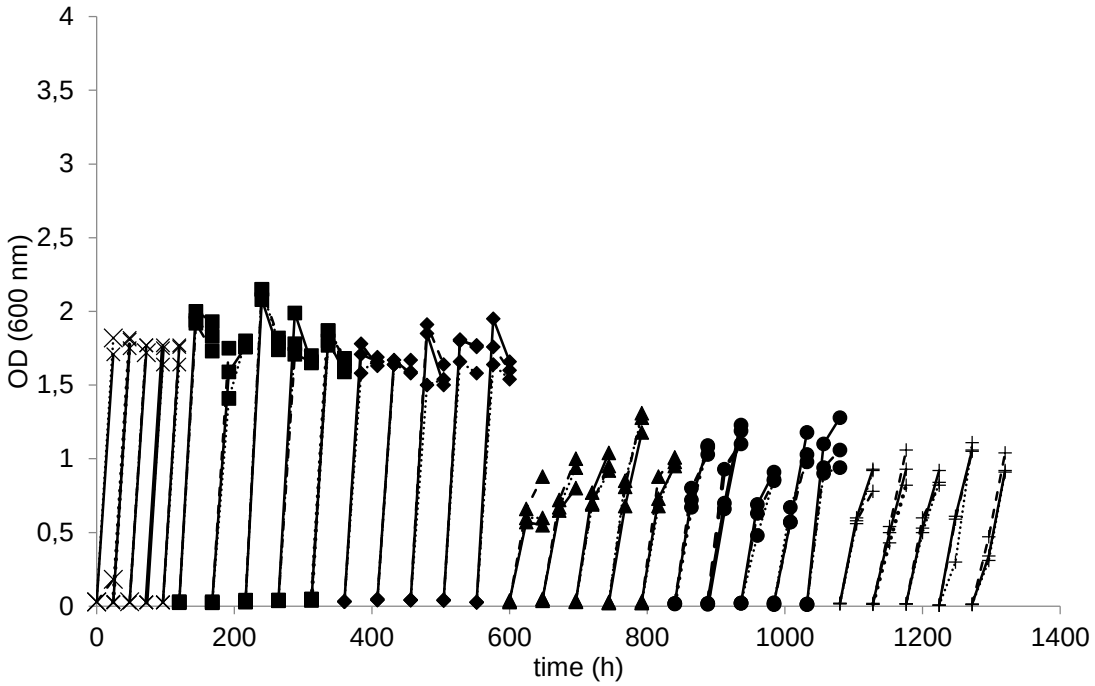

Figure 5.4 ADE-3. Adaptive evolution in serial batch cultures with 5 sequential transfers at each BMA concentration prior to increase. *E. coli* was grown in M9 minimal medium (10 mL) with BMA at 37°C and 200 RPM in 3 parallel FALCON® tubes (50 mL); tube 1 (Solid Line), tube 2 (dotted line), and tube 3 (dashed line). BMA concentration was gradually increased to 0.1% BMA (x), 0.5% BMA (■), 1 % BMA (♦), 5 % (▲), 10% (●), and 20% (+) using each separate tube as seed culture for subsequent transfer.

Another long term evolution experiment for the generation of BMA tolerant was performed (ADE-4). It was initially used to test whether or not a culture previously grown with 0.1% v/v BMA will grow well when subcultured to a medium containing 10% v/v BMA (Fig. 5.5). Considerable growth was observed after 24 h, which suggests that the previous culture may have contained strains that could tolerate BMA at 10% v/v or strains that were able to evolve and obtain tolerance for 10% v/v BMA (Cairns & Foster, 1991; Foster, 1993; Luria & Delbrück, 1943; Rosenberg, 2001). After another sequential transfer to a medium with 10% v/v BMA, the culture reached a higher cell density after 24 h than the previous culture. The experiment was further continued to investigate whether or not a superior BMA tolerant strain can be generated from an extended evolution in the presence of 20% v/v BMA with the use of the culture with the highest cell density as inoculum for the subsequent culture (Barrick et al., 2009; Blount et al., 2008; Dragosits & Mattanovich, 2013; Elena & Lenski,

2003). This was accomplished by transferring the culture grown at 10% v/v BMA for forty five sequential transfers in a medium containing 20% v/v BMA (Fig. 5.5).

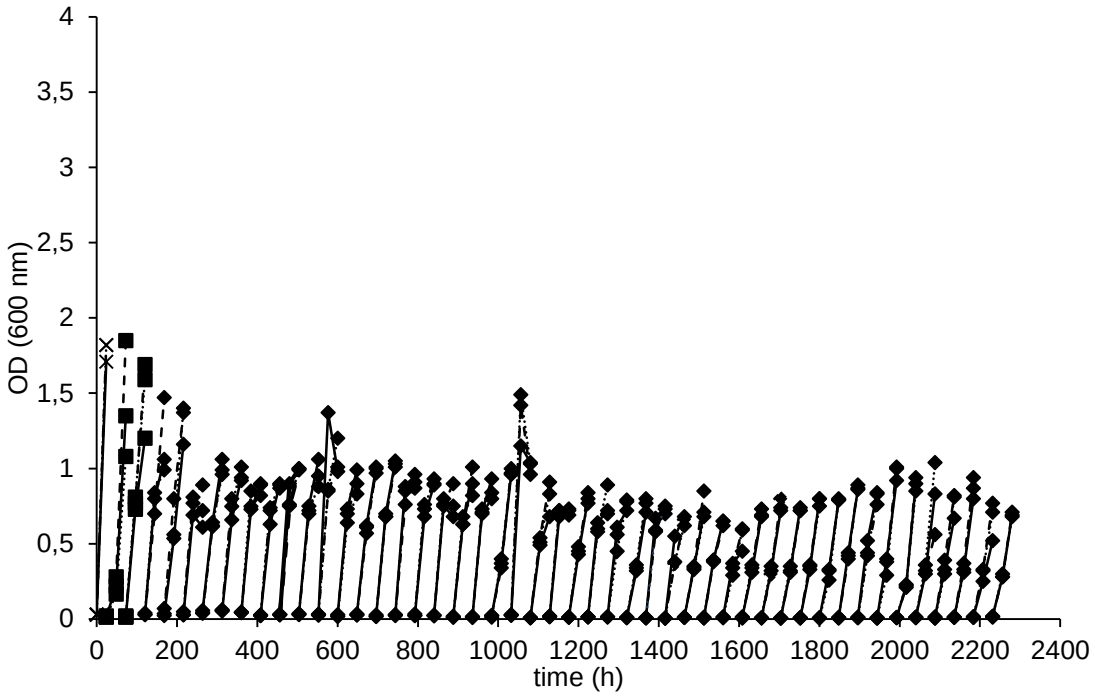

Figure 5.5 ADE-4. Adaptive evolution in serial batch cultures with 1, 2, and 45 sequential transfers at 0.1%, 10%, and 20% v/v BMA, respectively. *E. coli* cultures were grown in M9 minimal medium (10 mL) with BMA at 37°C and 200 RPM in 3 parallel FALCON® tubes (50 mL); tube 1 (Solid Line), tube 2 (dotted line), and tube 3 (dashed line). BMA concentration was increased from 0.1% BMA (x) to 10% BMA (■) and 20% BMA (♦) using culture with highest cell density as starting culture for each of the tubes.

5.3.2 Adaptive evolution in continuous cultures

An evolution experiment was also performed in a stirred bioreactor (Fig. 2.1) in a continuous culture (ADE-5) to generate more BMA tolerant *E. coli* strains and potentially expand their diversity. The use of a stirred bioreactor would allow increased dispersion of BMA throughout the culture and be more comparable to an actual industrial bioprocess as compared to the set-up used for evolution in sequential batch transfers (Kadic & Heindel, 2014). A chemostat culture, one of the most widely used mode of continuous culture for adaptive evolution (Dragosits & Mattanovich, 2013; Manch et al., 1999; Notley-McRobb & Ferenci, 1999; Reyes et al., 2012; Weikert et al., 1997), was applied to generate *E. coli* strains with tolerance for BMA at 20% v/v.

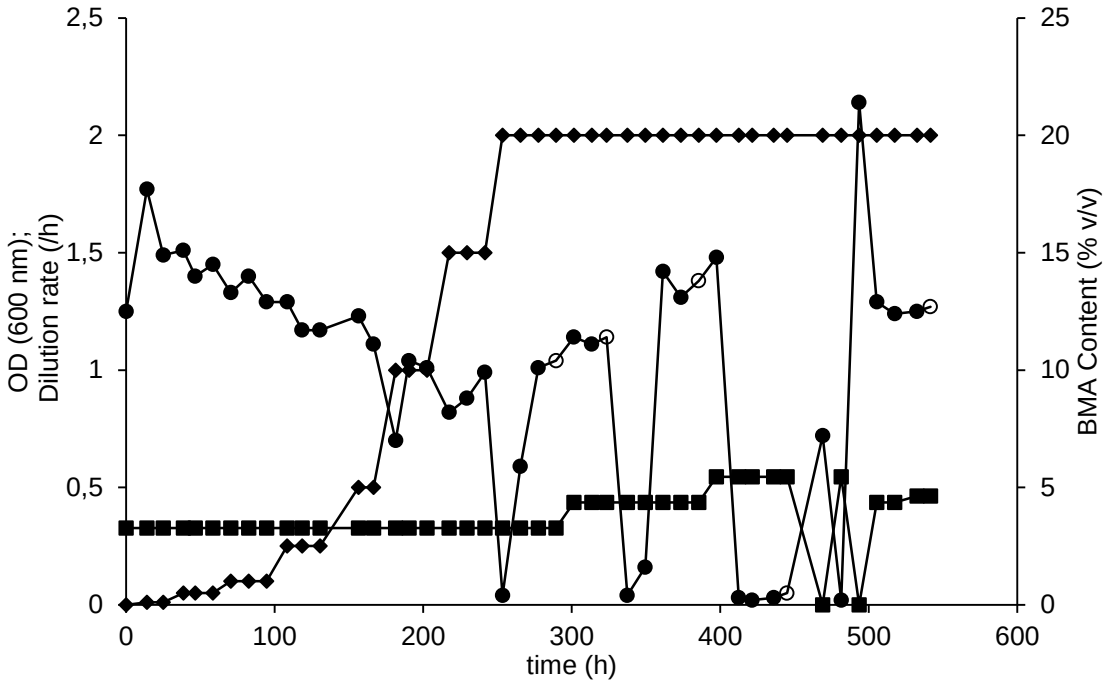

Figure 5.6 ADE-5. Adaptive evolution and selection of BMA tolerant E. coli in a chemostat. A Chemostat culture of *E. coli* in a mini-bioreactor (55 mL working volume) was grown in M9 minimal medium containing 1 g/L glucose at 37°C and aeration rate of ~ 0.3 L/h with BMA concentration (♦) gradually increased from 0 to 20% v/v and dilution rate (■) varied from 0.33 to 0.55 /h. Cell density (●) was reported as OD at 600 nm. Samples for strain isolation were taken at various points (○).

ADE-5 was established at a starting dilution rate of 0.33 /h (Fig. 5.6). The BMA concentration was gradually increased in a step-wise manner from 0 to 20% v/v under a constant dilution rate. A stable cell density was achieved from 0 to 5% BMA, but the cell density declined with further increases in BMA concentration to 10% and 20% v/v. A dip in cell density occured as the BMA concentration was increased but recovered within 24-36 h. The occasional dip in cell density observed after increase in BMA concentration is likely due to inhibition of BMA at the increased concentration to cell growth, which could have facilitated selection of strains with the ability to tolerate BMA at the exposure concentration and grow at a rate equal to or greater than that of the dilution rate (Dragosits & Mattanovich, 2013; Dykhuizen & Hartl, 1983; Harder et al., 1977).

After a stable cell density was attained at 20% v/v BMA, the dilution rate of the chemostat culture was increased between 0.33 and 0.55 /h to select for fast growing strains (Dykhuizen & Hartl, 1983; Harder et al., 1977; Weikert et al.,

1997). A huge decline in cell density was observed with every increase in dilution rate until 0.46 /h, but recovered within 12-36 h (Fig. 5.7). However, once the dilution was further elevated to 0.55 /h, the culture washed out and did not recover even after 36h, so the feed media and BMA flow into the bioreactor were stopped to allow recovery.

Once the cell density recovered to the previous level, the medium and BMA feed was started again (dilution rate 0.55 /h). However, the culture washed out again. Once more, the cells were allowed to grow and recover to its previous cell density. The dilution rate was adjusted to 0.44 /h and 0.46 /h before the evolution experiment was halted. The chemostat culture was stopped due to a recurring blockage at the effluent side arm, which was likely caused by the polymerized BMA. Nevertheless, it is apparent that the critical dilution rate of the evolved population grown with 20% v/v BMA is between 0.46 and 0.55 /h (Dykhuizen & Hartl, 1983; Harder et al., 1977). BMA tolerant *E. coli* strains from the selected population were isolated from samples taken at the various dilution rates tested for further characterization.

5.4 Growth characterization of BMA tolerant strains

Individual colonies were isolated by plating onto LB plates an aliquot sample from each of the ADE experiments and from a flask culture with 20% v/v BMA from preliminary growth tests for the parental strain where growth was observed (not shown). Single colonies were picked from the plates and stored for further characterization. The individual BMA tolerant isolates were grown in the presence of 20% v/v BMA to assess the growth characteristics of each strain and allow comparison of fitness under BMA stress. Growth curves (Appendix Figures 11.3, 11.4, and 11.5) were used to estimate the growth characteristics of each strain (Table 5.2) (Kovárová-Kovar & Egli, 1998).

Table 5.2 Growth kinetic parameters of BMA tolerant strains from various ADE experiments

Strain; % BMA (v/v)	Max Cell Density (OD at 600 nm)	Growth Rate (/h)	Lag time (h)	Source
Wild Type; 0	3.793 ± 0.561	0.728 ± 0.011	2	(Grenier et al., 2014)
LM-2 ; 20	0.883 ± 0.006	0.578 ± 0.050	4	(Disley, 2018)
RNM-2 ; 20	1.720 ± 0.137	0.532 ± 0.028	6	F.C.
RNM-3 ; 20	2.087 ± 0.200	0.566 ± 0.010	4	ADE-1
RNM-5 ; 20	1.576 ± 0.255	0.625 ± 0.052	5	ADE-3
RNM-6 ; 20	1.553 ± 0.076	0.620 ± 0.029	4	ADE-3
RNM-7 ; 20	1.500 ± 0.132	0.613 ± 0.033	4	ADE-3
RNM18 ; 20	2.733 ± 0.091	0.709 ± 0.015	5	ADE-4
RNM-8 ; 20	1.150 ± 0.026	0.712 ± 0.015	5	ADE-5
RNM-19 ; 20	1.727 ± 0.060	0.701 ± 0.007	5	ADE-5
RNM-20 ; 20	1.557 ± 0.031	0.719 ± 0.041	5	ADE-5
RNM-21 ; 20	1.157 ± 0.057	0.703 ± 0.030	5	ADE-5
RNM-22 ; 20	1.382 ± 0.066	0.701 ± 0.015	4	ADE-5
RNM-23 ; 20	1.396 ± 0.061	0.722 ± 0.019	4	ADE-5

Note: F.C. – a flask culture of *E. coli* BW25113 with 20% v/v BMA

Preliminary flask culture experiments with 20% BMA yielded a number of tolerant strains. Of these, strain RNM-2 was the best growing strain and was able to grow in the presence of 20% BMA but with lower cell density, lower growth rate, and longer lag time as compared to the parental strain grown without BMA (Table 5.2). The BMA tolerant strains, RNM-3 and RNM-4, isolated from ADE-2 had similar growth rates as RNM-2, but achieved higher cell densities and shorter lag times (Table 5.2). Strains RNM-5, RNM-6, and RNM-7 were isolated from ADE-3, with the longer evolution period as compared to the ADE-2 isolates, and exhibited a comparable cell density to RNM-2. The isolates from ADE-3 were able to grow with similar lag times but with superior growth rates as compared to RNM-2 and the strains isolated from ADE-2.

The BMA tolerant strain taken from ADE-4, which was the longest evolution experiment (Fig. 5.5), was able to achieve the highest cell density and maximum growth rates among the batch culture-ADE isolates. This suggests that longer ADE may have facilitated further evolution, selection, and enrichment of superior BMA tolerant strains (Barrick et al., 2009; Blount et al., 2008; Dragosits & Mattanovich, 2013; Elena & Lenski, 2003). Isolates from the chemostat evolution (ADE-5) grown with 20% v/v BMA were able to achieve very high growth rates, which were comparable to the parental strain grown in

the absence of BMA. However, these strains attained lesser cell densities as compared to the parental strain grown in the absence of BMA and the isolates from ADE-1 and ADE-4 (Table 5.2).

In comparison to the BMA tolerant *E. coli* strain isolated from a prior study (Disley, 2018), the BMA tolerant strains generated in this study *via* adaptive evolution were all able to reach a higher cell densities when grown in the presence of BMA (20% v/v). Growth rate-wise, the strains generated from ADE-4 and ADE-5 (chemostat culture) were superior.

In general, the BMA tolerant strains grown in the presence of 20% v/v BMA had longer lag times, lower cell densities, and growth rates lower or comparable to the parental strain grown without BMA (Table 5.2). Some of the isolates had very similar growth patterns and kinetic parameters, which could be due to isolation of an exactly the same strains, strains with similar changes/mutations, or completely different strains just growing in a similar manner (Atsumi et al., 2010; Barrick et al., 2009; Elena & Lenski, 2003; Lennen et al., 2019; Minty et al., 2011). The contrasting attributes of the BMA tolerant strains likely resulted from the difference in modes of evolution, selection, and enrichment (Dragosits & Mattanovich, 2013). In the sequential batch culture ADE, cells were allowed to grow until stationary phase prior to sequential transfer and isolation. Thus, likely to give a higher probability of selecting tolerant strains with ability to achieve higher cell densities. On the other hand, ADE in chemostat culture provided a selection based on growth rate, allowing cells with maximum growth rates equal to or higher than the dilution rate to remain in the culture (Dragosits & Mattanovich, 2013; Dykhuizen & Hartl, 1983; Fraleigh et al., 1989; Harder et al., 1977).

5.5 Genomic DNA sequencing

In order to get an idea of the genetic basis of the enhanced tolerance for BMA in the isolated strains and potentially clues on the mechanism of BMA tolerance, the genomic DNA sequence of the BMA tolerant strains generated from the ADE experiments were analysed and compared to the parental strain *E. coli* BW25113 (Tables 5.3). Each of the BMA tolerant strains had at least two alterations in its genomic DNA sequence, confirming that all had acquired

mutations during the adaptive evolution process (Atsumi et al., 2010; Barrick & Lenski, 2013; Dragosits & Mattanovich, 2013; Foster, 1993; Gordo & Sousa, 2010; Luria & Delbrück, 1943; Minty et al., 2011; Rosenberg, 2001; Royce et al., 2013a). The mutations acquired include point mutations, indels (insertions/deletions), and deletions that caused silent, missense, frameshift, and nonsense mutations in the affected genes (Griffiths et al., 1999). Except for the indels, the effect on the amino acid sequence of the proteins coded by the affected genes on the mutations acquired were also determined using a DNA to the amino acid sequence translation tool ExPASy (Artimo et al., 2012) (Appendix Tables 11.4 and 11.5).

Table 5.3 List of mutations acquired by the BMA tolerant strains

Strain	Mutations acquired	Source
RNM-2	*acrR*(E91fs) *rob*(R156H) *rpoC*(L361R) *ilvN*(C41Y) *ygbK*(A294E) *lpxM*(267_272 del) *ompT*(indels)	F.C.
RNM-3	*acrR*(E91fs) *rob*(R156H) *ilvN*(C41Y) *phoP*(L11F) *acrB*(V448L) *icd*(398Dfs) *ΔymfD ΔymfE Δlit ΔintE ΔxisE Δymf lΔymfJ ΔcohE ΔcroE ΔymfL ΔymfM ΔoweE ΔaaaE ΔymfR ΔbeeE ΔjayE ΔymfQ ΔstfP ΔtfaP ΔtfaE ΔstfE ΔpinE ΔmcrA yhdE*(indels)	ADE-1
RNM-5	*acrR*(N214fs) *soxR*(L139X) *mscK*(indels) 580116(G>T)	ADE-3
RNM-6	*acrR*(Y77fs) *soxR*(A146del)	ADE-3
RNM-7	*acrR*(E91fs) *rob*(A70V) *ompT*(indels)	ADE-3
RNM-18	*acrR*(A191fs) *rob*(A70T) *creA*(V85V) *yohJ*(L109R) *dnaK*(V377G) 927777(C>T) *cra*(I270fs) *clsA*(A448fs) *rpoC*(212_217 del) *opgH*(R95P) *cpxA*(P177Q) *ompX*(indels) *atpI*(indels) Δ*psuT ΔpsuG ΔpsuK ΔfruA ΔfruK ΔfruB ΔsetB*	ADE-4
RNM-8	*acrR*(K53fs) *rob*(R156H) *rpoB*(T1037P) *torY*(A87T)	ADE-5
RNM-19	*acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *ompR*(R15S) *acrB*(T379I) *yieL*(indels) *mioC*(indels)	ADE-5
RNM-20	*acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *ompR*(R15S) *yhiN*(indels) *pitA*(indels)	ADE-5
RNM-21	*acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *rpoC*(A787V) *ompR*(R15S) *acrB*(V901I) *ΔyhhJ ΔrbbA ΔyhiI ΔyhiJ ΔyhiL ΔyhiM ΔyhiN ΔpitA*	RNM-21
RNM-22	*acrR*(K53Yfs) *rob*(R156H) *rpoB*(T1037P) *groL*(P279L) *torY*(A87T) 1197659(C>A)	ADE-5
RNM-23	*acrR*(E74fs) *soxR*(R20L) *rpoC*(R1075C) *torY*(A87T) 2133236(T>A) 3915915(T>G)	ADE-5

Note: F.C. – a flask culture of *E. coli* BW25113 with 20% v/v BMA

5.5.1 Mutations in strain RNM-2

Strain RNM-2 acquired point mutations in *rob*, *rpoC*, *ilvN*, and *ygbK,* an insertion mutation in *acrR*, an 18 bp deletion in *lpxM* , and an indel in *ompT* (Table 5.3 and Appendix Tables 11.3, and 11.4). The point mutations observed in *rob*, *rpoC*, *ilvN*, and *ygbK* caused a single amino acid change at residue 156 (Arg→His), 361 (Leu→Arg), 41 (Cys→Tyr), and 294 (Ala→Glu) for Rob, RpoC, IlvN, and YgbK, respectively. The insertion mutation in *acrR* led to a change in amino acid sequence starting residue 91 and resulted in truncation of the protein at residue 101, instead of residue 215. The deletion mutation in *lpxM* caused removal of amino acid residues 267-272 but left the remaining amino acid sequence the same as the WT protein (Appendix Tables 11.3 and 11.4).

5.5.2 Mutations in strain RNM-3

In strain RNM-3, point mutations in *rob*, *ilvN*, *phoP*, and *acrB*, an insertion mutation in *acrR*, an 15,096 bp deletion that affected the genes *icd*, *ymfD*, *lit*, *intE*, *ymfI*, *cohE*, *ymfL*, *aaaE*, *beeE*, *ymfQ*, *tfaP*, *stfE*, *pinE*, and *mcrA*, and an indel mutation in *yhdE* were observed. The point mutations in *rob*, *ilvN*, *phoP*, and *acrB*, caused a single amino acid change at residue 156 (Arg→His), 41 (Cys→Tyr), 11 (Leu→Phe), and 448 (Val→Leu) for Rob, IlvN, PhoP, and AcrB, respectively. Insertion mutation in *acrR* caused a change in amino acid sequence starting residue 91 and resulted in truncation of the protein at residue 101, instead of residue 215. On the other hand, the 15,096 bp deletion affected the last 58 bp of icd and caused the removal of the entire gene of *ymfD*, *lit*, *intE*, *ymfI*, *cohE*, *ymfL*, *aaaE*, *beeE*, *ymfQ*, *tfaP*, *stfE*, *pinE*, and *mcrA* (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.3 Mutations in strain RNM-5

Strain RNM-5 gained a point mutation in *soxR*, an insertion mutation in *acrR*, an indel in *mscK*, and a nucleotide base change at the intergenic region in the position 580116 (G→T) (Grenier et al., 2014). The point mutation in *soxR* caused a change in a single amino acid at residue 139 (Val→Leu), while the insertion mutation in *acrR* caused a change in amino acid starting residue 214 and increased the length of AcrR to 249 residues. The change in nucleotide base at position 580116 affected the intergenic region 230 bp downstream and

19 bp downstream of *appY* and *ompT*, respectively (Table 5.3 and Appendix Tables 11.3, 11.4, and 11.5).

5.5.4 Mutations in strain RNM-6

A 3 bp deletion mutation in *soxR* and an insertion mutation in *acrR* were observed in strain RNM-6. The 3 bp deletion in *soxR* resulted in the removal of residue 146 (Ala) without changing the succeeding sequence. On the other hand, the insertion mutation in *acrR* caused a change in amino acid sequence starting residue 77 that resulted in a truncation at residue 84 for resulting protein (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.5 Mutations in strain RNM-7

The strain RNM-7 acquired a point mutation in *rob*, an insertion mutation in *acrR*, and an indel mutation in *ompT*. The point mutation in *rob* caused a change in a single amino acid at residue 70 (Ala→Val) for Rob, whilst the insertion mutation in *acrR* caused a change in amino acid sequence starting residue 91 that resulted in the truncation of the resulting protein at residue 96 (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.6 Mutations in strain RNM-18

Strain RNM-18 obtained point mutations in *rob*, *creA*, *yohJ*, *dnaK*, *opgH*, and *cpxA*, 1 bp deletion mutations in *acrR*, *cra*, *clsA*, an 18 bp deletion in *rpoC*, and indels in *ompX* and *atpI*. It also acquired an 8136 bp deletion mutation that affected the genes *psuT*, *psuG*, *psuK*, *fruA*, *fruK*, *fruB*, and *setB* and a nucleotide change in the DNA sequence at position 92777 (C→T). The point mutations in *rob*, *yohJ*, *dnaK*, *opgH*, and *cpxA* caused a single amino acid change at residue 70 (Val→Thr), 109 (Leu→Arg), 377 (Val→Gly), 95 (Arg→Pro), and 177 (Pro→Gln) for Rob, YohJ, DnaK, OpgH, and CpxA, respectively. In the case of the point mutation in *creA*, it did not cause a change in the amino acid residue for the affected region. The 1 bp deletion mutations in *acrR*, *cra*, and *clsA* led to a change in amino acid sequence starting residue 191 and increase in amino acid residues to 243, change in amino acid sequence starting at residue 270 and truncation at residue 319, and a change in amino acid sequence starting residue 448 and truncates at residue 465 for

AcrR, Cra, and ClsA, respectively. On the other hand, the 18 bp deletion in *rpoC* caused the removal amino acid residues at 211-217, while keeping the subsequent amino acid sequence unchanged. The 8,136 bp deletion caused the removal of the first 446 bp of *psuT* and deletion of the entire gene of *psuG*, *psuK*, *fruA*, *fruK*, *fruB*, and *setB*. The change in nucleotide base at position 927777 (C→T) affected the intergenic region 271 bp upstream and 274 bp upstream of *lrp* and *trxB*, respectively (Table 5.3 and Appendix Tables 11.3, 11.4, and 11.5).

5.5.7 Mutations in strain RNM-8

Strain RNM-8 acquired point mutations in *rob*, *rpoB*, and *torY* as well as an 11 bp deletion in *acrR*. The point mutations in *rob*, *rpoB*, and *torY* led to a change in a single amino acid at residue 156 (Arg→His), 1037 (Thr→Pro), and 87 (Ala→Thr) for Rob, RpoB, and TorY, respectively. On the other hand, the 11 bp deletion in *acrR* caused a change in the amino acid sequence starting at residue 53 and increase in amino acid residues from 215 to 244 for AcrR (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.8 Mutations in strain RNM-19

Strain RNM-19 had point mutations in *marR*, *rpoC*, *ompR*, and *acrB*, an insertion mutation in *acrR*, and indels in *yieL* and *mioC*. The point mutations in *marR*, *rpoC*, *ompR*, and *acrB* caused a change in a single amino acid at residue 84 (Val→Gly), 1075 (Arg→Cys), 15 (Arg→Ser), and 379 (Thr→Ile) for MarR, RpoC, OmpR and AcrB, respectively. On the other hand, the insertion mutation in *acrR* caused the truncation for the resulting protein at residue 34 (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.9 Mutations in strain RNM-20

Strain RNM-20 had point mutations in *marR*, *rpoC*, and *ompR*, an insertion mutation in *acrR*, and indels in *yieL*, *mioC*, *pitA*, and *stfP*. The point mutations in *marR*, *rpoC*, and *ompR*, caused a change in a single amino acid at residue 84 (Val→Gly), 1075 (Arg→Cys), and 15 (Arg→Ser), for MarR, RpoC, and OmpR, respectively. On the other hand, the insertion mutation in *acrR* caused

the truncation for the resulting protein at residue 34 (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.10 Mutations in strain RNM-21

Strain RNM-20 acquired point mutations in *marR*, *ompR*, and *acrB*, two point mutations in *rpoC*, an insertion mutation in *acrR*, and a 15,894 bp deletion that affected *yhhJ*, *rbbA*, *yhiI*, *yhiJ*, *yhiL*, *yhiM*, *yhiN*, and *pitA*. The point mutations in *marR*, and *ompR*, and *acrB* caused a change in a single amino acid at residue 84 (Val→Gly), 15 (Arg→Ser), and 901 (Val→Ile) for MarR, OmpR, and AcrB, respectively. On the other hand, the point mutations in *rpoC* caused changes in amino acid at residues 787 (Ala→Val) and 1075 (Arg→Cys). The insertion mutation in *acrR* caused the truncation for the resulting protein at residue 34. In the case of the 15,894 bp deletion, it resulted in the removal of the entire gene of *yhhJ*, *rbbA*, *yhiI*, *yhiJ*, *yhiL*, *yhiM*, *yhiN*, and *pitA* (Table 5.3 and Appendix Tables 11.3 and 11.4).

5.5.11 Mutations in strain RNM-22

Strain RNM-22 gained point mutations in *rob*, *rpoB*, and *groL*, and *torY*, an 11 bp deletion mutation in *acrR*, and a nucleotide change in the DNA sequence at position 1197659. The point mutations in *rob*, *rpoB*, *groL*, and *torY* caused a change in a single amino acid at residue 156 (Arg→His), 1037 (Thr→Pro), 279 (Pro→Leu), and 87 (Ala→Thr) for Rob, RpoB, GroL, and TorY, respectively. The 11 bp deletion mutation in *acrR* caused a change in the amino acid sequence starting at residue 53 and increase in amino acid residues from 215 to 244 for AcrR. The change in nucleotide base at position 1197659 (C→A) affected the intergenic region 45 bp downtream and 120 bp upstream of *asmA* and *yegH*, respectively (Table 5.3 and Appendix Tables 11.3, 11.4, and 11.5).

5.5.12 Mutations in strain RNM-23

Strain RNM-23 acquired point mutations in *soxR*, *rpoC*, and *torY*, an insertion mutation in *acrR*, and a nucleotide change in the DNA sequence at positions 2133236 and 3915915. The point mutations in *soxR*, *rpoC*, and *torY*, caused a change in a single amino acid at residue 20 (Arg→Leu), 1075 (Arg→Cys), and 87 (Ala→Thr), for SoxR, RpoC, and TorY, respectively. On the other hand, the

insertion mutation in *acrR* caused a change in the amino acid sequence starting at residue 74 and truncation at residue 84 for the resulting protein. The changes in nucleotide base at position 2133236 (T→A) and 3915915 (T→G) affected the intergenic region 4 bp downtream and 270 bp upstream of *asmA* and *yegH* and 115 bp upstream of *atpI* and 502 bp downstream of r*smG*, respectively (Table 5.3 and Appendix Tables 11.3, 11.4, and 11.5).

5.6 Correlation of mutations to growth in BMA

It can be observed that each of the BMA tolerant strains carry a mutation in *acrR* along with a mutation in *marR*, *soxR*, or *rob*, which suggests that the MarA-SoxS-Rob regulatory network could play a vital role for growth in the presence of BMA (Grkovic et al., 2002; Grkovic et al., 2001). In addition, each of the BMA tolerant strain grown in the presence of BMA (20% v/v) that has maximum growth rate of <0.70 /h acquired a mutation in either *rpoB* or *rpoC*. This suggests the potential role of the *rpoB* and *rpoC* mutation for the observed high growth rate for the strains isolated from the chemostat culture and strain RNM-18 (Tables 5.2 and 5.3).

5.7 Conclusions

ADE using sequential batch and chemostat cultures was successfully applied to generate various *E. coli* strains with improved growth in the presence of BMA up to 20% v/v. Selection and enrichment of strains with the ability to grow at a relatively high cell density and high rate in the presence of BMA (20% v/v) were achieved with ADE through sequential batch and chemostat cultures, respectively. The resequencing of the BMA tolerant strains generated *via* ADE revealed that various mutations were acquired along the evolution process. Presence of an *acrR* mutation along with either a *marR*, *soxR*, and *rob* mutation indicate the involvement of the MarA-SoxS-Rob regulatory network in BMA tolerance, possibly through the AcrAB-TolC efflux system.

6

Transcriptomics Analysis

6.1 Introduction

In Chapter 5, the generation of *Escherichia coli* strains with tolerance for BMA at 20% v/v *via* adaptive evolution (ADE) in sequential batch and chemostat cultures was done. The changes in genome sequence of selected BMA tolerant strains was also determined to obtain an insight into the molecular basis of the adaptive mutations acquired from the evolution process (Atsumi et al., 2010; Lang & Desai, 2014; Lennen et al., 2019; Minty et al., 2011; Nishant et al., 2009). Although genome resequencing demonstrated the genetic basis of evolution and, in part, the potential mechanism of BMA tolerance, it is not sufficient to unravel the complexity of tolerance mechanisms for BMA in *E. coli* (Ge et al., 2013; Horinouchi et al., 2010; Zhang et al., 2010). It will also be necessary to get an idea of how the adaptive mutations affected various cellular processes (Cao et al., 2017). This can be obtained from the changes in gene/protein expression and abundance of cellular metabolic products (Ge et al., 2013; Zhang et al., 2010). The changes can be quantitatively measured and functionally characterized with the aid of the emerging 'omic technologies, such as transcriptomics, proteomics, and metabolomics (Ge et al., 2013; Zhang et al., 2010).

Transcriptomics allows the analysis of the changes in gene expression from the strain of interest relative to the parental strain or reference condition; wherein, the whole set of mRNA (transcripts) generated by the cells under specific environmental conditions and biological state are measured (Aslam et al., 2017; Hoeijmakers et al., 2013; Lowe et al., 2017; Zhang et al., 2010). Proteomics and metabolomics are very similar to transcriptomics. Instead of mRNA, proteins (proteome) and cellular metabolic products (metabolome) are extracted, measured, and characterized for proteomics and metabolomics, respectively (Aslam et al., 2017; Graves & Haystead, 2002; Guo et al., 2013; Hill et al., 2015; Krastanov, 2010; Zhang et al., 2010). Ideally, a combination of the 'omic tools are used and integrated to gain a global overview of the cellular state and response for bioproduct tolerance (Manzoni et al., 2016;

Zhang et al., 2010). However, utilization of all the 'omic tools rarely happens. This can be partly attributed to the cost of analysis and the amount of data generated and processed (Zhang et al., 2010).

Both proteomics and metabolomics suffer coverage/quantification difficulties. The lack of protein amplification methods, difficulties in isolation of membrane proteins, detection of low abundance proteins and insoluble proteins are among the issues faced in proteomics. Similarly, only 15-30% of the entire mass spectra for metabolites can be identified and quantified. Thus, the amount and usefulness of information is limited. In contrast, transcriptomics benefit from the advancements of next-generation sequencing (NGS) technologies that enables amplification, sequencing, and identification of the transcripts (Misra et al., 2019). Due to the maturity and reliability of the RNA sequencing technology, transcriptomics analysis is used in this chapter to gain further understanding on the possible mechanism of BMA tolerance by the BMA tolerant *E. coli* strains generated from ADE (Chapter 5).

6.2 RNA sequencing

Six out of the 12 unique BMA tolerant strains generated from ADE (Chapter 5) were chosen based on the variety of acquired mutations and growth characteristics (Table 6.1) and analysed for the changes in their transcriptomes. In order to understand how the mutations altered the gene expression of the evolved strains, transcripts from the strains grown in minimal medium from OD600 0.05 to 0.3 were analysed and compared to the parental strain (Cao et al., 2017). The cultures grown until OD600 of 0.3 were added with BMA (20% v/v) and the changes in their transcriptomes 1 h after BMA addition was determined to get an overview of the BMA tolerant *E. coli* strains' response under BMA stress (Yung et al., 2016). Both approaches may help elucidate the underlying mechanisms of BMA tolerance for the evolved strains (Cao et al., 2017; Yung et al., 2016).

Table 6.1 Summary of strains used for the transcriptomics analysis

Strain number	Genotype	Source
RNM-2	*acrR(*E91fs) *rob*(R156H) *rpoC*(L361R) *ilvN*(C41Y) *ygbK*(A294E) *lpxM*(267_272 del) *ompT*(indels)	Flask culture
RNM-5	*soxR*(Leu139X) *acrR*(indels) *mscK*(indels) 580116(G>T)	ADE-2 (Batch)
RNM-18	*rob*(A70T) *creA*(V85V) *yohJ*(L109R) *dnaK*(V377G) *927777*(C>T) *cra*(I270fs) *acrR*(A191fs) *clsA*(A448fs) *rpoC*(K215fs) *opgH*(R95P) *cpxA*(P177Q) *ompX*(indels) *atpI*(indels) Δ*psuT* Δ*psuG* Δ*psuK* Δ*fruA* Δ*fruK* Δ*fruB* Δ*setB*	ADE-4 (Batch)
RNM-21	*acrR*(L34) *marR*(V84G) *rpoC*(R1075C) *rpoC*(A787V) *ompR*(R15S) *acrB*(V901I) *ΔyhhJ ΔrbbA ΔyhiI ΔyhiJ ΔyhiL ΔyhiM ΔyhiN ΔpitA*	ADE-5 (Chemostat)
RNM-22	*acrR*(K53Yfs) *rob*(R156H) *rpoB*(T1037P) *groL*(P279L) *torY*(A87T) 1197659(C>A)	ADE-5 (Chemostat)
RNM-23	*acrR*(E74fs) *soxR*(R20L) *rpoC*(R1075C) *torY*(A87T) 2133236(T>A) 3915915(T>G)	ADE-5 (Chemostat)

6.3 Differentially expressed genes

The RNA sequencing results was reported as read counts, while the relative abundance to a particular strain or condition was reported as fold changes or $\log_2$ fold change (logFC). A differentially upregulated gene with a logFC value of ≤1.0 and *p*-value of <0.05 was classified as differentially upregulated gene, while a gene with value of ≥-1.0 and *p*-value of <0.05 was classified as differentially downregulated gene (Yung et al., 2016). These logFC values chosen correspond to at least a 2-fold change in the gene mRNA relative to the reference strain or condition. The mutations gained by the BMA tolerant strains resulted in the differential expression of hundreds to thousands of genes with respect to the parental strain (Table 6.2 and Appendix Table 11.6).

Excluding RNM-2, there seems to be a correlation between the number of mutations/affected genes and the number of differentially expressed genes with respect to the parental strain (Tables 6.1 and 6.2). This pattern can be observed with the RNM-18 and RNM-21, which had the highest number of affected genes (14-20) and highest number of differentially expressed genes (1017-1107). This was followed by RNM-22 and 23, which had 6 mutations each and 827-863 differentially expressed genes. Finally, RNM-5, which only acquired 4

mutations, had the lowest number of differentially expressed genes at 181. By contrast, RNM-2 had 7 affected genes, but the number of differentially expressed genes only totalled 192. It is possible that most of the mutations acquired by RNM-2 only had a subtle effect on gene expression and/or that the overall effect from the combination of mutations balanced out the perturbations in gene expression (Chong et al., 2014).

Addition of BMA at 20% v/v evoked a huge response from the BMA tolerant *E. coli* cells (Table 6.2 and Appendix Table 11.7), which accounted for 25-50% of the total annotated genes for *E. coli BW25113* (Grenier et al., 2014). The strain RNM-18 had the largest total number of differentially expressed genes (2219), while RNM-2 had the smallest (1155).

Table 6.2 Summary of the number of differentially expressed genes

BMA tolerant strain	Number of differentially expressed genes					
	with reference to the parental strain			After BMA addition		
	Total	Up	Down	Total	Up	Down
RNM-2	192	83	109	1155	500	655
RNM-5	181	62	119	1494	724	770
RNM-18	1017	395	622	2219	1041	1178
RNM-21	1107	532	575	1729	861	868
RNM-22	863	373	490	2173	1055	1118
RNM-23	827	319	508	1956	939	1017

Differentially expressed genes were identified as genes with at least a logFC of ≤1.0 and ≥-1.0 for up- and down-regulated genes, respectively with a p-value of < 0.05 as cutoff (Yung et al., 2016).

Although all the genes differentially expressed can potentially give an insight on the possible mechanisms of BMA tolerance, it will be necessary to distinguish between the strain specific response and response fundamental for BMA tolerance. This can be accomplished by identifying the differentially expressed genes that are common to each of the strains, which is likely to include the genes crucial for BMA tolerance. The differentially expressed genes from each strain (Appendix Tables 11.6 and 11.7) were compared to determine the number (Fig. 6.1) and identity (Table 6.3) of the genes common in the six strains.

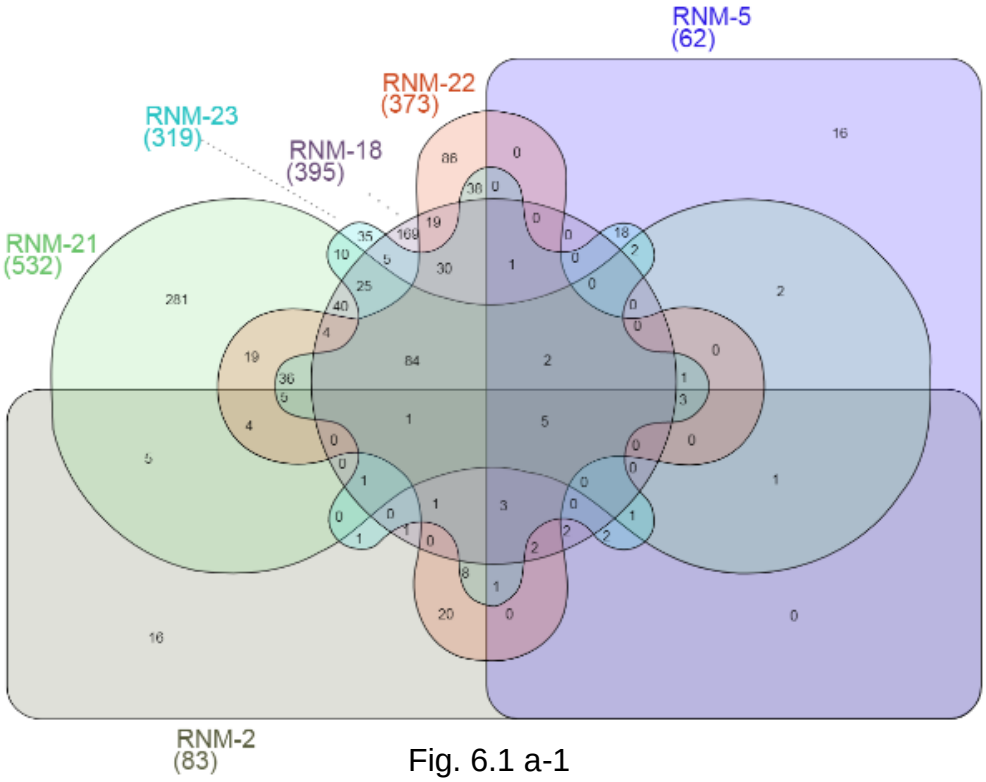

Fig. 6.1 a-1

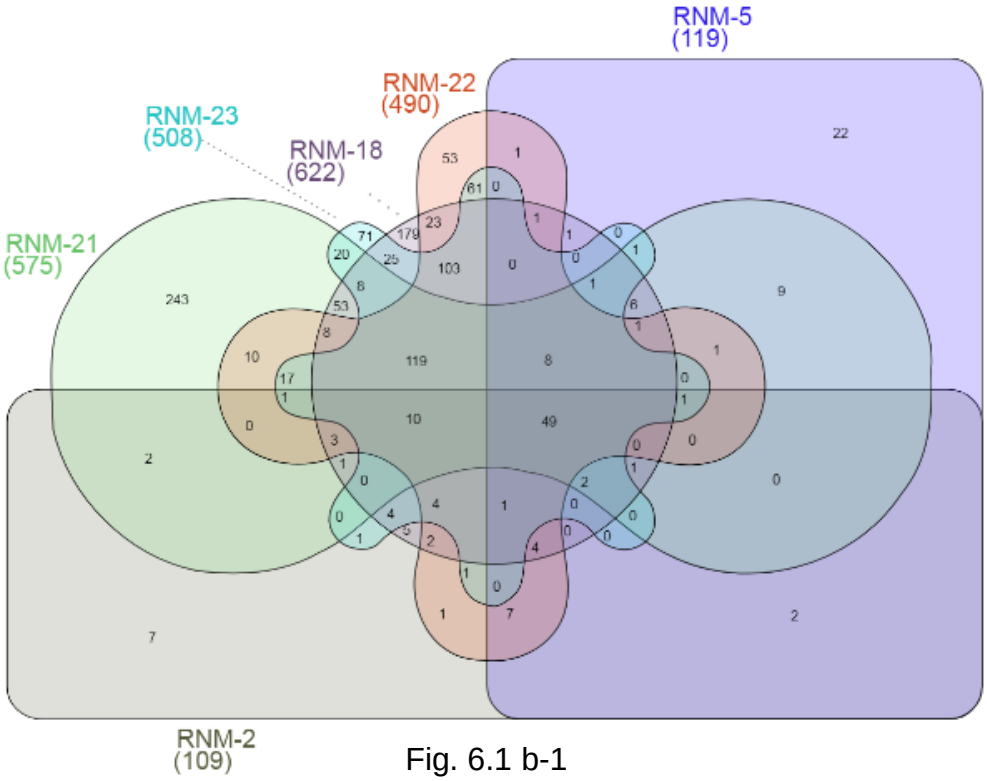

Fig. 6.1 b-1

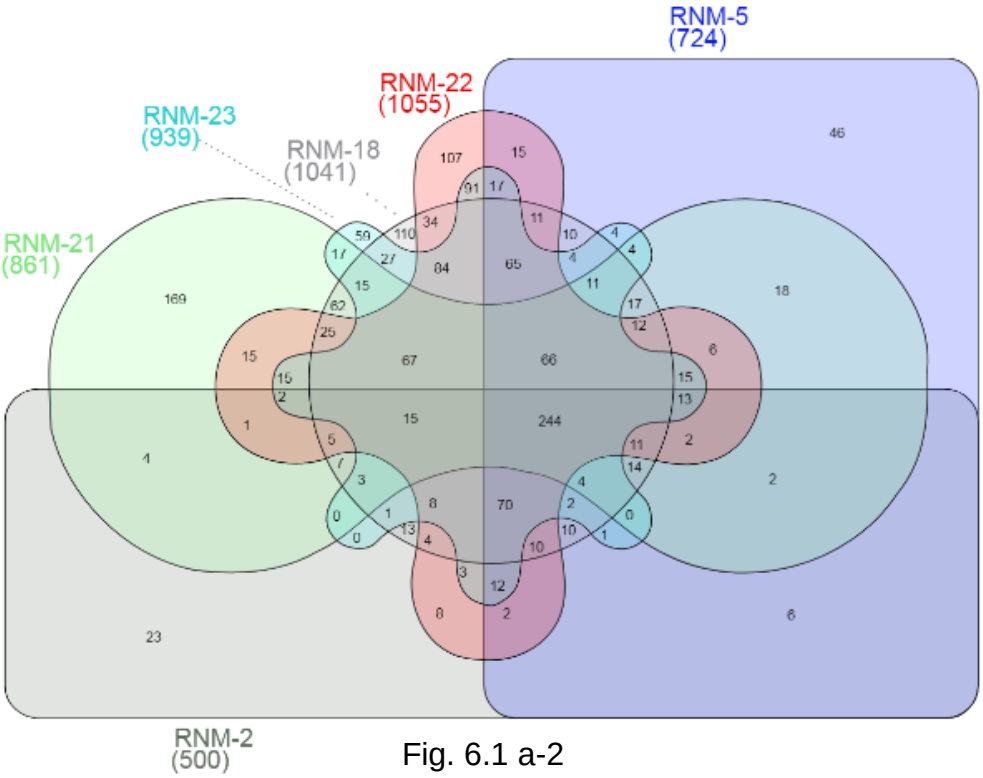

Fig. 6.1 a-2

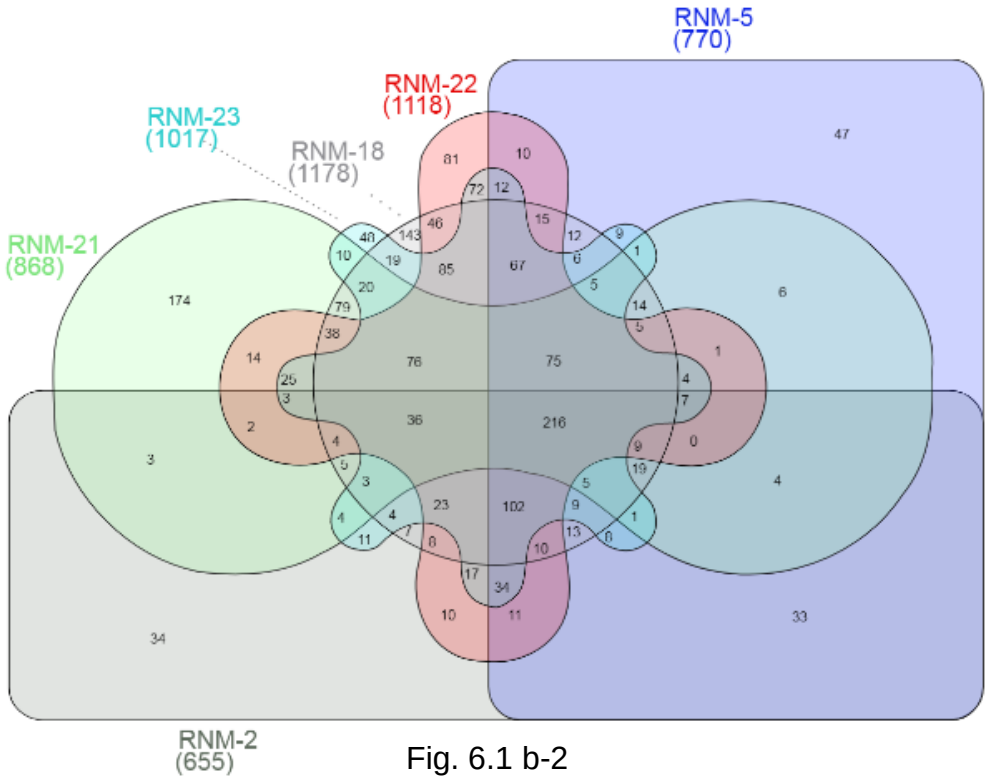

Fig. 6.1 b-2

Figure 6.1 Venn diagrams for the differentially expressed genes from the 6 BMA tolerant strains. Legend: a – upregulated, b – downregulated genes; 1 – relative to the parental strain, 2 – after BMA addition.

Among the differentially expressed genes from six different BMA tolerant strains with respect to the parental strain, only 5 and 49 genes were commonly upregulated and downregulated, respectively (Fig. 6.1-1; Table 6.3). On the other hand, only 244 and 216 genes were commonly upregulated and downregulated, respectively after BMA addition (Fig. 6.1-2; Table 6.3). Although this thoroughly narrows down the list of differentially expressed genes that are likely to include those indispensable for BMA tolerance, it will still take a tremendous time and effort to individually look up the differentially expressed genes and somehow decipher the possible interrelation of the associated genes in a systematic and comprehensible manner (Bindea et al., 2009).

Table 6.3 List of differentially up or down regulated genes common in 6 BMA tolerant strains

Condition	Gene names
with reference to parental strain	***acrA acrB inaA nfsA ybjC*** *arrS cheA cheB cheR cheW cheY cheZ dctR fimA fimC fimF fimG fimH fimI flgH flgK flgL flhE fliA fliC fliD fliI fliJ fliQ fliS fliT fliZ flxA gadA gadB gadC gadE glsA hdeA hdeB hdeD mdtE mdtF motA motB slp tap tar tsr ybaT ycgR yhiD yhiM yhjH*
After addition of 20% v/v BMA	***aceE aceF adhP adiY agp ahr aldB araH ariR aroF aroL bhsA bssR bssS cbpA cbpM cdd cfa chrR clpB cpsG csgA csgB cspD csrB csrC curA dacC degQ deoB deoC deoD dicF dkgA dmlA dnaK dps ecnB elaB elbB eutD eutE eutG eutM fbaB fucU fxsA gabD gabP gabT gadA gadB gadC galU garR ggt glgS glk glmY glmZ glxR grcA grxA gstB gudD hchA hdeA hdeB hdhA higA higB hokD hspQ ibpA ibpB ilvC ivy katE ldhA ldtA ldtE leuD loiP lolA mcbA mlaC mliC mqsA mqsR mscS msrA narU narV narW narY narZ nuoK ompX omrA omrB osmB osmC osmE osmY paoB paoD patD pfkA pfkB pgl pgpC pheL preT psiF pspA pspB pspC pspD pspE pspG pstS ptwF putA qmcA qorA qorB raiA rclA relB relE rmf rof rpoH rpoS rrlC rrlH rrsH ryfA ryjA sbmC sfmA slt slyB sodB sodC spy sra ssrA talA tisB tktB treF tyrA udp ushA uspA uspB uspD uspF uspG wrbA yaeH yagG yagH yahK yahO yaiY yajL ybbJ ybeD ybeL ybfA ybgS ybhF ybhG ybhL ybhR ybhS ybiB ybiH ybjP ybjQ ycaC yccJ ycfJ ychH yciE yciF yciG ycjF ydcF ydeI ydeP ydgU ydhR ydiZ yeaO yeaQ yegP yfdQ yfdY yfeO yffR yfiL ygaM ygaU ygdI ygdR yghA yghU ygiW yhaK yhbO yhbW yhcN yhhA yhjX yiaG yibT yihQ yjdJ yjfY ylil ymdF ymgA ymgC ymgE yneM ynfD ynfM yniA yodD yohC yohJ yohK ypeC ypfM yphA yqaE yqjD yqjG ytfK ytjA zraP*** *alaW alaX alsR amiC argC argQ argU argV argX argY argZ arpA artP asnU asnV carA cdh cmk coaA cobC cspA cspB cspF cspG cspH cysT dinF dnaB dsrA dusB efeB efeO efeU entD entS epmB essD fecA fecB fecC fecE fecI fecR fepC fepE fepG fes fhuB fhuC fhuD fhuF flgB flgJ fliF fliI fliM folK ftsB galS glnU glnW gltU gltV gluQ glyU glyW glyY gnsA gpp gpt gspM gtrA hda hisR holD hyfA iclR infA insA insJ intZ ispD kdgT leuT leuW leuX leuZ lpxH lpxP lysQ lysT lysV lysW lysY lysZ mdtL mepS metT metU metV metY mioC mltD mnmA mreC mreD ompF opgE pcnB pheV pncB potA ppdD priA proM prs purN pyrD queA queD rcnA recG recQ renD rimI rimM rlmA rlmC rlmG rlmH rluB rluC rluE rnpA rph rpmB rpsP rpsT rpsU rseC rsfS rspR rsxA rsxB rsxC rtn rttR sdiA secG serU serV suhB tgt tonB trmH trmL trmN tsaD tyrT tyrV ubiX udk valT valY valZ waaA yacC yadS yaeI ybaN ybeF ybfE ybfP ybgC ycgX ycjW ydbD ydcD yddA ydfU ydiE ydiY yeaP yecJ yecT yegK yeiB yeiW yfhL yfhR yfiP yfjV ygfF yggI ygiQ yhdJ yhjV yibQ yidD yidX yihG yiiQ yijF yjhD yjhR ymcE ymjA ynaE yneG ynfN ynjI yoaA yohO yojI yqjH yraJ***

Notes: Text in bold-italics are differentially upregulated genes, while regular-italics are differentially downregulated genes.

To facilitate grouping of functionally related differentially expressed genes, an enrichment analysis based on the GO terms biological process, molecular function, and cellular component (Ashburner et al., 2000; The Gene Ontology Consortium, 2018) and regulating transcription factors

(Gama-Castro et al., 2016) was performed with the aid of the enrichment analysis tool ClueGO (Bindea et al., 2009) and Funrich (Pathan et al., 2015), respectively. Heat maps for genes regulated by significantly enriched transcription factors and genes of potential significance to the transcriptomics analysis were generated using GENE-E (Broad Institute) to facilitate visualization of the changes in gene expression.

Based on the GO terms biological process, a number of differentially expressed genes relative to the parental strain are related to cell motility, cellular monovalent inorganic cation homeostasis, cellular nitrogen compound metabolic process, chemotaxis, drug transmembrane transport, regulation of locomotion, and response to pH (Table 6.4).

Table 6.4 Summary of significantly enriched GO terms based on biological process for the differentially expressed genes with respect to the parental strain

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0048870; cell motility	17; 25.37; 9.02×10^{-22}	*cheY cheZ flgH flgK flgL fliC fliD fliI fliJ fliS fliT fliZ motA motB tsr ycgR yhjH*
GO:0030004; cellular monovalent inorganic cation homeostasis	3; 30; 0.0031	*gadA gadB gadC*
GO:0034641; cellular nitrogen compound metabolic process	6; 0.16; 1.58×10^{-5}	*dctR fliA fliI fliT fliZ gadE*
GO:0006935; chemotaxis	12; 29; 1.04×10^{-15}	*cheA cheB cheR cheW cheY cheZ fliJ motA motB tap tar tsr*
GO:0006855; drug transmembrane transport	4; 8; 0.029	***acrA acrB*** *mdtE mdtF*
GO:0040012; regulation of locomotion	3; 15; 0.027	*cheZ fliZ ycgR*
GO:0009268; response to pH	5; 20; 5.05×10^{-5}	*glsA hdeA hdeB hdeD ybaT*

Notes: Text in bold-italics are differentially upregulated genes, while regular-italics are differentially downregulated genes.

Glutamate decarboxylase activity, motor activity, nucleic acid binding, and transmembrane receptor activity emerged as the most significantly enriched GO terms for molecular function (Table 6.5).

Table 6.5 Summary of significantly enriched GO terms based on molecular function for the differentially expressed genes with respect to the parental strain

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0004351; glutamate decarboxylase activity	2; 66.67; 0.0044	*gadA gadB*
GO:0003774; motor activity	2; 25.0; 0.038	*flgH fliJ*
GO:0003676; nucleic acid binding	4; 0.14; 0.001	*dctR fliA fliZ gadE*
GO:0004888; transmembrane signalling receptor activity	4; 36.36; 1.68×10^{-5}	*fliZ tap tar tsr*

Notes: Text in bold-italics are differentially upregulated genes, while regular-italics are differentially downregulated genes.

As for the GO terms cellular component, the most significantly enriched differentially expressed genes belonged to bacterial-type flagellum, bacterial type flagellum filament, and pilus (Table 6.6).

Table 6.6 Summary of significantly enriched GO terms based on cellular component for the differentially expressed genes with respect to the parental strain

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0009288; bacterial-type flagellum	10; 35.71; 7.71×10^{-14}	*cheZ flgH flgK flgL flhE fliC fliD fliJ fliQ ycgR*
GO:0009420; bacterial-type flagellum filament	2; 66.67; 0.0036	*fliC fliD*
GO:0042995; cell projection	15; 13.89; 9.61×10^{-16}	*cheZ fimA fimF fimG fimH fimI flgH flgK flgL flhE fliC fliD fliJ fliQ ycgR*
GO:0009289; Pilus	5; 6.25; 0.0065	*fimA fimF fimG fimH fimI*

Notes: Text in bold-italics are differentially upregulated genes, while regular-italics are differentially downregulated genes.

On the other hand, the enrichment analysis for which transcription factors that regulate the differentially expressed genes relative to the parental strain revealed potential involvement of Rob, SoxS, MarA, EnvR, MprA, AcrR, OxyR, PhoP, GadX, GadW, GadE, H-NS, FliZ, FlhDC, RcsB, GadE-RcsB, YdeO, TorR, MatA, Lrp, AdiY, SutR, CsgD, EvgA, and CpxR regulons (Fig. 6.2).

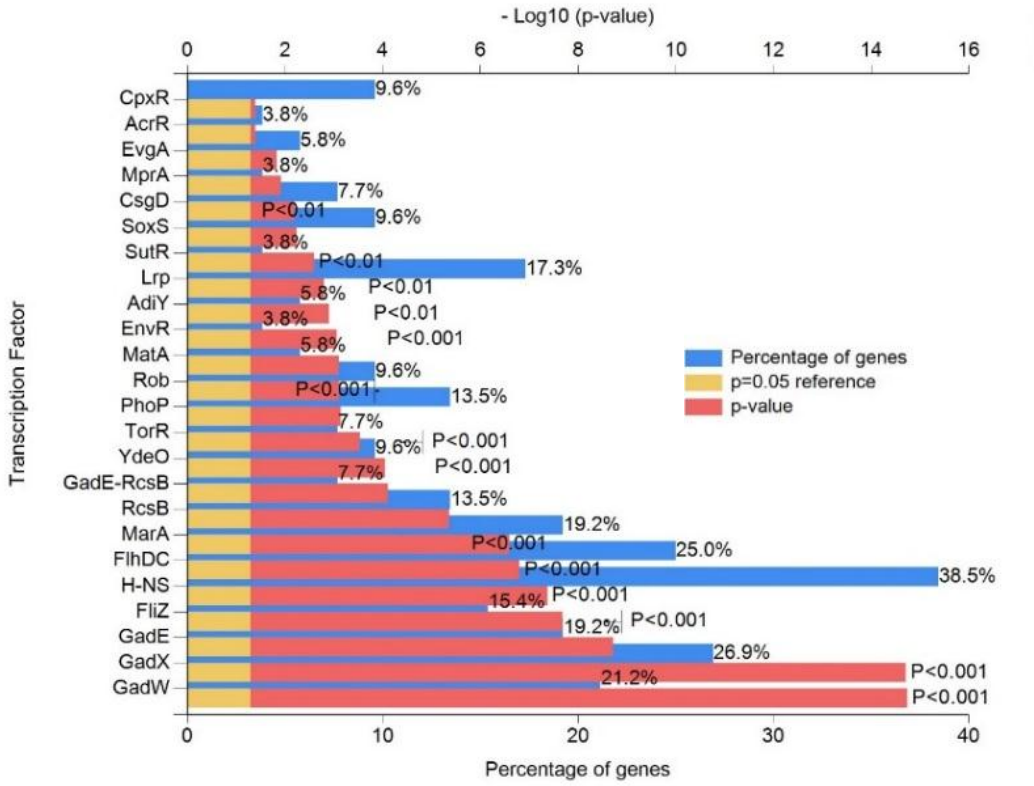

Figure 6.2 Significantly enriched regulating transcription factors of the differentially expressed genes relative the parental strain.

The enriched transcription factors (Fig. 6.2) were closely related to the significantly enriched GO terms (Tables 6.4, 6.5, and 6.6). MarA, SoxS, Rob, AcrR, EnvR, MprA, and CpxR regulate multidrug resistance related genes in *E. coli* (Dorel et al., 2006; Grkovic et al., 2002; Grkovic et al., 2001; Hirakawa et al., 2008; Lomovskaya et al., 1995), while PhoP, GadX, GadW, GadE, GadE-RcsB, H-NS, YdeO, AdiY, and EvgA regulate genes related to acid resistance (Castanié-Cornet et al., 2010; Eguchi et al., 2011; Giangrossi et al., 2005; Hommais et al., 2004; Sayed et al., 2007; Stim-Herndon et al., 1996; Tramonti et al., 2002). Transcription factors involved in the regulation of genes related to chemotaxis, motility, flagellum and pili biosynthesis, and biofilm formation that were significantly enriched include RcsB, H-NS, Fliz, FlhDC, MatA, Lrp, CsgD, and CpxR (Calvo & Matthews, 1994; Claret & Hughes, 2002; Donato & Kawula, 1999; Dorel et al., 2006; Huang et al., 2006; Lehti et al., 2012; Loferer et al., 1997; Pesavento & Hengge, 2012; Soutourina et al., 1999).

All of the differentially upregulated genes with respect to the parental strain were part of the of the MarA-SoxS-Rob regulatory system (Gama-Castro et al.,

2016; Paterson et al., 2002) (Fig. 6.3). Among them, only *acrA* and *acrB* were associated with the significantly enriched GO terms (Table 6.4).

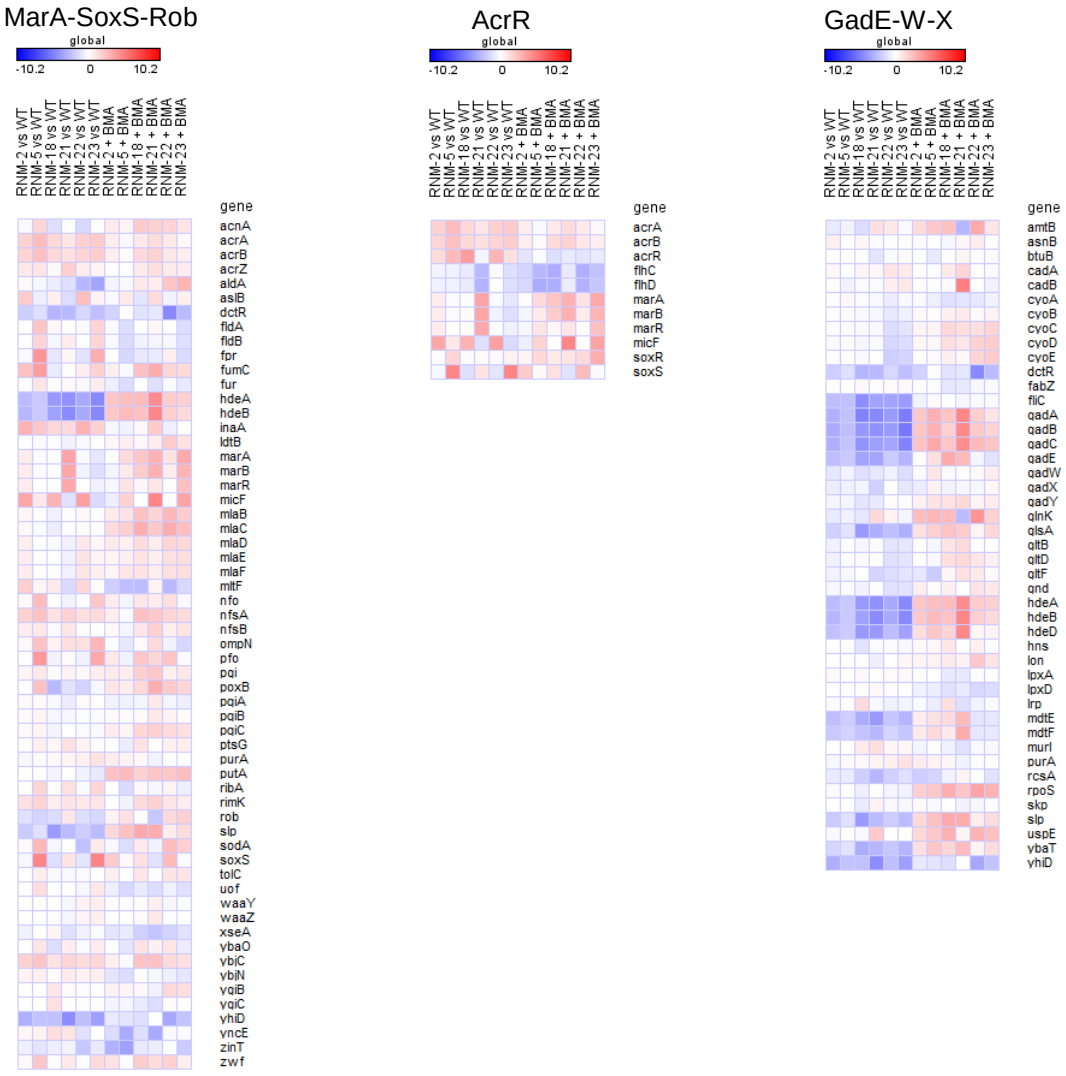

Figure 6.3 Heat maps for genes regulated by MarA-SoxS-Rob, AcrR, and GadE-X-W. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

Another key result from the enrichment analysis is the emergence of differentially downregulated genes related to chemotaxis, motility, flagellum and pili biosynthesis, biofilm formation, and acid resistance (Tables 6.4-6.6; Fig. 6.2-6.4).

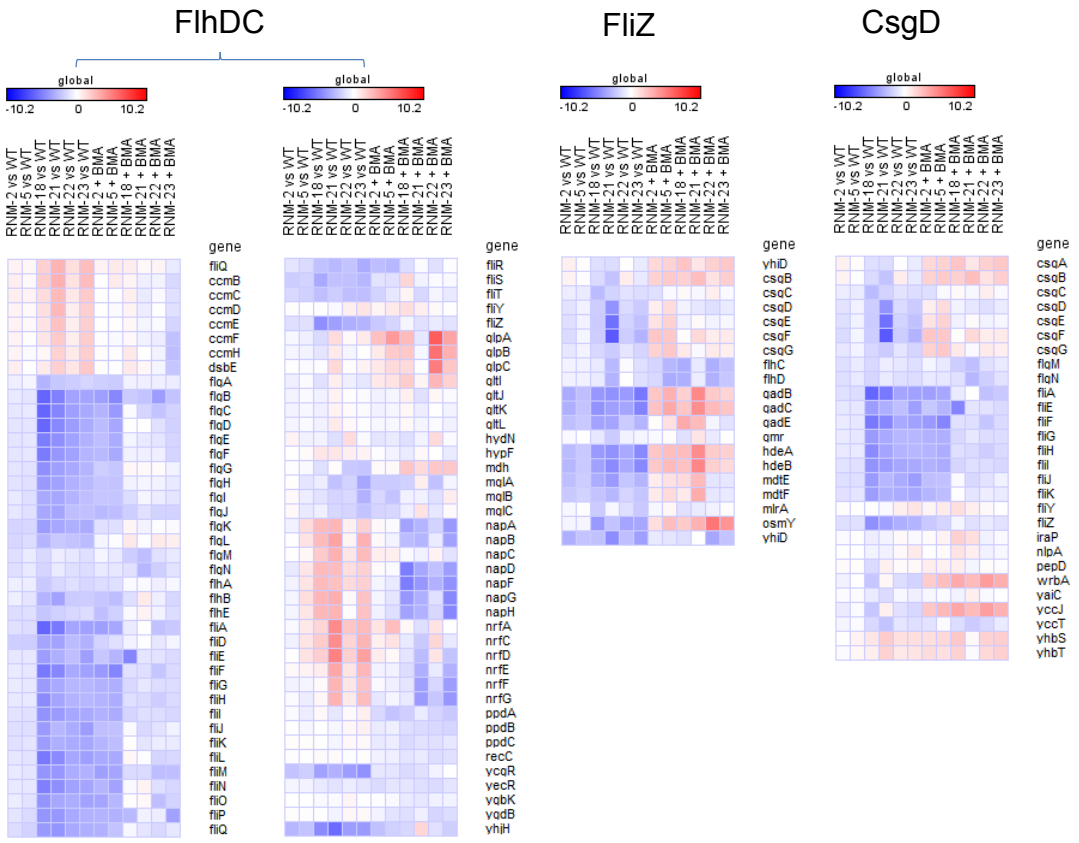

Figure 6.4 Heat maps for genes regulated by FlhDC, FliZ, and CsgD. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

Addition of BMA resulted in the differential expression of genes related to the DNA metabolic process, RNA processing, cation homeostasis, nucleic acid metabolic process, nucleotide catabolic process, organonitrogen compound metabolic process, protein secretion, response to ethanol, response to oxidative stress, response to pH, response to temperature, and ribonucleotide metabolic process (Table 6.7). The differentially expressed genes were also linked to various molecular functions that include DNA binding, RNA binding, intramolecular transferase activity, iron-chelate transport, nucleotidyl-transferase activity, oxidoreductase activity acting on the aldehyde or oxo group of donors, purine ribonucleotide binding, ribonucleoprotein complex binding (Table 6.9), which are either a members of the cytoplasmic part or the integral component of membrane (Table 6.9).

Table 6.7 Summary of significantly enriched GO terms based on biological process for the differentially expressed genes after BMA addition

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0006259; DNA metabolic process	20; 1.23; 1.01×10^{-11}	*argU* ***cspD*** *dnaB* ***dnaK*** *efeB hda holD insA intZ* ***mcbA*** *priA recG recQ rlmC* ***rpoH*** *rpsP* ***sbmC*** *yacC yhdJ* ***ymgE***
GO:0006396; RNA processing	21; 14.58; 2.62×10^{-4}	*dusB gluQ mnmA pcnB queA rimM rlmA rlmC rlmG rlmH rluB rluC rluE rnpA* ***rof*** *rph tgt trmH trmL trmN tsaD*
GO:0055080; cation homeostasis	21; 25.61; 6.27×10^{-9}	***dps*** *efeB efeU fecA fecB fecC fecE fecI fecR fepC fepE fepG fes fhuB fhuC fhuD fhuF* ***gadA gadB gadC*** *yqjH*
GO:0090304; nucleic acid metabolic process	74; 2.52; 3.04×10^{-7}	***adiY*** *alsR argU* ***clpB*** *cspA cspB* ***cspD*** *cspF cspG cspH* ***curA*** *dnaB* ***dnaK*** *dusB efeB entS fecI galS gluQ hda* ***higA higB*** *holD iclR insA intZ* ***mcbA*** *mnmA* ***mqsA mqsR*** *pcnB priA* ***pspA pspB pspC putA*** *queA recG recQ* ***relB relE*** *rimM rlmA rlmC rlmG rlmH rluB rluC rluE rnpA* ***rof*** *rph* ***rpoH rpoS*** *rpsP rspR* ***sbmC*** *sdiA tgt trmH trmL trmN tsaD yacC* ***yajL*** *ybeF ybfE* ***ybiH*** *ycjW* ***ydiZ*** *yhdJ* ***yiaG*** *yihG* ***ymgE***
GO:0009166; nucleotide catabolic process	6; 50; 0.0017	***deoB deoC*** *gpp gpt* ***udp ushA***
GO:1901564; organonitrogen compound metabolic process	82; 7.26; 1.55×10^{-4}	***aceE aceF*** *amiC argC* ***aroF aroL*** *carA* ***cdd*** *cmk coaA cobC* ***dacC deoD elbB*** *entD* ***eutD eutE eutG eutM fbaB*** *fliI folK* ***gabD gabP gabT gadA gadB ggt glk*** *gluQ* ***glxR*** *gpp gpt* ***grcA gstB*** *gtrA* ***gudD*** *hda* ***higB ilvC*** *infA* ***ldhA ldtA ldtE leuD*** *mepS mioC mltD* ***mqsR nuoK paoB patD pfkA pfkB pgl*** *pncB* ***preT*** *prs purN* ***putA*** *pyrD queA queD* ***raiA relE rmf*** *rpmB rpsP rpsT rpsU rsfS rsxB* ***slt sra talA*** *tgt tyrA udk* ***udp yajL yghU yhbO***
GO:0009271; phage shock	4; 10; 0.0013	***pspA pspB pspD pspG***
GO:0009306; protein secretion	2; 0.59; 0.032	*fliI secG*
GO:0045471; response to ethanol	3; 100; 0.030	***adhP aldB uspB***
GO:0006979; response to oxidative stress	19; 21.35; 1.68×10^{-6}	***ariR*** *glyU* ***katE msrA osmC qorA rclA*** *rseC* ***sodB sodC uspF wrbA yajL ychH ydeI ygiW yhbO yhcN yodD***
GO:0009268; response to pH	7; 28; 0.023	***hchA hdeA hdeB ydeP yhbO yhcN yodD***
GO:0009266; response to temperature stimulus	17; 19.1; 6.35×10^{-5}	***clpB*** *cspA cspB cspG* ***dnaK eutD hspQ ibpA ibpB ldhA*** *lpxP pncB* ***pspA raiA rpoH yajL yhbO***
GO:0009119; ribonucleoside metabolic process	21; 17.8; 7.91×10^{-6}	***aceE aceF*** *carA* ***cdd*** *coaA* ***fbaB*** *fliI* ***glk*** *gpp gpt* ***ldhA nuoK paoB pfkA pfkB*** *pyrD queA queD tgt udk* ***udp***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Table 6.8 Summary of significantly enriched GO terms based on molecular function for the differentially expressed genes after BMA addition

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0003677; DNA binding	52; 2.17; 1.78×10^{-8}	***adiY*** *alsR argU* ***ariR cbpA*** *cspA cspB* ***cspD*** *cspF cspG cspH dnaB* ***dps*** *efeB fecI galS hda* ***higA hspQ*** *iclR insA insJ intZ mnmA* ***mqsA osmE pgpC*** *priA* ***pspB pspC putA*** *recG recQ* ***relB rpoH rpoS*** *rpsP rspR* ***sbmC*** *sdiA trmN tsaD ybeF ybfE* ***ybgS ybiB ybiH*** *ycjW yhdJ* ***yiaG*** *ynaE yoaA*
GO:0003723; RNA binding	24; 9.41; 0.032	*cspA cspB* ***cspD*** *dusB gluQ hda* ***higB*** *infA mnmA pcnB* ***qorA raiA relE*** *rlmC rluB rluC rluE* ***rmf*** *rnpA rph rpsT trmH trmL* ***ybiB***
GO:0016866; intramolecular transferase activity	8; 19.51; 0.042	***cpsG deoB*** *epmB* ***leuD*** *rluB rluC rluE* ***tyrA***
GO:0015623; iron-chelate-transporting ATPase activity	7; 58.33; 2.64×10^{-5}	*fecB fecC fepC fepG fhuB fhuC fhuD*
GO:0016779; nucleotidyltransferase activity	9; 1.40; 0.0054	***galU*** *holD ispD* ***lolA*** *pcnB rph* ***rpoH*** *yacC yeaP*
GO:0016903; oxidoreductase activity, acting on the aldehyde or oxo group of donors	11; 18.64; 0.0049	***aceE aceF aldB*** *argC* ***eutE gabD*** *gtrA* ***paoB patD putA ydeP***
GO:0032555; purine ribonucleotide binding	35; 2.11; 3.63×10^{-5}	***aroL*** *artP carA* ***clpB*** *cmk coaA dnaB* ***dnaK*** *fecE fepC fhuC fliI folK* ***glk*** *gluQ* ***lolA*** *mnmA pcnB* ***pfkA pfkB*** *potA priA prs recG recQ* ***rof sbmC*** *udk* ***uspF*** *yadS* ***ybhF*** *yddA yeaP yoaA yojI*
GO:0043021; ribonucleoprotein complex binding	8; 20.51; 0.030	*infA* ***raiA relE*** *rimM rlmH* ***rmf*** *rsfS* ***yqjD***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Table 6.9 Summary of significantly enriched GO terms based on cellular component for the differentially expressed genes after BMA addition

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0044444; cytoplasmic part	105; 8.60; 5×10^{-9}	***aceE aceF adiY*** *argC carA* ***cdd cfa chrR clpB*** *cmk coaA cobC* ***cpsG*** *cspA cspB* ***cspD*** *cspG* ***deoB deoC deoD dkgA*** *dnaB* ***dnaK*** *dusB* ***elaB elbB eutG fbaB*** *fhuF* ***gabT gadA gadB galU glk*** *gluQ gnsA gpt* ***grcA gstB hchA*** *hda hdhA ibpA iclR* ***ilvC*** *infA ispD* ***katE ldhA leuD lolA*** *mioC mnmA* ***msrA osmC patD*** *pcnB* ***pfkA pfkB pgl*** *pncB ppdD prs* ***pspA*** *purN pyrD* ***qorA qorB*** *queA* ***raiA*** *recG rimM rlmG rluB rluC rluE rph rpmB* ***rpoH*** *rpsP rpsT rpsU rsfS* ***sodB sra*** *suhB* ***talA*** *tgt t****ktB*** *trmN tsaD udk* ***udp uspD wrbA yajL ybeD ybiB ycaC yccJ ydhR ygaM yhbW*** *ymjA yqjH*
GO:0016021; integral component of membrane	114; 3.11; 7.61×10^{-12}	***araH*** *artP cdh* ***cfa*** *cysT* ***dacC degQ*** *dinF efeU* ***elaB*** *entD entS essD fecA fecC fecR fepC fepE fepG fhuB fliF ftsB* ***fxsA gabP gadC glk*** *gspM gtrA* ***hokD*** *kdgT* ***lolA*** *lpxP mdtL mepS* ***mlaC*** *mreC mreD* ***mscS narU narV nuoK*** *ompF* ***ompX*** *opgE* ***pgpC*** *potA ppdD* ***pspB pspC pspD pspG pstS qmcA raiA*** *rcnA* ***relE*** *rlmH rph* ***rpoS*** *rseC rsxA rtn secG* ***ssrA tisB*** *tonB trmN* ***uspB*** *waaA yadS* ***yagG yaiY*** *ybaN* ***ybbJ ybfA ybhG ybhL ybhR ybhS ycfJ ychH ycjF*** *ydcD yddA* ***ydgU yeaQ*** *yeiB* ***yfdY yfeO*** *yfhR yfjV* ***ygaM yhcN*** *yhjV* ***yhjX*** *yidD yidX yihG ymcE* ***ymgE yneM ynfM*** *ynjI* ***yohC yohJ yohK*** *yohO yojI* ***yphA yqaE yqjD*** *yraJ* ***ytjA zraP***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Enrichment analysis for transcription factors revealed that some of the differentially expressed genes after BMA addition were under the regulation of PspF, RcsB, McbR, CecR, CytR, FliZ, RelB-RelE, RelB, BluR, MqsA, GadW, UvrY, CsiR, HigB-HigA, HigA, DeoR, GadX, AdiY, H-NS, TorR, Fis, Fur, HypT, or PurR (Fig. 6.5). These transcription factors are linked to various stress response systems in *E. coli* such as phage shock response (PspF) (Flores-Kim & Darwin, 2016), multidrug resistance (CecR) (Yamanaka et al., 2016), acid resistance (BluR, GadW, GadX, AdiY, TorR, H-NS, and Fis) (Bordi et al., 2003; Bradley et al., 2007; Giangrossi et al., 2005; Gong et al., 2003; Ma et al., 2002; Tschowri et al., 2009), and oxidative stress response (HypT, Fis, and Fur) (Bradley et al., 2007; Drazic et al., 2013; Tardat & Touati, 1993).

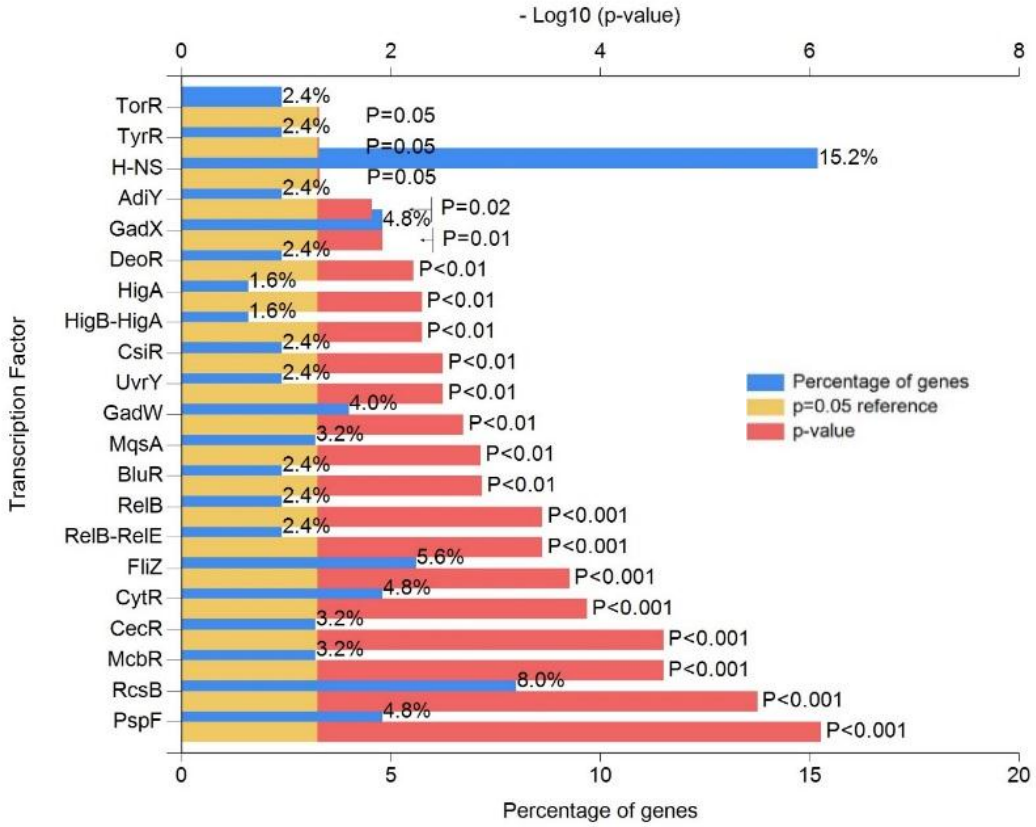

Figure 6.5 Enrichment analysis for the regulating transcription factors of the differentially expressed genes after BMA addition.

Transcription factors with roles in regulation of genes involved in chemostaxis, motility, and biofilm formation (H-NS, RcsB, BluR, FliZ, McbR, MqsA, and Fis) (Bradley et al., 2007; Krin et al., 2010; Pesavento & Hengge, 2012; Tschowri et al., 2009; Yamaguchi et al., 2009; Zhang et al., 2008) and DNA and RNA biosynthesis, transport, and utilization (CytR, DeoR, and PurR) (Cho et al.,

2011; Munch-Petersen & Jensen, 1990; Sernova & Gelfand, 2012) were also well represented.

The seemingly complex involvement of various regulatory networks, stress response systems, and cellular processes in response to exposure to BMA is in accordance with reports from recent efforts to understand the cellular response upon exposure of bacteria with various other industrially relevant chemical products such as biofuels and other solvent like molecules (Brynildsen & Liao, 2009; Rau et al., 2016; Shimizu, 2013b; Yung et al., 2016). This is exemplified by the most significantly perturbed regulon under the transcription of Fis (Figs. 6.5 and 6.6) that regulates a diverse set of genes from various operons that are related to transport, acid stress response, oxidative stress response, cell structure, chemotaxis, motility, biofilm formation, virulence, carbon compound metabolism, central intermediary metabolism, amino acid metabolism, and nucleotide metabolism (Bradley et al., 2007).

Fis is abundant during cell growth, but repressed during stationary phase under the control of the stringent response (Shimizu, 2013b). The regulation of the stringent response is highly linked to CsrA. CsrA is a dimeric RNA binding protein that plays a huge role in the regulation of translation and/or stability of the bound transcript, which effectively affects global gene expression including those related to virulence factors, quorum sensing, motility, biofilm formation, carbon metabolism, and etc. (Edwards et al., 2011). The binding of CsrA to its regulated transcript can be disrupted upon interaction with the non-coding RNAs CsrB and CsrC, which consequently affects gene expression of CsrA regulated genes (Edwards et al., 2011; Mondragón et al., 2006; Suzuki et al., 2002).

Both *csrB* and *csrC*, both under the regulation of BarA-UvrY (Edwards et al., 2011; Mondragón et al., 2006), were observed to be differentially upregulated after addition of BMA (Table 6.3; Fig. 6.6). BarA-UvrY regulatory system can be activated by the abundance of guanosine pentaphosphate ((p)ppGpp), which can be synthesized by RelA or hydrolysed to its tetraphosphate form by SpoT (Edwards et al., 2011; Shimizu, 2013b).

Fis

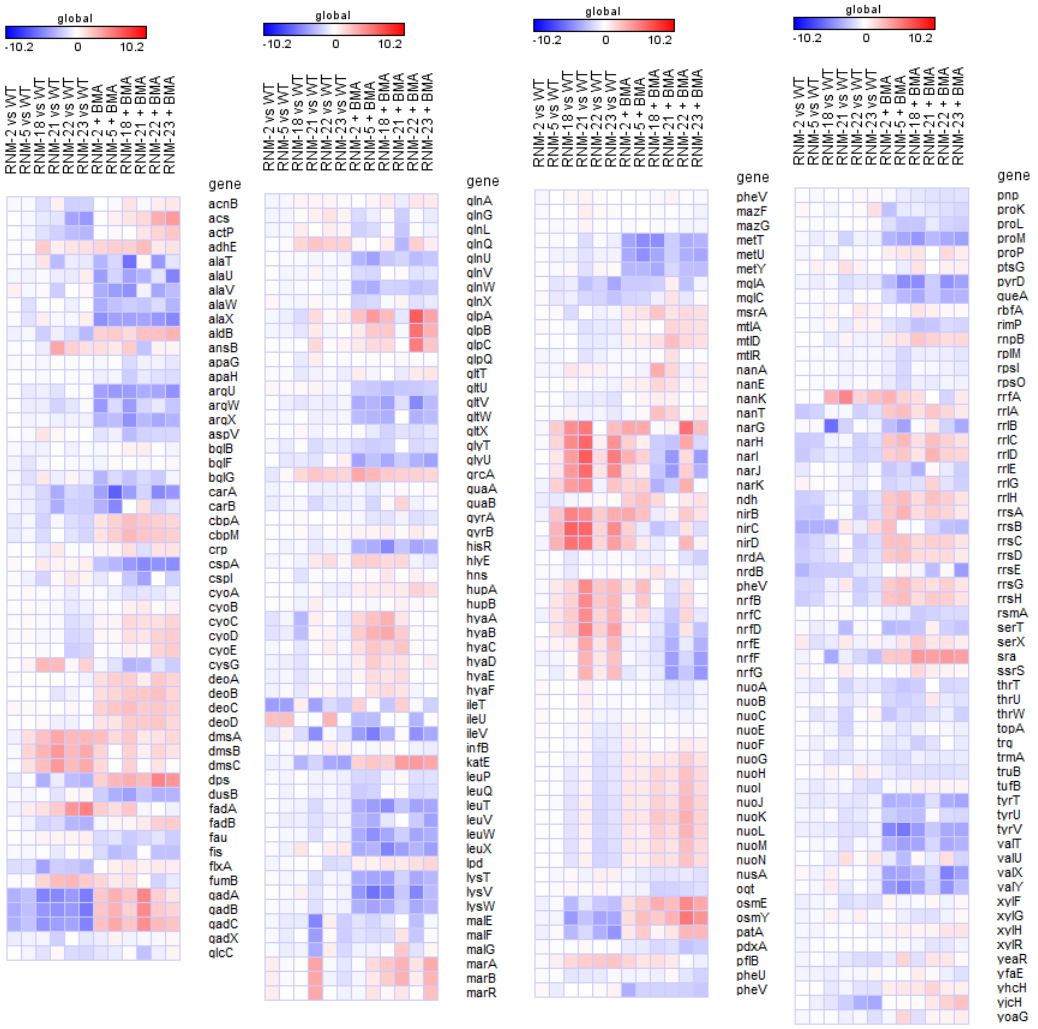

Figure 6.6 Heat map for genes regulated by Fis. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

Gene expression of *relA* was relatively unperturbed, while *spoT* was slightly downregulated (Fig. 6.7 a). The slight downregulation of *spoT* may have contributed to the increase in (p)ppGpp level due to reduced hydrolysis and subsequent activation of BarA-UvrY for the increased expression of *csrB* and *csrC* (Fig. 6.7 UvrY). The escalation in CsrB and CsrC levels may have contributed to the observed distortion in gene expression of genes for various cellular processes under the regulation of Fis (Edwards et al., 2011).

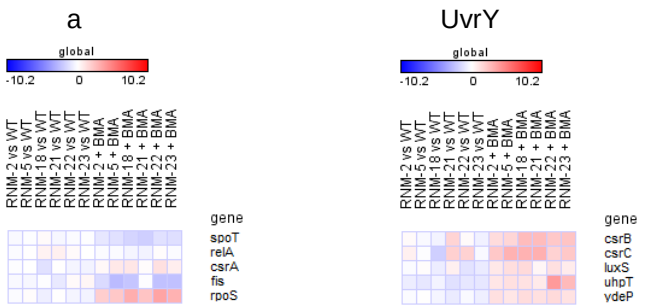

Figure 6.7 Heat map of genes related to (a) stringent control and UvrY. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

RpoS is another important global regulator that has been reported to be positively regulated by the level of (p)ppGpp. However, the mechanism of its regulation is unclear (Hengge-Aronis, 2002; Shimizu, 2013b). RpoS is a sigma factor that acts as a master regulator for various stress response systems in *E. coli* that includes oxidative, lethal heat shocks, hyperosmolarity, acid, ethanol, near UV- irradiation and potentially yet to be identified stress (Hengge-Aronis, 2002). The expression of *rpoS* was observed to be differentially upregulated after addition of BMA (Table 6.3 and Fig. 6.7), which could have contributed to the activation/deactivation genes from various stress response systems in *E. coli* (Table 6.7). Among them, the oxidative stress was the most significantly enriched (Table 6.7).

The oxidative stress response acts to relieve the cells from the harmful reactive oxygen species (ROS) generated during cell growth such as hydrogen peroxide (H_2O_2), superoxide anions ($O2^{-}\cdot$), hydroxyl radicals ($OH^{-}\cdot$), and etc. as well as the damage caused by these ROS (Chiang & Schellhorn, 2012; Ezraty et al., 2017). The enzymes encoded by the differentially upregulated genes *katE, sodB*, and *sodC* directly converts H_2O_2 (Seaver & Imlay, 2001; Sorkin & Miller, 1997) and $O2^{-}\cdot$ (Sorkin & Miller, 1997) into H_2O and H_2O_2, while *osmC*, *qorA*, and *wrbA* reduces organic hydroperoxides (Lesniak et al., 2003) and quinones (Maruyama et al., 2003; Patridge & Ferry, 2006). Furthermore, protein damage through oxidation of sulfur in methionine and reaction with glyoxals can be repaired by the enzymes encoded by *msrA* (St John et al., 2001), *yajL* (Abdallah et al., 2016), and *yhbO* (Abdallah et al., 2007; Abdallah et al., 2016). Another

gene that could play an important role in the oxidative stress response is the differentially downregulated gene, *rseC* (Table 6.8). The gene product of *rseC*, along with *rsxABCDGE*, has been proposed to be responsible in re-reduction of [2Fe-2S] cluster in SoxR, which restores SoxR into its inactive state (Koo et al., 2003). Since *rseC* is downregulated, it could lessen inactivation of SoxR and allow SoxR mediated oxidative stress response to remain active. However from the enrichment analysis of transcription factors and heat map (Figs. 6.3 and 6.4), the SoxS-MarA-Rob oxidative stress response related genes (e.g. *sodA*, *fur*) are not consistently up or downregulated after BMA addition. This suggests that the oxidative stress response exhibited by the BMA tolerant strains might not be reliant on SoxRS regulated oxidative stress response (Chiang & Schellhorn, 2012).

An important aspect in the oxidative stress response is the iron homeostasis (Chiang & Schellhorn, 2012). Iron in either Fe^{2+} or Fe^{3+} configuration facilitates the formation of hydroxyl radicals and hyperoxide radicals via the Fenton and the Haber-Weiss reactions (Andrews et al., 2003; Touati, 2000). Thus, excessive iron would favour further production of ROS that can damage cellular components and interrupt vital processes. In order to limit further production of ROS, cells regulate the amount of iron within the cell that can participate in the formation of more ROS (Andrews et al., 2003; Cornelis et al., 2011; Lau et al., 2015; Touati, 2000). A number of genes that are related to transport of Fe^{3+} with the aid of siderophores (*efeB*, *efeU*, *fecA*, *fecB*, *fecC*, *fecI*, *fecR*, *fepC*, *fepE*, *fepG*, *fhuB*, *fhuC*, and *fhuD*) (Andrews et al., 2003; Cao et al., 2007; Ochs et al., 1995) and their release within the cell (*fes* and *fhuF*) (Caza et al., 2015; Matzanke et al., 2004) were differentially downregulated, while a gene (*dps*) that is involved with the sequestration and storage of Fe^{2+} (Nair & Finkel, 2004) was differentially upregulated (Tables 6.7 and 6.8; Fig. 6.8). A number of these genes and their operons are regulated by HypT and Fur (Fig. 6.8), which acts as their repressor when activated. HypT is activated via methionine oxidation in HypT itself (Drazic et al., 2013), while Fur binds with its corepressor Fe^{2+} to increase its repression activity (Andrews et al., 2003). The changes in the gene expression of these iron-homeostasis related genes could have aided in limiting the iron available for further production of ROS.

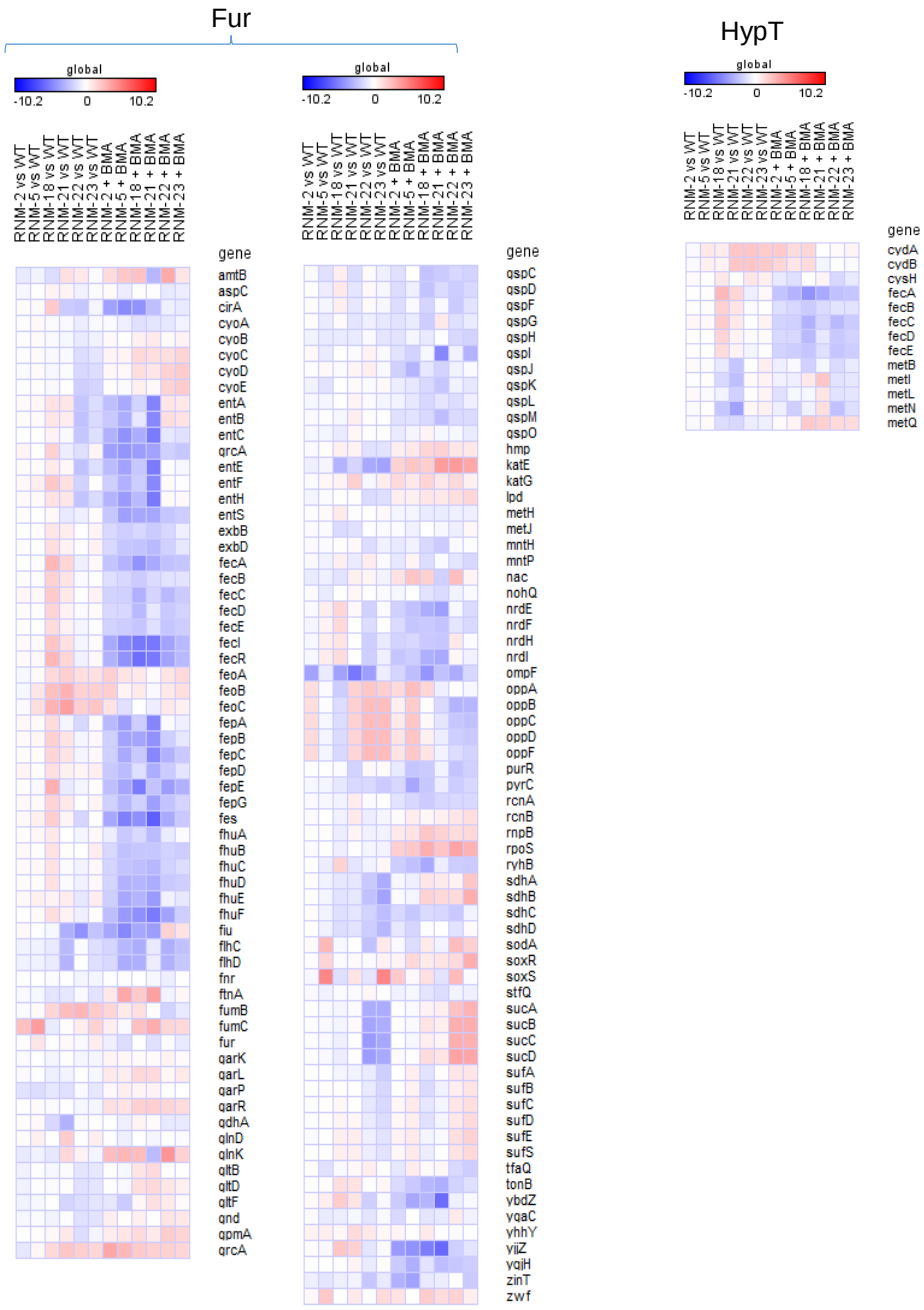

Figure 6.8 Heat map for genes regulated by Fur and HypT. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

The next most significantly represented stress response is the response to temperature stimulus (Table 6.7). Genes that encode for heat shock proteins (*clpB, dnaK, hspQ, ibpA, ibpB, ldhA, eutD,* and *rpoH*) and cold shock proteins (*cspA*, *cspB*, and *cspG*) were differentially upregulated and downregulated, respectively. Thus, suggesting the potential role of the heat shock response in BMA tolerance (Brynildsen & Liao, 2009; Cao et al., 2017; Rau et al., 2016). Heat shock response allow cells to adapt and survive during exposure to

various environmental stress conditions such as heat shock, metabolically harmful substances, and complex metabolic processes that cause protein damage or other cellular components (e.g. membranes and nucleic acids) (Arsène et al., 2000; Richter et al., 2010; Whitley et al., 1999). The heat shock related proteins include those that aid with repair/degradation of denatured proteins (*clpB*, *dnaK*, *hspQ*, *ibpA*, and *ibpB*) (Bertelsen et al., 2009; Kuczynska-Wisnik et al., 2002; Shimuta et al., 2004; Zolkiewski, 1999), energy generation (*ldhA* and *eutD*) (Bologna et al., 2010; Bunch et al., 1997), and activation of the heat shock response itself (*rpoH*) (Arsène et al., 2000).

The third most significantly enriched stress response system was the phage shock response (Table 6.7), which also had its regulating transcription factor PspF significantly enriched (Fig. 6.5). PspF is responsible for the activation of *pspABCDE* and *pspG* operons (Fig. 6.9).

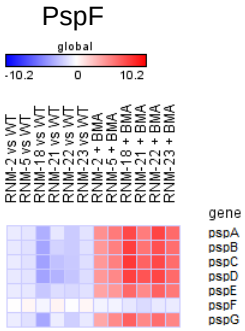

Figure 6.9 Heat map for genes regulated by PspF. Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

The response to pH was the fourth most significantly enriched stress related GO (Table 6.7). This set includes the acid stress resistance genes such as *hdeA*, *hdeB*, and *ydeP*. Aside from these genes, *gadA*, *gadB*, and *gadC*, which are known for their role in acid resistance, were also upregulated (Fig. 6.6). This suggested the possible role of acid stress response in BMA tolerance. The proteins encoded by *hdeA* and *hdeB* function as molecular chaperones that prevents low pH protein aggregation at the periplasm (Kern et al., 2007). YdeP, an oxidoreductase, overexpression has been reported to improve acid resistance (Masuda & Church, 2003). The genes *gadA* and *gadB* encode the enzyme isoforms with the ability to convert glutamate to gamma-aminobutyrate

(GABA), while consuming H^+ in the reaction. Thus, they aid in pH maintenance (Braun et al., 2017). On the other hand, GadC acts as an antiporter for glutamate and GABA (Capitani et al., 2003).

The last most significantly enriched stress response GO term of the biological process category is the response to ethanol (Table 6.8). The genes *adhP* (Thomas et al., 2013) and *aldB* (Ho & Weiner, 2005) encode enzymes that catalyze oxidation-reduction reactions of short alcohols and aldehydes, respectively. On the other hand, *uspB* encodes a universal stress protein induced upon exposure of *E. coli* to ethanol (Farewell et al., 1998). Their increased expression can aid with energy generation from ethanol as well detoxification by reduction of ethanol concentration (Ho & Weiner, 2005; Thomas et al., 2013). However, their role in BMA tolerance is uncertain. The overexpression of these genes can also be a consequence from the activation of RpoS regulated genes as *aldB* and *uspB* are known to be under the regulation of RpoS (Farewell et al., 1998; Ho & Weiner, 2005).

Aside from the stress response systems enriched from the GO terms, the emergence of CecR (also known as YbiH) from the transcription factor enrichment analysis (Fig. 6.4) suggests the involvement of another multidrug resistance mechanism (Yamanaka et al., 2016) might be activated in the presence of BMA.

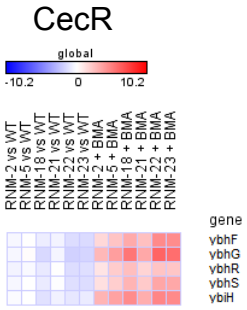

Figure 6.10 Heat map for genes regulated by CecR (YbiH). Gene logFC of BMA tolerant strain relative to parental strain (columns 1-6) and BMA tolerant strain after BMA addition relative to before BMA addition (columns 7-12).

The CecR regulon contains *ybhFRS*, *ybhG*, and *ybiH*, which encode an ATP-binding cassette (ABC)-type transporter, a predicted inner membrane of

unknown function, and the transcription factor itself, respectively (Yamanaka et al., 2016).

Genes that are involved in DNA replication were also differentially expressed after exposure of BMA tolerant strains to BMA. A number of genes that encode proteins known to inhibit DNA replication were upregulated (*cspD* and *higB*) (Uppal et al., 2014; Wood & Wood, 2016), while genes that promote DNA replication were observed to be downregulated (*dnaB*, *priA*, *recG*, *recQ*, and *yoaA*) (Brown et al., 2015; Cooper et al., 2015; Dou et al., 2004; Harami et al., 2015; LeBowitz & McMacken, 1986; Manhart & McHenry, 2013) (Tables 6.7 and 6.8). Various genes that can be linked to protein synthesis were also differentially expressed (Tables 6.7 and 6.8). Downregulation of genes related to transfer RNA maturation (*mnmA*, *trmN*, *trmL*, *tsaD*, *dusB*, *queA*, and *rlmC*) (Benítez-Páez et al., 2010; Bou-Nader et al., 2017; Desmolaize et al., 2011; Deutsch et al., 2012; Dineshkumar et al., 2002; Fislage et al., 2012; Kambampati & Lauhon, 2003), ribosomal RNA maturation (*rlmC*, *rlmG*, *rluB*, *rluC*, and *rluE*) (Conrad et al., 1998; Czudnochowski et al., 2014; Desmolaize et al., 2011; Pan et al., 2007; Sergiev et al., 2006), and translational initiation (*infA*) (Milón et al., 2012) were observed. On the other hand, genes that are linked to inihibition of protein synthesis (*relE* and *rmf*) (Bøggild et al., 2012; Yoshida et al., 2009) were upregulated. The observed differential expression in DNA and RNA related processes suggests that exposure to BMA inhibits DNA replication and protein synthesis, which could consequentially inhibit various cellular processes including growth.

A notable significantly represented GO term related to molecular function is the oxidoreductase acitivity acting on the aldehyde or oxo group donors (Table 6.8). The upregulated genes (*aceE*, *aceF*, *aldB*, *eutE*, *gabD*, *paoB*, *patD*, *putA*, and *ydeP*) (Becker & Thomas, 2001; Bologna et al., 2010; Langendorf et al., 2010; Masuda & Church, 2003; Neumann et al., 2009; Patel et al., 2014; Schneider & Reitzer, 2012; Xu & Johnson, 1995) encode enzymes involved in oxidation and energy generation, while the downregulated genes (*argC* and *gtrA*) (Baicha & Vogel, 1962; Kim et al., 2009) encode enzymes involved in reduction

reactions. The differential expression of these genes suggests potential role of energy generation for BMA tolerance.

Changes in the transcriptome of the parental strain upon exposure to BMA was also determined as a control experiment. A total of 2316 genes were differentially expressed with 1081 and 1235 differentially upregulated and downregulated, respectively (Appendix Table 11.7). The differentially upregulated genes in the parental strain after BMA addition were compared to the differentially upregulated genes common to the six BMA tolerant strains with respect to the wild-type and after BMA addition. On the other hand, the differentially downregulated genes in the parental strain after BMA addition were compared to the differentially downregulated genes common to the six BMA tolerant strains with respect to the wild-type and after BMA addition. This was done to determine the difference in the response of the parental strain and BMA tolerant strains upon exposure to BMA. It could potentially give an insight of the additional response necessary to enable growth of the parental strain in the presence of BMA (20% v/v). A total of 81 genes were differentially expressed in the BMA tolerant strains but not in the parental strain after BMA addition (Table 6.10).

Table 6.10 List of differentially expressed genes exclusive to the BMA tolerant strains

Condition	Gene names
with reference to parental strain	*cheW cheY cheZ fimA fliC flxA gadC hdeB slp tar tsr yhiM*
After addition of 20% v/v BMA	***aceE aceF adhP agp ahr aldB cbpA cfa chrR csgA csgB eutD eutE eutG eutM fbaB gabD gabP gabT gadA gadB gadC ggt glxR gstB hdeA hdeB katE leuD mscS narU narW narY narZ nuoK patD pfkA pfkB pgl psiF putA rclA sfmA talA tktB ushA yahK yahO ycaC ydiZ yfdQ yghU yiaG ymgA ymgC yniA*** *amiC ftsB hyfA kdgT lpxP pncB ppdD rcnA rseC ybeF yegK yijF yjhR ynfN yohO*

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

An enrichment analysis based on the GO terms biological process, molecular function and cellular for these genes was also done to facilitate grouping of genes with similar functionality (Tables 6.11, 6.12, and 6.13).

Table 6.11 Summary of significantly enriched GO terms based on biological process for the differentially expressed genes exclusive to the BMA tolerant strains

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0030004; cellular monovalent inorganic cation homeostasis	3; 30; 0.015	***gadA gadB gadC***
GO:0006935; Chemotaxis	5; 12.20; 0.006	*cheW cheY cheZ tar tsr*
GO:0030198; extracellular matrix organization	2; 100; 0.015	***csgA csgB***
GO:0009450; gamma-aminobutyric acid catabolic process	3; 75; 6.34×10^{-4}	***gabD gabP gabT***
GO:0046496; nicotinamide nucleotide metabolic process	8; 14.04; 8.97×10^{-6}	***ace aceF adhP agp ahr*** *amiC* ***cfa eutD eutE eutG eutM fbaB gabD gabP gabT gadA gadB glxR leuD*** *lpxP* ***narU narW narY narZ nuoK patD pfkA pfkB pgl*** *pncB* ***putA talA ushA*** *ybeF* ***ydiZ yiaG***
GO:0042126; nitrate metabolic process	4; 18.18; 0.008	***narU narW narY narZ***
GO:0090304; nucleic acid metabolic process	4; 0.14; 5.40×10^{-8}	***putA*** *ybeF* ***ydiZ yiaG***
GO:0055114; oxidation-reduction process	22; 2.30; 0.005	***aceE aceF adhP ahr aldB chrR eutE eutG gabD glxR gstB*** *hyfA* ***katE narW narY narZ patD rclA yahK yghU***
GO:0045471; response to ethanol	2; 66.67; 0.041	***adhP aldB***
GO:0044712; single-organism catabolic process	17; 3.51; 2.72×10^{-4}	***aceE aceF adhP agp*** *amiC* ***eutD eutE eutG eutM fbaB gabD gabP gabT gadA gadB ggt glxR gstB leuD nuoK patD pfkA pfkB pgl*** *pncB* ***putA talA ushA yghU***
GO:0044281; small molecule metabolic process	30; 2.43; 2.65×10^{-5}	***aceE aceF adhP agp*** *amiC* ***cfa eutD eutE eutG eutM fbaB gabD gabP gabT gadA gadB ggt glxR gstB leuD narU narW narY narZ nuoK patD pfkA pfkB pgl*** *pncB* ***putA talA ushA yghU***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Based on the GO terms biological process, the differentially expressed genes exclusive to the BMA tolerant strains are related to cellular monovalent inorganic cation homeostasis, chemotaxis, extracellular matrix organization, gamma-aminobutyric acid catabolic process, nicotinamide nucleotide metabolic process, nitrate metabolic process, nucleic acid metabolic process, oxidation-reduction process, response to ethanol, single organism catabolic process, and small molecule metabolic process (Table 6.11). Alcohol dehydrogenase, aldehyde dehydrogenase, glutamate decarboxylase activity, nucleic acid binding, protein histidine kinase binding, purine ribonucleoside binding, and pyruvate dehydrogenase activity were the most significantly enriched GO terms in terms of molecular function (Table 6.12). The enriched GO terms cellular

component include cell projection, cytoplasmic part, intrinsic component of membrane, and nitrate reductase complex (Table 6.13).

Notable stress related systems associated with the genes that were exclusively differentially upregulated in BMA tolerant strains after BMA addition include glutamate dependent acid resistance (*gadA*, *gadB*, *gadC*, *gabD*, *gabP*, *gabT*, and *patD*) (Braun et al., 2017; Brechtel et al., 1996; Capitani et al., 2003; Langendorf et al., 2010; Liu et al., 2005; Schneider et al., 2013), oxidative stress (*katE*) (Schellhorn & Hassan, 1988), osmotic stress (*mscS*) (Bass et al., 2002), and modification of unsaturated fatty acid into a cyclopropane fatty acid (*cfa*) (Chang et al., 2000).

Table 6.12 Summary of significantly enriched GO terms based on molecular function for the differentially expressed genes exclusive to the BMA tolerant strains

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0004022; alcohol dehydrogenase (NAD) activity	4; 28.57; 5.274×10^{-4}	***adhP ahr eutG yahK***
GO:0004029; aldehyde dehydrogenase (NAD) activity	5; 35.71; 9.70×10^{-6}	***aceE aceF aldB eutE gabD patD putA***
GO:0004351; glutamate decarboxylase activity	2; 66; 0.017	***gadA gadB***
GO:0003676; nucleic acid binding	4; 0.14; 5.27×10^{-8}	*cbpA putA ybeF yiaG*
GO:0043424; protein histidine kinase binding	2; 100; 0.006	*tar tsr*
GO:0035639; purine ribonucleoside triphosphate binding	2; 0.12; 5.15×10^{-4}	***chrR pfkA pfkB***
GO:0004738; pyruvate dehydrogenase activity	2; 100; 0.006	***aceE aceF***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Table 6.13 Summary of significantly enriched GO terms based on cellular component for the differentially expressed genes exclusive to the BMA tolerant strains

GO term	Count; % associated genes; Corrected P-value	Associated differentially expressed genes
GO:0042995; cell projection	7; 6.48; 0.001	*cheZ* ***csgA csgB*** *fimA fliC ppdD* ***sfmA***
GO:0044444; cytoplasmic part	24; 1.97; 0.005	***aceE aceF cfa*** *cheW cheY cheZ* ***chrR eutG fbaB gabT gadA gadB gstB katE leuD patD pfkA pfkB pgl*** *pncB ppdD* ***talA tktB ycaC***
GO:0031224; intrinsic component of membrane	17; 0.46; 4.31×10^{-5}	***cfa*** *fimA ftsB* ***gabP gadC*** *kdgT lpxP* ***mscS narU nuoK*** *ppdD rcnA rseC tar tsr yhiM yohO*
GO:0009325; nitrate reductase complex	2; 33.33; 0.022	***nary narZ***

Notes: Text in bold-italics are differentially upregulated genes, regular-italics are differentially downregulated genes.

Potential importance of increasing energy generation and essential building block synthesis in BMA tolerance can also be observed with the differentially upregulated genes related to glucose utilization under the Embden-Meyerhof-Parnas (EMP) pathway (*pfkA*, *pfkB*, and *fbaB*) (Dadinova et al., 2016; Lovingshimer et al., 2006) and pentose phosphate pathway (*pgl* and *talA*) (Song et al., 2006; Zimenkov et al., 2005), generation of acetyl-COA for the TCA cycle (*aceE* and *aceF*) (Patel et al., 2014), luecine biosynthesis (*leuD*) (Fultz et al., 1979), nitrate/nitrite utilization (*narU*, *narW*, *narY*, and *narZ*) (Blasco et al., 1990; Clegg et al., 2002), and various oxidation reduction processes (*adhP, ahr, eutG, yahK, aldB, eutE, putA, chrR, gstB,* and *nuoK*) (Akhtar et al., 2013; Becker & Thomas, 2001; Bologna et al., 2010; Chrysostomou et al., 2015; Eswaramoorthy et al., 2012; Ho & Weiner, 2005; Pick et al., 2013; Stojiljkovic et al., 1995; Thomas et al., 2013; Torres-Bacete et al., 2012).

6.4 Conclusions

The transcriptomics analysis revealed key evolutionary outcomes from the adaptive evolution of *E. coli* for BMA tolerance as well as the response of BMA tolerance strains upon exposure to BMA (20% v/v), which suggest potential mechanisms for BMA tolerance. BMA tolerant strains exhibited increased expression of *acrAB* and reduced expression of acid resistance, chemotaxis, motility, flagellum and pili biosynthesis, and biofilm formation related genes. The former were possibly involved in expulsion of BMA entering the cell, while the latter as an energy saving mechanism. Exposure to BMA (20% v/v) may have stimulated activation of the oxidative stress, heat shock, phage shock, and acid stress response systems and membrane modifying, energy generating, and essential building block synthesizing enzymes, whilst possibly inhibited DNA replication and protein synthesis in BMA tolerant strains. Comparison of the parental strain and BMA tolerant strains response to BMA (20% v/v) exposure revealed that the parental strain's inability to grow in the presence of BMA (20% v/v) may be due to the lack in increased expression of key genes involved in membrane modification, osmotic stress, glutamate dependent acid resistance, oxidative stress, energy generation, and essential building blocks synthesis.

7

Genome Shuffling and BMA Production

7.1 Introduction

The BMA tolerant *E. coli* strains generated *via* ADE (Chapter 5) has shown potential for use as host cell. However, most of the evolved *E. coli* strains were only able to achieve moderate cell density and marginal growth rate or low cell density at a very high growth rate in the presence of BMA (20% v/v) with reference to growth of the parental strain in the absence of BMA. The emergence of the BMA tolerant strain RNM-18 showed the potential to achieve both high cell density and growth rate. In comparison to the parental strain grown in the absence of BMA, RNM-18 grown in the presence of BMA was able to grow as fast but only achieved 72% of the maximum cell density. The reduced cell density suggests growth inhibition (Theophel et al., 2014) and possibly inefficient use of cellular resources (Trinh et al., 2008).

It might still be possible to further improve the evolved strains ability to grow in the presence of BMA (20% v/v) through further evolution and/or application of any of the random mutagenesis techniques, reverse/inverse metabolic engineering, rational/targeted engineering (Chapter 2). Although results from the genome resequencing and transcriptomics analysis can be used for the reverse/inverse metabolic engineering or the rational/targeted approach, it will likely take considerable amounts of time and effort to identify and test which genes/proteins are able to contribute to enhanced growth in the presence of BMA (20% v/v). As a diverse set of mutations has already been acquired by various BMA tolerant strains from adaptive evolution (Chapter 5), further evolution or application of other random mutagenesis techniques may or may not contribute further. Thus, rather than application of further mutagenesis, the current set of mutations can be exploited to generate new combinations of mutations through genome shuffling (Biot-Pelletier & Martin, 2014; Gong et al., 2009; Winkler et al., 2010). In this chapter, genome shuffling was used to further improve growth of the evolved strains in the presence of BMA (20% v/v). BMA production in the parental strain and ADE generated BMA tolerant strains was also tested.

7.2 Genome Shuffling

The twelve unique BMA tolerant *E. coli* strains generated from adaptive evolution (Table 5.4) were used as starting population to generate new combinations of mutations *via* genome shuffling. The resulting population from genome shuffling was grown in M9 minimal medium containing BMA (20% v/v) for 2 and 10 sequential batch cultures to facilitate selection and enrichment of better growing strains (Dunlop et al., 2011). The growth in minimal media containing BMA (20% v/v) of the isolates from the enriched genome shuffled strains were compared to RNM-18, which was the best strain isolated from ADE (Chapter 5), to test for improvements. All of the tested strains had slightly lower growth rates, while three strains (RNM-28, RNM-29, and RNM-30) isolated after enrichment with 10 sequential batch transfer were able to achieve higher cell densities as compared to RNM-18 (Fig. 7.1). The isolated strain RNM-29 achieved the highest cell density, which was 32.5% more than that of strain RNM-18.

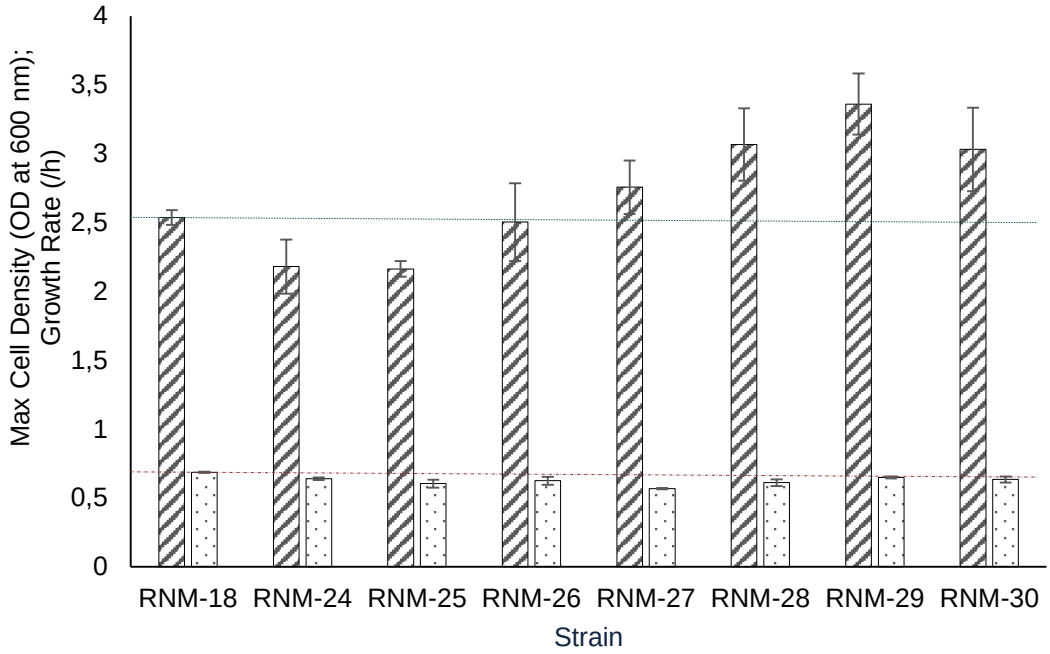

Figure 7.1 Comparison of max cell density (dashed line) and growth rate (long dash dot dot line) of BMA tolerant isolates from genome shuffling and strain RNM-18. Strains were grown in M9 minimal medium at 37°C and 200 RPM using 250 mL conical flasks with 50 mL media. Legend: Max cell density (diagonal bars), growth rate (dotted bars).

7.2.1 Genome resequencing

The genome sequence of the best strain (RNM-29) isolated from genome shuffling and enrichment was analysed to determine the combination of mutations acquired (Table 7.1). Strain RNM-29 had point mutations in *rob*, *rpoC*, *ilvN*, and *ygbK,* a 1 bp deletion in *pepA*, an 18 bp deletion in *lpxM,* and insertion mutation in *acrR* (Table 7.2 and Appendix Tables 11.3, and 11.4). The point mutations observed in *rob*, *rpoC*, *ilvN*, and *ygbK* caused a single amino acid change at residue 156 (Arg→His), 361 (Leu→Arg), 41 (Cys→Tyr), and 294 (Ala→Glu) for Rob, RpoC, IlvN, and YgbK, respectively. The 1 bp deletion in *pepA* led to a change in amino acid sequence starting residue 76 and resulted in truncation of the protein at residue 99, instead of residue 503. The 18 bp deletion mutation in *lpxM* caused removal of amino acid residues 267-272, but left the remaining amino acid sequence the same as the WT protein. The insertion mutation in *acrR* led to a change in amino acid sequence starting residue 91 and resulted in truncation of the protein at residue 101, instead of residue 215 (Appendix Tables 11.3 and 11.4).

Table 7.1 Summary of the combination of mutations acquired after genome shuffling and enrichment.

Strain	Mutations acquired
RNM-29	*acrR*(E91fs) *rob*(R156H) *ygbK*(A294E) *ilvN*(C41Y) *rpoC*(L361R) *pepA* (I76fs) *lpxM*(267_272 del)

The profile of the mutations present in strain RNM-29 is closest to strain RNM-2 due to the common mutations in *acrR*, *rob*, *ygbK*, *ilvN*, *rpoC*, and *lpxM*. This suggests that the parental strain of RNM-29 was RNM-2, which lost the mutation in *ompT* and acquired a deletion mutation for *pepA*. Aside from the reversion of the mutations in *ompT*, there was no gene conformation that was common with the other strains. The deletion mutation in *pepA* was not observed previously in any of the strains. It is possible that deletion mutation was acquired along the genome shuffling experiment or the enrichment procedure.

7.3 1-Butanol susceptibility

The susceptibility of *E. coli* BW25113 and strain RNM-18 for n-butanol was tested to get an idea of the butanol concentration that can be used for BMA production without completely inhibiting cell growth (Fig. 7.2). It can be observed that *E. coli* BW25113 can still grow until 1-butanol concentration of 0.875% v/v (145 mM), while strain RNM-18 can only grow with 0.5% v/v 1-butanol (83 mM). This suggests that the BMA tolerant strain RNM-18 is more susceptible towards 1-butanol as compared to the parental strain and could potentially limit the working 1-butanol concentration for BMA production.

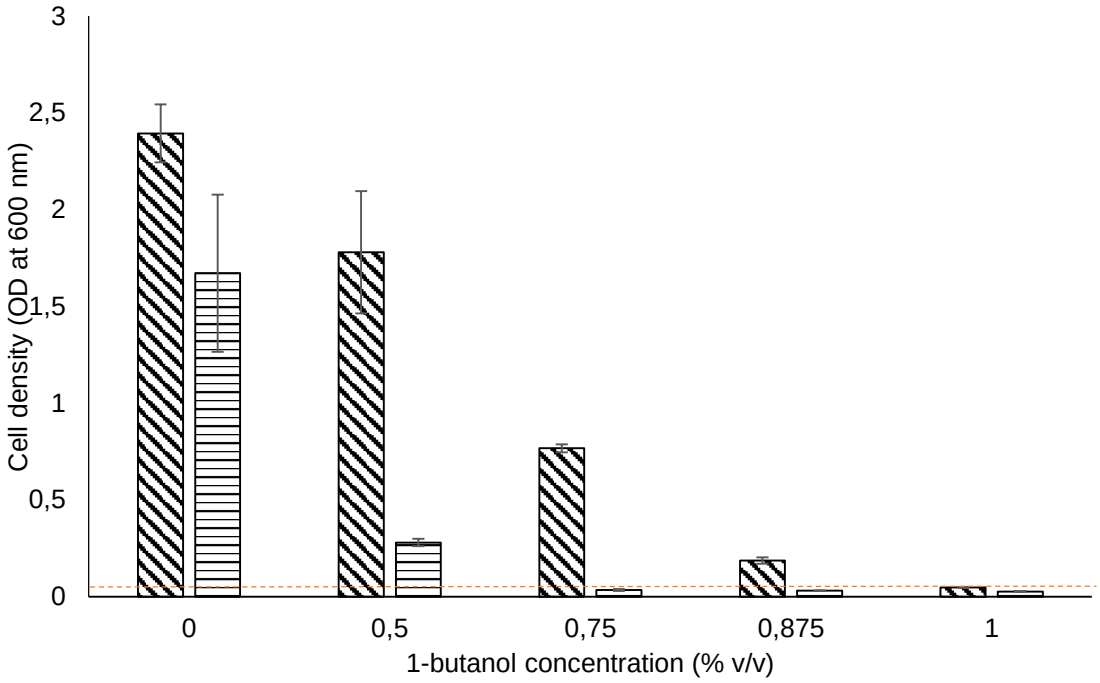

Figure 7.2 Determination of 1-butanol susceptibility of *E. coli* strains. Cultures were grown for 36 h in M9 minimal medium (5 mL) supplemented with 10 g/L glucose at 37°C and 200 RPM in 30 mL sealed glass vials containing n-butanol at a starting OD600 of 0.05 (dashed line). OD600 after 36 h is indicated in the figure. Legend: *E. coli* BW25113-WT (diagonal bars) and RNM-18 (horizontal bars).

7.4 BMA production

The production of BMA using *E. coli* BW25113-WT and the 6 strains (RNM-2, RNM-5, RNM-18, RNM-21, RNM-22, and RNM-23) used for the transcriptomics analysis (Table 6.1) as the host strain was done to test whether or not the strains with improved BMA tolerance can produce higher titres of BMA as compared to the parental strain. The strains where chosen for the diversity in the profile of affected genes from the acquired mutations and characteristics of growth in the presence of BMA. BMA production was tested *via* a biotransformation to convert 3-methyl-2-oxobutanoate/2-ketoisovalerate (KIV) and 1-butanol to BMA (Fig. 7.3). The host strains were transformed with the plasmid pBAD-MMA050_mACX4 corrected (3), which can be induced with arabinose to express the necessary enzymes for the production of BMA from KIV (Fig. 7.3).

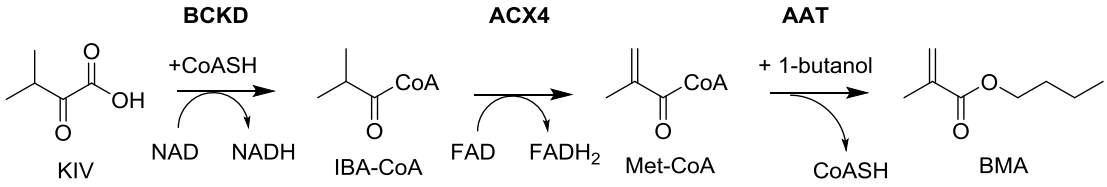

Figure 7.3 Scheme for BMA production from 2 –ketoisovalerate
Legend: BCKD = branched-chain alpha-keto acid dehydrogenase complex, Acx4 = acyl-coenzyme A oxidase 4, AAT = alcohol acyl transferase, KIV = 2 – ketoisovalerate, IBA-CoA = isobutyryl-COA, Met-CoA = methacrylyl-CoA, CoASH = Coenzyme A, NAD/NADH = nicotinamide adenine dinucleotide, FAD/$FADH_2$ = flavin adenine dinucleotide

The host strains were allowed grow with aeration for 12 h to achieve high cell density prior to the induction for expression of the enzymes required for BMA production from KIV. Cell densities (OD at 600 nm) of 10-13 were attained (Table 7.2). BMA production was initiated by induction with arabinose (200 µg/mL) and addition of 1-butanol (15 mM). BMA production was observed at 0.005-0.043 mM within 6 h, but remained at this range 24 h after induction (Table 7.3). The highest BMA production was observed with strain RNM-18 and *E. coli* BW25113-WT. Aside from BMA, other butyl esters were also observed to be produced (Appendix Figs. 11.7). The major byproducts observed include butyl isobutyrate (BIB), butyl acetate (BA), and butyl isovalerate (BIV) (Figs. 11.9-11.11).

Table 7.2 Cell density and viability *E. coli* strains during BMA production test

Time after induction (h)	Strain	Cell Density (OD at 600 nm)	Cell Viability (CFU/mL)
0	WT	10.32 ± 0.98	$4.15x10^{9} \pm 6.46x10^{8}$
	RNM-2	10.52 ± 0.17	$3.60x10^{8} \pm 2.27x10^{8}$
	RNM-5	10.23 ± 0.15	$2.47x10^{8} \pm 1.73x10^{8}$
	RNM-18	12.70 ± 0.66	$1.06x10^{9} \pm 2.15x10^{8}$
	RNM-21	10.32 ± 0.98	$1.45x10^{10} \pm 4.70x10^{9}$
	RNM-22	12.78 ± 0.95	$1.24x10^{9} \pm 4.63x10^{8}$
	RNM-23	11.18 ± 0.23	$2.23x10^{10} \pm 1.78x10^{10}$
6	WT	11.38 ± 0.68	$1.31x10^{11} \pm 6.16x10^{10}$
	RNM-2	13.15 ± 0.09	$5.30x10^{8} \pm 2.88x10^{8}$
	RNM-5	11.95 ± 0.60	$1.99x10^{9} \pm 1.34x10^{9}$
	RNM-18	12.12 ± 0.58	$2.37x10^{9} \pm 4.57x10^{8}$
	RNM-21	11.38 ± 0.68	$3.03x10^{10} \pm 1.54x10^{10}$
	RNM-22	11.62 ± 0.98	$1.43x10^{9} \pm 5.91x10^{8}$
	RNM-23	11.92 ± 0.36	$3.80x10^{9} \pm 1.95x10^{9}$
24	WT	10.35 ± 0.23	$2.14x10^{10} \pm 9.19x10^{9}$
	RNM-2	12.30 ± 0.65	$1.33x10^{8} \pm 6.79x10^{7}$
	RNM-5	11.07 ± 0.20	$2.75x10^{8} \pm 1.70x10^{8}$
	RNM-18	10.75 ± 0.53	$2.84x10^{10} \pm 1.58x10^{10}$
	RNM-21	11.38 ± 0.68	$5.61x10^{10} \pm 4.04x10^{10}$
	RNM-22	11.02 ± 0.72	$8.30x10^{8} \pm 2.64x10^{8}$
	RNM-23	11.12 ± 0.47	$3.00x10^{9} \pm 1.95x10^{9}$

Test of BMA of production with *E. coli BW25113* (WT) or BMA tolerant strains + pKIV_ara using Lund media (with yeast extract; 0.8 g/L) with glycerol (10 g/L) as carbon source, KIV (20 mM), 1-butanol (15 mM), and ampicillin (100 µg/mL) in 250 mL of conical flask. Cell viability was measured in LB agar with ampicillin (100 µg/mL).

Unlike BMA, the production of BIB, BA, and BIV increased between 6 and 24 h after induction. BIB, BA, and BIV titres were observed to reach 2.2 mM, 5.8 mM, and 0.4 mM, respectively. The highest production for BIB, BA, and BIV were observed after 24 from *E. coli* BW25113-WT and strain RNM-18, whilst the highest combined butyl esters at 8.4 mM was observed to be produced from *E. coli* BW25113-WT. Selectivity-wise, BMA was produced the lowest among the four butyl esters in all of the host strains after 24 h of production (Table 7.3). The strains *E. coli* BW25113-WT, RNM-18, and RNM-21 produced BA, BIB, BIV, and BMA in decreasing order. In the case of strains RNM2, RNM-5, RNM-22, and RNM-23, BIB, BA, BIV, and BMA were produced in decreasing order.

Table 7.3 BMA and other butyl esters production in *E. coli* strains

Strain	Concentration (mM)					Selectivity (%)			
	BMA	BIB	BA	BIV	Total	BMA	BIB	BA	BIV
6 h after induction									
WT	0.039 ± 0.011	1.838 ± 0.383	0.842 ± .540	0.195 ± .083	2.914 ± .201	1.33	63.07	28.89	6.69
RNM-2	0.009 ± 0.002	0.079 ± 0.002	0.028 ± 0.002	0.007 ± 0.001	0.122 ± 0.001	7.38	64.75	22.95	5.74
RNM-5	0.017 ± 0.005	0.563 ± 0.179	0.043 ± 0.011	0.037 ± 0.006	0.659 ± 0.054	2.58	85.43	6.53	5.61
RNM-18	0.043 ± 0.034	1.182 ± 0.574	0.329 ± 0.286	0.257 ± 0.094	1.810 ± 0.196	2.38	65.30	18.18	14.20
RNM-21	0.028 ± 0.002	1.243 ± 0.094	0.621 ± 0.067	0.128 ± 0.009	2.022 ± 0.035	1.38	61.47	30.71	6.33
RNM-22	0.006 ± 0.001	0.102 ± 0.022	0.017 ± 0.001	0.005 ± 0.001	0.131 ± 0.007	4.58	77.86	12.98	3.82
RNM-23	0.018 ± 0.012	0.365 ± 0.167	0.044 ± 0.016	0.018 ± 0.016	0.445 ± 0.051	4.04	82.02	9.89	4.04
24 h after induction									
WT	0.034 ± 0.005	2.219 ± 0.122	5.755 ± .545	0.367 ± .043	8.375 ± .201	0.41	26.50	68.72	4.38
RNM-2	0.010 ± 0.001	0.201 ± 0.036	0.029 ± 0.003	0.021 ± 0.001	0.260 ± 0.011	3.85	77.31	11.15	8.08
RNM-5	0.016 ± 0.004	0.895 ± 0.277	0.064 ± 0.018	0.180 ± 0.007	1.156 ± 0.084	1.38	77.42	5.54	15.57
RNM-18	0.036 ± 0.027	1.522 ± 0.745	4.781 ± 3.33	0.409 ± 0.068	6.748 ± 1.029	0.53	22.55	70.85	6.06
RNM-21	0.024 ± 0.001	1.718 ± 0.078	3.315 ± 0.456	0.27 ± 0.008	5.327 ± 0.140	0.45	32.25	62.23	5.07
RNM-22	0.005 ± 0.001	0.198 ± 0.044	0.015 ± 0.001	0.02 ± 0.005	0.238 ± 0.013	2.10	83.19	6.30	8.40
RNM-23	0.018 ± 0.007	0.872 ± 0.439	0.083 ± 0.019	0.146 ± 0.017	1.119 ± 0.133	1.61	77.93	7.42	13.05

Test of BMA of production with *E. coli BW25113* (WT) or BMA tolerant strains + pKIV_ara using Lund media (with yeast extract; 0.8 g/L) with glycerol (10 g/L) as carbon source, KIV (20 mM), 1-butanol (15 mM), and ampicillin (100 µg/mL) in 250 mL of conical flask. Legend: BMA = butyl methacrylate, BIB = butyl isobutyrate, BA = butyl acetate, BIV = butyl isovalerate

The results from BMA production *via* biotransformation revealed that the current attainable BMA titres (0.04 mM) is very far from achieving phase separation (<5.6 mM). In addition to that, the BMA tolerant strains were not able to produce more BMA as compared to the parental strain. However, the halt in BMA production was likely not due to BMA/butyl esters toxic effect to the host strain. The cell viability of the host strains were observed to be stable, which suggests that BMA nor the butyl ester combination did not reach a lethal concentration. In addition, the esterification *via* the AAT can still be observed to be active after 6h due to the observed increase in the titres of the other butyl esters. The abundance of the other butyl esters observed from the biotransformation

suggests that the AAT used in the current production system is not specific for BMA production (Sato et al., 2017). Thus, the BMA production may be limited due to a low selectivity for BMA production by the AAT and/or possibly a low availability of methacrylyl-COA. In this regard, the optimal BMA production may have not yet been reached. Hence, it will be difficult to fully assess the actual potential of the parental strain and BMA tolerant strains ability to produce BMA.

7.5 Conclusions

The application of genome shuffling followed by enrichment allowed the generation of a BMA tolerant strains with improved growth in the presence of 20% v/v BMA. In the case of BMA production, the use of the BMA tolerant strains as the host strain did not improve BMA titres. However, the current production system does not seem to be limited by the toxicity of BMA. Thus, it will be necessary to address the selectivity of the AAT and limitation of BMA titres before one can fully assess the potential of the evolved strains as hosts for BMA production.

8

Overall Discussions

The results from genome resequencing and RNA sequence may have given a glimpse on the potential mechanism of BMA toxicity added externally as well as the mechanism of BMA tolerance in *E. coli*. Enhancement in BMA tolerance of *E. coli* was likely due to the mutations acquired along the evolution process (Tables 5.3, 5.4, and 5.5). Presence of an *acrR* and of either a *marR*, *soxR*, or *rob* mutation (Table 5.3) in each of the genome sequenced BMA tolerant strains and the genome shuffled strain generated in this study and a *soxR* mutation in each of the BMA tolerant strain isolated from the preceding work (Disley, 2018), suggests the potential role of the MarA-SoxS-Rob regulatory network in BMA tolerance. Furthermore, the differential upregulation of *acrA* and *acrB* in each of the RNA sequenced strains (Table 6.3) indicates that the AcrAB-TolC efflux pump might play a key role in BMA tolerance. The AcrAB-TolC efflux pump has been implicated as a vital contributor in the enhanced resistance of *E. coli* for antibiotics (Du et al., 2014; Müller Reinke & Pos Klaas, 2015) and organic solvents (Table 2.6) (Aono, 1998; Shah et al., 2013; White et al., 1997). The *acrR* mutations acquired by the BMA tolerant strains may have contributed to the activation of *acrAB*, if the changes in its structure caused a reduction in DNA binding/repressor activity (Li et al., 2007; Routh et al., 2009). The reduction in repression by AcrR can lead to enhanced activation of *acrAB*.

In addition to the *acrR*, the mutations in *marR*, *soxR*, and *rob* also have the potential to activate *acrAB* (Grkovic et al., 2002; Grkovic et al., 2001). MarR is a repressor of *marA*, which encodes MarA that is known to activate *acrAB* (Grkovic et al., 2001). It is possible that the mutation acquired in *marR*, which was located along the DNA and salicylate binding region (Alekshun et al., 2001), altered the structure of MarR to reduce its DNA binding/repressor activity and facilitate the observed increased activation of *marA* (Fig. 6.3 MarA-SoxS-Rob) in the BMA tolerant strain RNM-21. Consequently, the increased expression in *marA* may have contributed to the observed upregulation in *acrAB* (Fig. 6.3 MarA-SoxS-Rob).

SoxR is an activator of *soxS* that encodes SoxS, which is also capable of *acrAB* activiation (Grkovic et al., 2002; Grkovic et al., 2001). Two of the *soxR* mutations (RNM-5 and RNM-6) from the BMA tolerant strains affected the Fe-S cluster domain, while the other caused an alteration at the DNA binding domain (Appendix Table 11.5). Similar mutations in these regions of SoxR have been reported in solvent tolerant strains (Chander & Demple, 2004; Chander et al., 2003; Hidalgo et al., 1997; Nakajima et al., 1995; Nunoshiba & Demple, 1994). Alteration or truncation at the C-terminal domain at amino acid residue 136 and above allowed constitutive expression of *soxS*, (Chander & Demple, 2004; Nakajima et al., 1995; Nunoshiba & Demple, 1994). On the other hand, the mutation found in strain RNM-23 in *soxR* at the DNA binding site may have affected SoxR through its DNA binding affinity in the *soxS* promoter region (Chander & Demple, 2004; Chander et al., 2003) and/or redox potential (Bains & Warren, 2016; Hidalgo et al., 1997; Olson et al., 2013). Thus, it is possible that the mutations observed in *soxR* and their corresponding alterations for SoxR to cause the observed upregulation of *soxS* and contribute to the observed activation of *acrAB* (Fig. 6.3 MarA-SoxS-Rob).

Rob is also known as an activator of *acrAB*. Rob is believed to form clusters through self-sequestration that hinders its DNA binding activity and liberated into its active form upon contact with its activators (e.g. dipyridyl and bile salts) (Azam et al., 2000; Griffith et al., 2009; Rosenberg et al., 2003; Rosner et al., 2002). No increase in expression of *rob* was observed for the BMA tolerant strains (RNM-2, RNM-18, and RNM-22) that acquired mutations in *rob* (Table 5.4 and Fig. 6.3 MarA-SoxS-Rob). It might be possible that the mutations acquired in *rob* allowed the increase of free/active Rob, that would have a similar effect to overexpression (Gee et al., 2000; Li et al., 2014b; Teng et al., 2010) (Azam et al., 2000; Griffith et al., 2009; Rosner et al., 2002) and consequently contribute to the observed increased expression of *acrAB* (Fig. 6.3 MarA-SoxS-Rob).

The other notable observation in the BMA tolerant strains was the presence of either an *rpoB* or *rpoC* mutation in strains that were able to grow at a maximum growth rate of <0.70 /h in the presence of BMA (20% v/v) (Tables 5.2 and 5.3). The *rpoB* and *rpoC* genes encode the 1342 amino acid-residues ß (Wang et

al., 1997) and 1407 amino acid-residues ß' (Nedea et al., 1999) subunit of the RNA polymerase, respectively. The *E. coli* RNA polymerase is responsible for its DNA transcription and main target of transcriptional regulation. Its core enzyme is composed of 2 α, ß, ß', and ω subunits (Finn et al., 2000; Molodtsov et al., 2013; Murakami, 2013; Nedea et al., 1999; Wang et al., 1997), where the subunits ß and ß' form the catalytic subunit (Finn et al., 2000). Mutations in *rpoB and rpoC* at diverse gene locations have been observed in various evolved strains under minimal media (Conrad et al., 2010), antibiotic (Brandis et al., 2012), thermal (Rodríguez-Verdugo et al., 2016; Rodríguez-Verdugo et al., 2015), or osmotic pressure stress (Utrilla et al., 2016; Xiao et al., 2017).

In general, the *rpoB* or *rpoC* mutations from adaptive evolution caused alterations in the RNA polymerase subunit's structure and protein-protein interaction (Utrilla et al., 2016) as well as changes in gene expression (Brandis et al., 2012; Conrad et al., 2010; Rodríguez-Verdugo et al., 2016; Rodríguez-Verdugo et al., 2015; Utrilla et al., 2016; Xiao et al., 2017). The changes in gene expression led to restoration of expression for hundreds of genes towards the pre-stress level (Rodríguez-Verdugo et al., 2016; Rodríguez-Verdugo et al., 2015) and improved metabolic and energy efficiency (Utrilla et al., 2016; Xiao et al., 2017). The *rpoB* or *rpoC* mutants were able to grow faster in the stress conditions as compared to the wild-type/parental strain (Brandis et al., 2012; Conrad et al., 2010; Rodríguez-Verdugo et al., 2016; Rodríguez-Verdugo et al., 2015; Utrilla et al., 2016; Xiao et al., 2017). Likewise, the strains that acquired either an *rpoB* or *rpoC* mutation/s, except for strain RNM-2, had growth rates (measured with 20% v/v BMA) comparable to the wild-type/parental strain grown in the absence of BMA. It is possible that mutations acquired in *rpoC* or *rpoB* in these BMA tolerant strains had the similar effects on its structure and gene expression, which could allow the strains to grow at a rate similar to non-stress conditions.

The mutations that were not found in each of the BMA tolerant strains may still have contributed towards BMA tolerance. However, since these mutations always appear together with other mutations related to solvent tolerance; it is likely that they are not as vital as the mutations in the MarA-SoxS-Rob regulatory network. Thus, they may not necessarily be direct contributors

towards BMA tolerance but could act as compensatory mutations for the loss in fitness (Brandis et al., 2012; Conrad et al., 2010; Rodríguez-Verdugo et al., 2016; Rodríguez-Verdugo et al., 2015; Utrilla et al., 2016; Xiao et al., 2017). Consequently, the differences in the combination of mutations acquired by each of the BMA tolerant strains and their effect on the overall gene expression (Table 5.3 and Appendix Tables 11.6 and 11.7) may have contributed to the differences in the length of lag phase, maximum observed cell density, and/or growth rate observed with the BMA tolerant *E. coli* strains grown in the presence of BMA (20% v/v).

As BMA (20% v/v) was added externally, the first line of defence for the cell is its cell envelope that serves as a barrier protecting essential cell components and regulating the transport of molecules between the cell's internal and external environment (Murínová & Dercová, 2014; Silhavy et al., 2010; Weber & de Bont, 1996). Organic solvent like molecules, such as BMA, with a log of the octanol and water partition coefficient (log P) value below 4 are known to have high affinity for the membrane bilayer (Sardessai & Bhosle, 2002). The log P value of BMA (2.9) and solubility studies of BMA in model membrane components (Personal communication from Dr. Boyan Bonev and Lucite International) suggests that BMA can accumulate in the cell membrane and potentially cause membrane damage.

Based on the results of the transcriptomics analysis, the interaction of BMA with the cellular membrane may have promoted various membrane related stresses such osmotic stress and membrane stored curvature elastic (SCE) stress (Bass et al., 2002; Flores-Kim & Darwin, 2016; Jovanovic et al., 2010; Jovanovic et al., 2006) and possibly the disruption of membrane integrity and fluidity (Murínová & Dercová, 2014), proton motive force (PMF) (Flores-Kim & Darwin, 2016; Jovanovic et al., 2010; Jovanovic et al., 2006), and the difference of the intracellular and extracelluar pH (ΔpH) (Farha et al., 2013; Meyer-Rosberg et al., 1996). In addition to membrane related perturbations, BMA could have interacted with other cellular components (lipids, protein, DNA, and RNA) and cause direct damage by denaturation and indirect damage (Asakura et al., 1978; Murínová & Dercová, 2014; Segura et al., 2012) *via* the ROS (Pérez-Gallardo et al., 2013). The various damaging effects of BMA could have

contributed to the likely inhibition in DNA replication, protein synthesis and cell growth (Chapters 5 and 6).

In order to alleviate the deleterious effects of BMA, with the help of the mutations gained from evolution the BMA tolerant strains activated/deactivated various genes that allowed cellular membrane modification, active efflux of BMA, reduction in the amount of ROS, maintenance of pH, repair of damaged cellular components, and adjustment of energy metabolism and biosynthesis of essential building blocks for cellular components (Chapters 5 and 6).

As membrane composition is an important adaptation mechanism for bioproduct tolerance, changes in membrane composition may have contributed to the observed BMA tolerance (Ramos et al., 1997; Sikkema et al., 1995). The differential upregulation of *cfa* (Table 11.6) in the BMA tolerant strains after exposure to BMA (20% v/v) suggests an adaptation mechanism through modification of an unsaturated fatty acid into a cyclopropane fatty, which are known to have an increased bulk as compared to the unsaturated fatty acid counterpart (Courtois et al., 2004; Murínová & Dercová, 2014; Perly et al., 1985; Royce et al., 2014). Perhaps, this mechanism would allow to accommodate BMA in the membrane to restore membrane fluidity and integrity (Courtois et al., 2004; Perly et al., 1985). Mutations acquired in genes related to lipid biosynthesis also have the potential to alter membrane composition and properties. This include *lpxM* and *clsA* that encode the Lipid A biosynthesis myristoyltransferase (LpxM) (Emiola et al., 2015) and cardiolipin synthase A (ClsA) (Romantsov et al., 2018), respectively. It is possible that the mutations acquired in the lipid synthesis related genes resulted in an altered activity, which could potentially affect the abundance of myristate or cardiolipin phospholipid in the cell membrane. The mutation in *opgH*, which encodes the glucans biosynthesis glucosyltransferase H (OpgH) is another alteration that has the potential to change membrane composition and property. OpgH is one of the enzymes responsible for the biosynthesis of the backbone of osmoregulated periplasmic glucans (OPGs) that are associated with regulation of motility and secretion of exopolysaccharides.

The active efflux of BMA was likely accomplished *via* the AcrAB-TolC efflux system (Aono, 1998; Shah et al., 2013; White et al., 1997), which can be linked from the observed differential upregulation of *acrAB* in the evolved strains (Fig. 6.3 MarA-SoxS-Rob). Other potential contributors to the active efflux of BMA are the uncharacterized proteins encoded by *ybhFSR* and *ybhG*, which were both differentially upregulated upon exposure of the *E. coli* strains to BMA (20% v/v) (Fig. 6.7). The gene *ybhFSR* and *ybhG* encode an ATP-binding cassette (ABC)-type transporter and a predicted inner membrane protein of unknown function, respectively. Deletion of the former increased *E. coli* susceptibility for the antibiotic cefoperazone, while deletion of the latter made *E. coli* more sensitive to the antibiotic chloramphenicol as compared to the control strain (Yamanaka et al., 2016). Considering that the AcrAB-TolC efflux system also extrude a broad range of antibiotics at the outer leaflet of the inner membrane (Seeger et al., 2006) and/or from the periplasm (Murakami et al., 2006) *via* AcrB, either YbhFSR or YbhG might help transport BMA from the cytoplasm towards the periplasmic space. This could allow *E. coli* to maintain the BMA concentration within the cell at a tolerable level. However, further characterization of these proteins and investigation of the potential synergy with the AcrAB-TolC efflux system would be required.

Reduction in the amount of ROS and their potential damage may have been achieved through the action of the enzymes that directly converts H_2O_2 (KatE, SodB, and SodC) (Seaver & Imlay, 2001; Sorkin & Miller, 1997) and $O2^{-}\cdot$ (Sorkin & Miller, 1997) into H_2O and H_2O_2, as well as reduction of organic hydroperoxides (OsmC) (Lesniak et al., 2003) and quinones (QorA and WrbA) (Maruyama et al., 2003; Patridge & Ferry, 2006). Another route to lessen the amount of ROS and its potential damage is by reducing its generation. This may have been accomplished with the possible $Fe^{3+/2+}$ limitation within the cell (Chapter 6). On the other hand, the intracellular pH may have been maintained *via* the glutamate dependent acid resistance (Braun et al., 2017; Brechtel et al., 1996; Capitani et al., 2003; Langendorf et al., 2010; Liu et al., 2005; Schneider et al., 2013).

Repair of cellular components that may have been damaged directly or indirectly due to BMA exposure can be performed by various stress response

systems (Chapter 6). PspA might play a part in supressing proton leakage from damaged lipid membrane by forming PspA oligomers that binds directly to damaged liposomes (Kobayashi et al., 2007). Protein damage and denaturation may be taken care by molecular chaperones from the acid stress response (HdeA and HdeB) that act in the periplasm (Kern et al., 2007) and the molecular chaperons from the heat shock response (ClpB, DnaK, HspQ, IbpA, and IbpB) that act in the cytoplasm (Bertelsen et al., 2009; Kuczynska-Wisnik et al., 2002; Shimuta et al., 2004; Zolkiewski, 1999). Some of the protein damaged *via* oxidation of sulfur in methionine and reaction with glyoxals may be repaired by MsrA, YajL, and YbhO from the oxidative stress response (Abdallah et al., 2007; Abdallah et al., 2016; St John et al., 2001). Possible involvement of molecular chaperones for BMA tolerance can be extended with the mutations in *dnaK* and *groL* by two separate BMA tolerant strains (Bertelsen et al., 2009; Houry, 2001).

The adjustment of energy metabolism and biosynthesis of essential building block for cellular components may be realized by the enhanced glucose utilization (Dadinova et al., 2016; Lovingshimer et al., 2006; Song et al., 2006; Zimenkov et al., 2005), generation of acetyl-COA for the TCA cycle (Patel et al., 2014) , nitrate/nitrite utilization (Blasco et al., 1990; Clegg et al., 2002), amino acid biosynthesis (Fultz et al., 1979), and oxidation reduction processes (Table 6.11). In addition to that, the adjustment in resource allocation *via* reduced synthesis of non-vital cellular components could be beneficial. This may have been realized in part from the reduced expression of genes related to cell motility and chemotaxis (Table 6.4) in the evolved strains, which has been suggested as a resource and energy saving mechanism (Gauger et al., 2007).

The importance of adjusting cellular metabolism to aid in BMA tolerance can also be recognized from the mutations found in strain RNM-18 for the regulatory proteins Cra and CpxA, which have the potential to alter gene expression related to cellular metabolism. Cra functions as a cyclic AMP (cAMP)-independent dual regulator for carbon metabolism (Saier & Ramseier, 1996). It serves as a positive regulator for TCA and glyoxylate shunt related genes, but acts as a negative regulator for the pentose phosphate (PP) and Entner

Doudoroff (ED) pathway (Sarkar et al., 2008). On the other hand, CpxA is part of the of the CpxA/CpxR two-component signal transduction system that responds to envelope stress. CpxR can be activated and deactivated by CpxA *via* phosphorylation and dephosphorylation, respectively. Activation of CpxR leads to repression of genes related to chemotaxis and biofilm formation, while it enhances the expression of multidrug resistance, chaperone proteins, and proteases (Dorel et al., 2006; Yamamoto & Ishihama, 2006). It is also very interesting to note that the potential key difference in response to BMA exposure for the BMA tolerant strains and the parental strain are mostly compromised of genes related to energy generation and biosynthesis of essential building block for cellular components. Thus, further highlighting its importance for BMA tolerance.

The BMA tolerant strain RNM-18 was found to be more susceptible to 1-butanol as compared to the parental strain. Similar effects on short chain alcohol susceptibility in strains that had an increase in *acrAB* expression have been reported (Ankarloo et al., 2010). The increased susceptibility of the BMA tolerant strains to 1-butanol could potentially further limit the working 1-butanol concentration for BMA production. However, this drawback can be circumvented by controlled addition of 1-butanol that would allow BMA production without inhibiting the host strain. In addition to the process engineering adjustment, 1-butanol tolerance can be enhanced by application of the various host strain engineering approaches presented in Chapter 2.

Actual BMA production in the parental strain and BMA tolerant strains stalled at around 0.035-0.04 mM (Table 7.4). Although the production of BMA in the BMA tolerant strains did not show any improvements in the BMA titres, it does not necessarily indicate that they are not suitable host strains for BMA production. Proper assessment for the potential of the BMA tolerant strains as host for BMA production can only be possible, if the limitations in the current production system can be addressed. The principle of improving bioproduct tolerance is to break the limitations in product titres due to product toxicity or inhibition towards the host strain. Results from the BMA production *via* biotransformation does not suggest that the BMA titre was limited due to toxicity. In addition to that, the presence of other butyl esters at titres 10-100

fold higher than BMA makes it more difficult to assess effects of BMA production to the host strain. Thus, it will be necessary to demonstrate first that the parental strain's BMA production stalled due to its toxicity (Menchavez & Ha, 2019).

In this regard, improvements in the current production system should be implemented. This includes the use of an AAT that would be specific or very selective for BMA production and minimize production of other butyl esters, which may be achieved through enzyme engineering of the current AAT (Kaul & Asano, 2012; Otte & Hauer, 2015) or use of an alternative natural AAT (Kruis et al., 2019). Another potential limitation in the BMA production pathway is the availability of methacrylyl-COA for BMA production. It might be necessary to investigate the limitations of the enzyme ACX4 for BMA production. This could be potentially due to inhibition of BMA and/or possibly the other butyl esters to the enzyme (Gopalan & Srivastava, 1997) and/or limitation in the regeneration of the cofactor FAD (Hou et al., 2017).

It is also interesting to note that BIB titres reached 2 mM and may be close to attaining phase separation. Since BMA can be produced from BIB by a signle oxidative dehydrogenation step (Macho et al., 2004), it might be a potential alternative target product for the bioprocess instead of BMA. In this case, the process developed for BMA can still be applied for BIB production and recovery. BIB can then be converted to BMA *via* a catylytic oxidative dehydrogenation (Macho et al., 2004). Thus, bypassing the potential limitations for this step in the pathway. In addition to that, *E. coli BW25113*-WT can grow in the presence of BIB at 20% v/v (Appendix Fig. 11.16). Strains RNM-5 and RNM-23, which provided relatively titres for BIB, high selectivity for BIB, and low selectivity BA can be explored as potential host strains for BIB production (Table 7.4).

9

Conclusions and Recommendations for future work

9.1 Conclusions

The aim of this study was to generate a robust host strain for the commercial production of BMA. However, it remains to be seen whether or not this aim has been achieved. Nonetheless, this study has successfully generated *via* adaptive evolution and genome shuffling various *E. coli* strains with improved growth in the presence of BMA up to 20% v/v, which is within the BMA concentration required for the commercial viability of the proposed integrated process for MMA production. This corresponds to a 200-fold increase in the BMA titres that can be tolerated relative to the parental strain. The best strain generated from adaptive evolution was able to achieve a 3.1-fold and 1.2-fold improvements in cell density and growth rate, respectively as compared to the previously isolated BMA tolerant strain grown in the presence of BMA (20% v/v). A BMA tolerant strain generated from genome shuffling and enrichment gained a 1.3-fold enhancement in the cell density achieved, when grown in the presence of BMA (20% v/v), as compared to the best BMA tolerant strain isolated from adaptive evolution.

Understanding of the potential mechanisms of tolerance was facilitated by genome and RNA sequencing. The observed enhanced growth of the evolved strain in BMA (20% v/v) was likely due to the acquired mutations in *acrR*, *marR*, *soxR*, and *rob* and the involvement of the MarA-Sox-Rob regulatory network, especially the AcrAB-TolC efflux pump. In order to combat the deleterious effects and allow growth in the presence of BMA, the BMA tolerant strains may have adapted by adjusting their gene expression. The adjustments in gene expression potentially allowed cellular membrane modification, active efflux of BMA, reduction in the amount of ROS, maintenance of pH, repair of damaged cellular components, and adjustment of energy metabolism and biosynthesis of essential building blocks for cellular components.

The use of the BMA tolerant strains as the host strain did not improve BMA titres. However, the BMA titres obtained from the *E. coli* strains does not seem

to be limited by BMA toxicity to the host strain. Thus, it will be necessary to address the bottlenecks in the production pathway and demonstrate that the parental strain's BMA production is limited due to its toxicity. Hence, whether or not the enhanced BMA tolerance can lead to higher BMA titres can't be properly assessed at this moment.

9.2 Recommendations for future work

Although the results obtained were able to address the objectives set to help achieve the aim of this study, it may be necessary to verify some of the potentially vital observations or investigated further to enable future host and pathway engineering efforts.

In the case of BMA tolerance, a number of potentially important genes affected by mutations or change in gene expression have been identified. Firstly, the verification on the role of the mutations found in the evolved strains for BMA tolerance, especially the ones in *acrR*, *marR*, *soxR*, and *rob*, would help identify the beneficial mutations. This can be achieved through genome integration of the acquired mutation into the parental strain and tested for growth in the presence of BMA. Similarly, the role of the genes differently expressed relative to the parental strain and after BMA exposure in the BMA tolerant strains that are potentially vital to BMA tolerance can be verified by overexpression or silencing of the gene of interest. Notable differentially expressed genes include *acrAB*, *ybhFSR* and *ybhG*, which have a potential role in BMA efflux. It would also be interesting to see whether or not overexpression or silencing of the genes that were exclusively differentially expressed in the BMA tolerant strains (Table 6.10) would be enough to confer tolerance to the parental the strain. Likewise, it would be intriguing to test the differentially enriched transcription factors from the differentially expressed genes (from the transcriptomics analysis) for GTME/directed evolution to confer tolerance in *E. coli* towards BMA.

BMA production-wise, exploration of alternative AAT's or enzyme engineering of the current AAT should be done to minimize the other butyl ester byproducts. It would also be necessary to investigate the limitations of the enzyme ACX4 for BMA production, especially on the potential product inhibition and limitation

on the regeneration of the cofactor FAD. Once these limitations are addressed, the BMA tolerant strains generated in this study can be tested for BMA production. As an alternative to the BMA bioprocess route, it might be worthwhile exploring the BIB bioprocess route for BMA and MMA production.

In addition to the verification and further investigation of the results from the study, It might be worthwhile exploring direct production MMA from the renewable microbial process. In this case, the transesterification step would be unnecessary (Fig. 1.1). The approaches used in this study can be applied to develop an MMA tolerant host strain. Major considerations for this process would be the alternative recovery options, due to high solobulity of in H_2O (15 g/L) and close proximity of its boiling point to H_2O (101 ºC vs 100 ºC), and AAT that would be specific for MMA production.

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11. Appendices

11.1 Plasmids used in the study

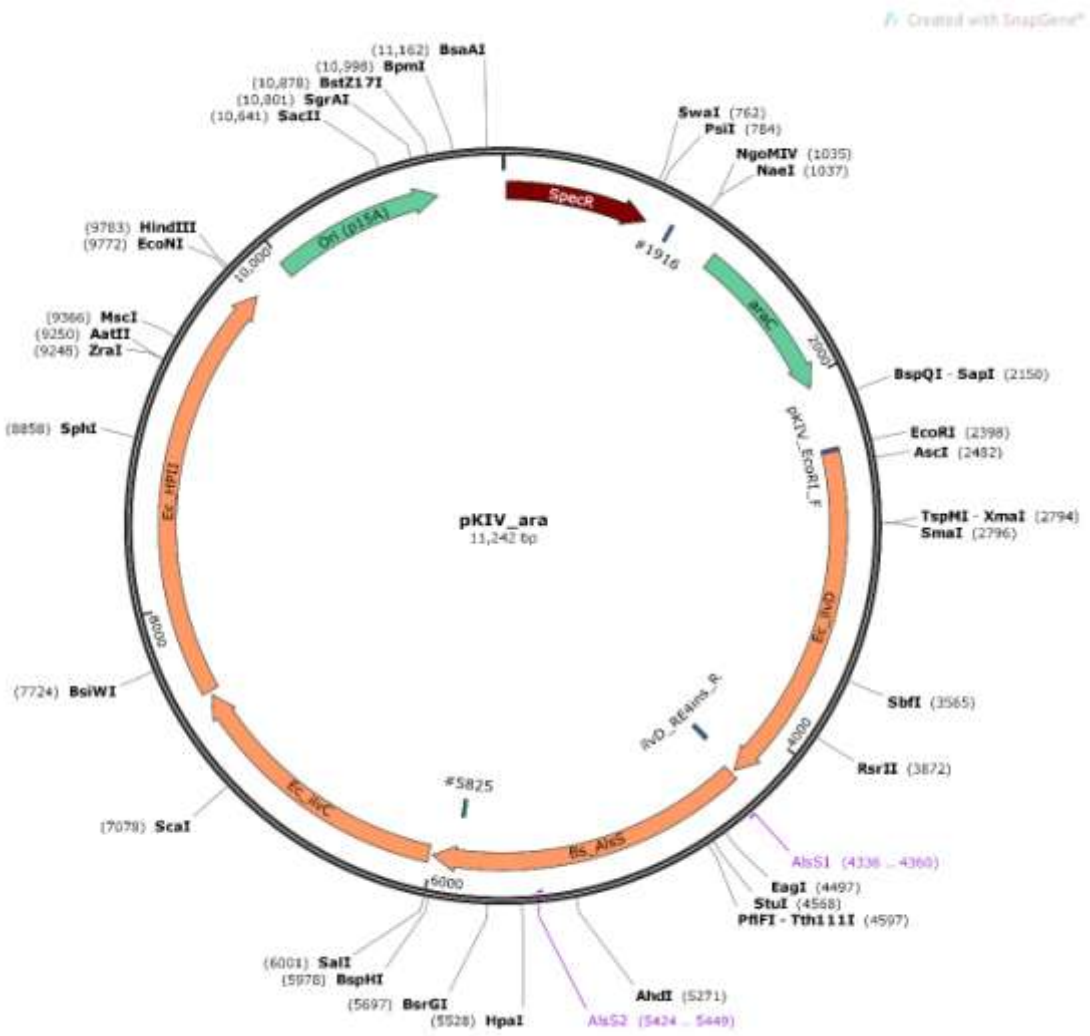

Fig. 11.1 Plasmid map of pKIV_ara

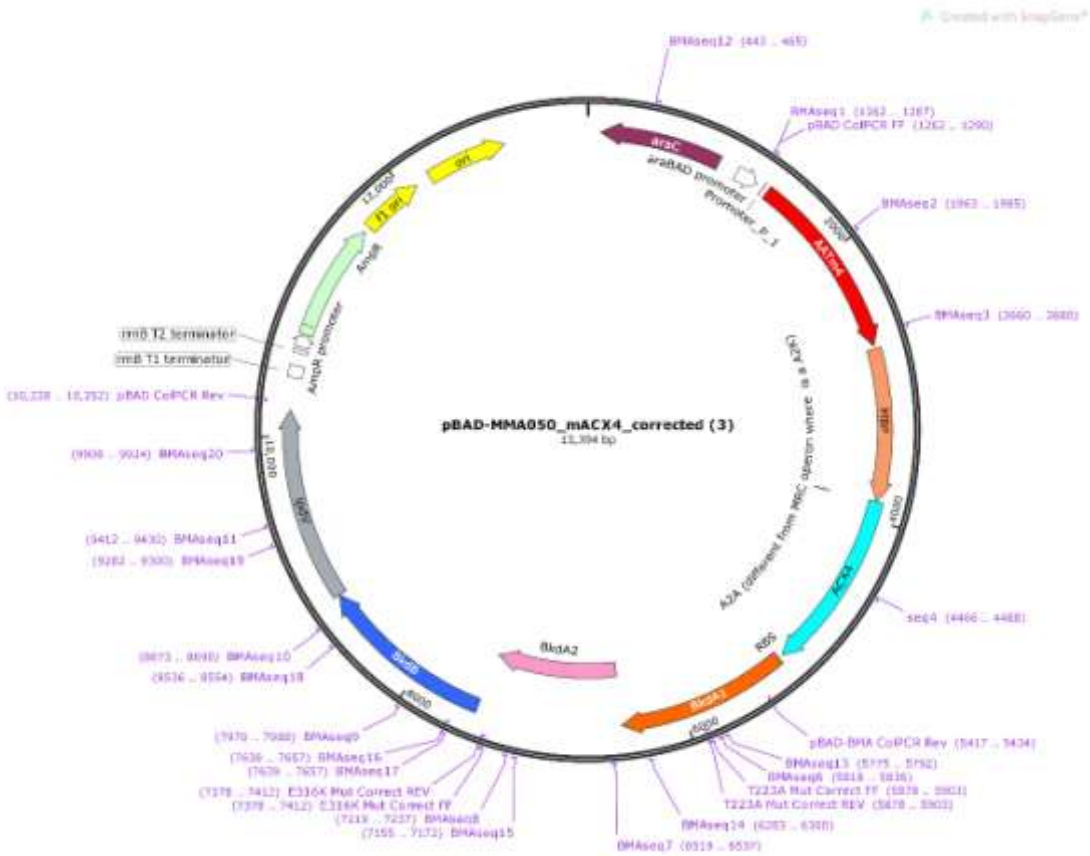

Fig. 11.1 Plasmid map of pBAD-MMA050_mACX4_corrected (3)

Table 11.1 Primers used in the study

Name	Sequence	Purpose
acrR-V-F	CTCGAGTGTCCGATTTCAAATTGG	Amp and Seq – *acrR*
acrR-V-R	CTCGAGTGTCCGATTTCAAATTGG	Amp and Seq – *acrR*
acrR-2-VM	GATCAGCCAGTCCACATCC	Seq – *acrR* (RNM-2)
acrR-5-VM	CTCCCGTAAATGCCTTGAATCAG	Seq – *acrR* (RNM-5)
acrR-6-VM	ACGAACAGTGGGGCTATGTC	Seq – *acrR* (RNM-6)
acrR-7-VM	AACTGGCCCGCCAATTG	Seq – *acrR* (RNM-7)
acrR-19-VM	GCTTTTTCGGGAGGAAGTGATAC	Seq – *acrR* (RNM-19)
acrR-21-VM	AGTGATACGGCAATGAGACGT	Seq – *acrR* (RNM-21)
acrR-23-VM	ATCAGCCAGTCCACATCCA	Seq – *acrR* (RNM-23)

Note: Amp = amplification, Seq = sequencing, - gene (source strain/plasmid)

11.3 Growth Curves

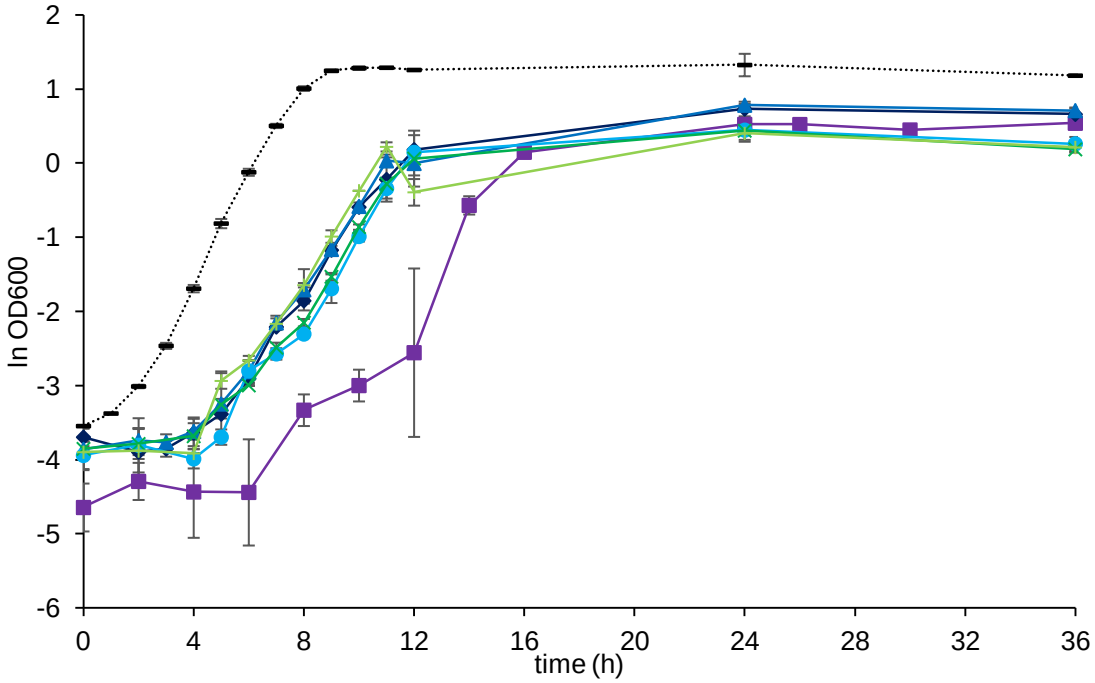

Figure 11.3 Growth characterization of isolates from ADE-1, ADE-2, and ADE-3. Growth of *E. coli* strains in M9 minimal medium (50 mL) contained in conical flasks (250 mL) at 37°C and 200 RPM (triplicates). The strains RNM-2 (■), RNM-3 (♦), RNM-4 (▲), RNM-5 (●), RNM-6 (x), and RNM-7 (+) were grown with BMA (20% v/v), while *E. coli* BW25113 –WT (-) was grown without BMA.

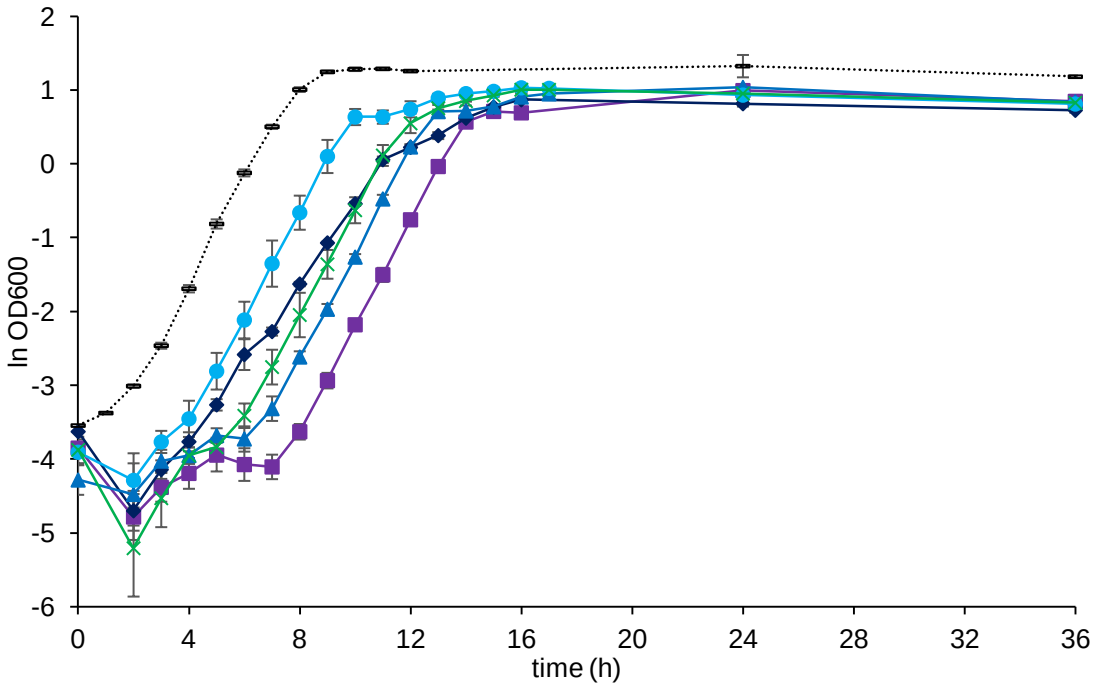

Figure 11.4 Growth characterization of isolates from ADE-4. Growth of *E. coli* strains in M9 minimal medium (50 mL) contained in conical flasks (250 mL) at 37°C and 200 RPM (triplicates). The strains RNM-14 (■), RNM-15 (♦), RNM-16 (▲), RNM-17 (●), and RNM-18 (x) were grown with BMA (20% v/v), while *E. coli* BW25113 –WT (-) was grown without BMA.

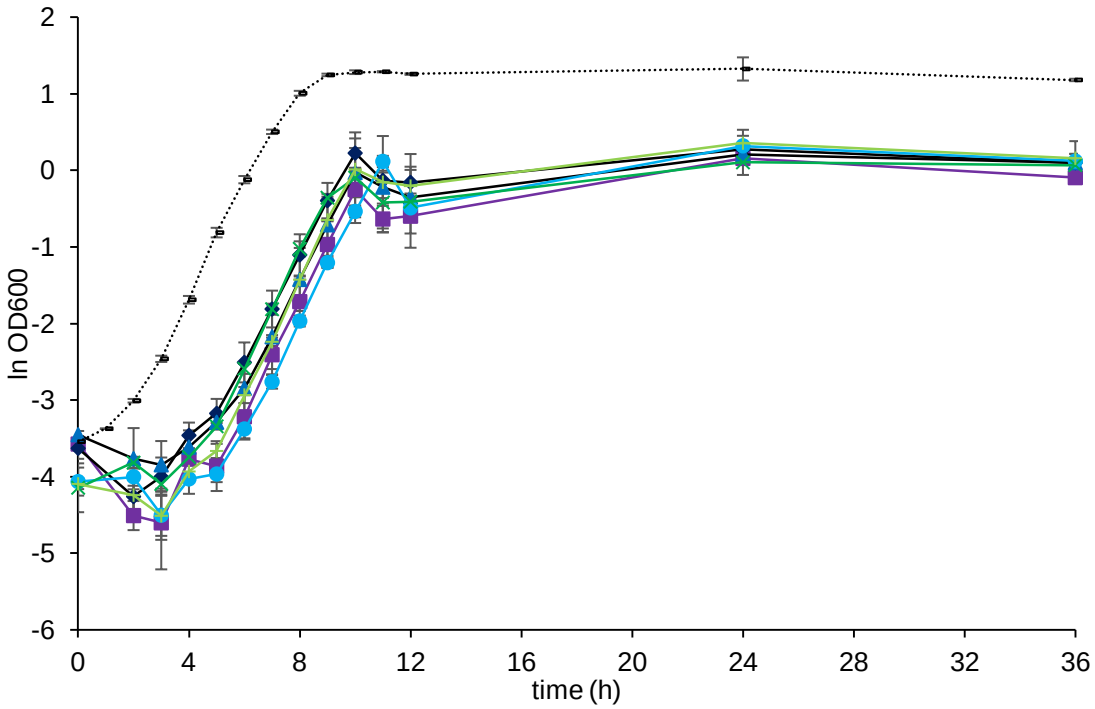

Figure 11.5 A Growth characterization of isolates from ADE-4 (A). Growth of *E. coli* strains in M9 minimal medium (50 mL) contained in conical flasks (250 mL) at 37°C and 200 RPM (triplicates). The strains RNM-8 (■), RNM-9 (♦), RNM-10 (▲), RNM-11 (●), RNM-12 (x), and RNM-13 (+) were grown with BMA (20% v/v), while *E. coli* BW25113 –WT (-) was grown without BMA.

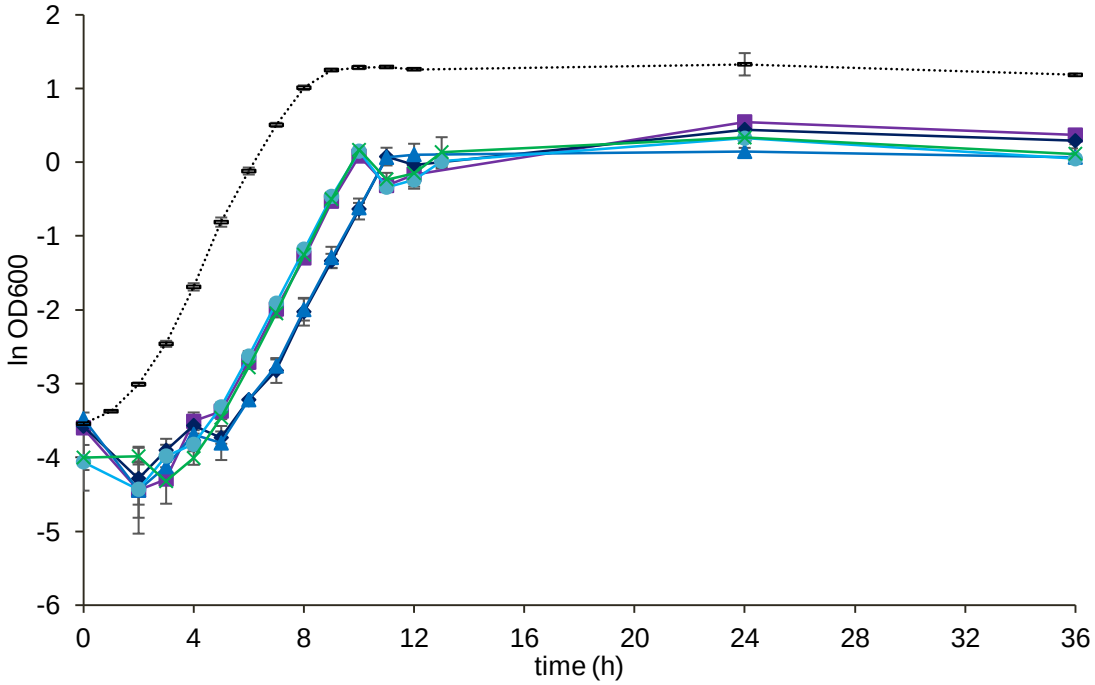

Figure 11.5 B Growth characterization of isolates from ADE-4 (B). Growth of *E. coli* strains in M9 minimal medium (50 mL) contained in conical flasks (250 mL) at 37°C and 200 RPM (triplicates). The strains RNM-19 (■), RNM-20 (♦), RNM-21 (▲), RNM-22 (●), and RNM-23 (x) were grown with BMA (20% v/v), while *E. coli* BW25113 –WT (-) was grown without BMA.

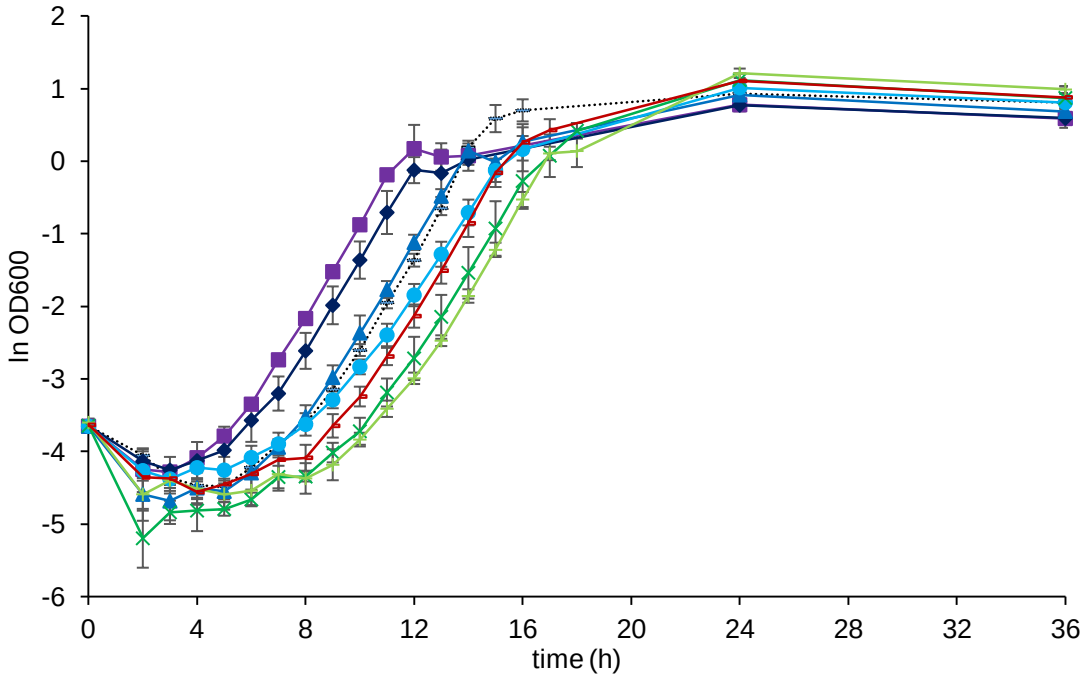

Figure 11.6 Growth characterization of isolates from genome shuffling. Growth of *E. coli* strains in M9 minimal medium (50 mL) contained in conical flasks (250 mL) at 37°C and 200 RPM (triplicates). The strains RNM-24 (■), RNM-25 (♦), RNM-26 (▲), RNM-27 (●), RNM-28 (x), RNM-29 (+), RNM-30 (-), and RNM-18 (—) were grown with BMA (20% v/v).

11.4 DNA translation

Table 11.2 List of codons for amino acids and their abbreviations

Amino acid	Three letter symbol	One letter symbol	DNA codons
Alanine	Ala	A	GCT;GCC;GCA;GCG
Arginine	Arg	R	CGT;CGC;CGA;CGG;AGA;AGG
Asparagine	Asn	N	AAT;AAC
Aspartic acid	Asp	D	GAT;GAC
Cysteine	Cys	C	TGT;TGC
Glutamic acid	Glu	E	GAA;GAG
Glutamine	Gln	Q	CAA;CAG
Glycine	Gly	G	GGT;GGC;GGA;GGG
Histidine	His	H	CAT;CAC
Isoleucine	Ile	I	ATT;ATC;ATA
Leucine	Leu	L	TTA;TTG;CTT;CTC;CTA;CTG
Lysine	Lys	K	AAA;AAG
Methionine	Met	M	ATG (Start)
Phenylalanine	Phe	F	TTT;TTC
Proline	Pro	P	CCT;CCC;CCA;CCG
Serine	Ser	S	TCT;TCC;TCA;TCG;AGT;AGC
Threonine	Thr	T	ACT;ACC;ACA;ACG
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAT;TAC
Valine	Val	V	GTT;GTC;GTA;GTG
STOP	N/A	N/A	TAA;TAG;TGA

Notes: DNA codons are read from 5’ end to 3’ end with 3 bp sequence read from a start codon to stop codon.

11.5 Mutations found from genome resequencing

Table 11.3 Summary of mutations in genes that encode for proteins that regulate gene expression

Gene	Gene Description	Cellular Location	Genomic Coordinate	Nucleotide Change/ Mutation Before → After	Effect of Nucleotide change; Length of Reference Amino Acid Sequence	Strain
acrR	acriflavine resistance regulator –acrAB operon repressor	Cyt	481486	insertion	Frameshift at AA 91 and truncates at AA 101; 215	2, 3
acrR	acriflavine resistance regulator –acrAB operon repressor	Cyt	481858	insertion	Frameshift at AA 214 and truncates at AA 249; 215	5
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481445	insertion	Frameshift at AA 77 and truncates at AA 84; 215	6
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481486	insertion	Frameshift at AA 91 and truncates at AA 96; 215	7
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481787	1 bp deletion	Frameshift AA at position 191 and truncates at AA 243; 215	18
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481361	11 bp deletion	frameshift at AA 53 and truncates at AA 244; 215	8, 22
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481310	insertion	truncates at AA 34; 215	19, 20, 21
acrR	acriflavine resistance regulator – acrAB operon repressor	Cyt	481437	insertion	Frameshift at AA 74 and truncates at AA 85; 215	23

Table 11.3 Continued…

rob	Right origin binding protein	Cyt	4624661	C→T	missense - Arg156His ; 289	2, 3, 8, 22, 29
rob	Right origin binding protein	Cyt	4624919	G→A	missense – Ala70Val ; 289	7
rob	Right origin binding (rob) protein	Cyt	4624920	C→T	missense – Ala70Thr ; 289	18
soxR	Superoxide response regulon activator	Cyt	4267812	T→A	Truncation-stops at Arg138 ; 154	5
soxR	Superoxide response regulon activator	Cyt	4267830	3 bp Deletion	Removal of Ala146 without changing the succeeding sequence ; 154	6
soxR	Superoxide response regulon activator	Cyt	4267455	G→T	Missense-Arg20Leu ; 154	23
marR	Multiple antibiotic resistance (mar) operon - repressor protein	Cyt	1613627	G→A	Missense-Val84Gly ; 144	19, 20, 21
ompR	Outer membrane porin protein - activator	Cyt	3529901	G→T	missense – Arg15Ser ; 239	19, 20, 21
phoP	Magnesium starvation regulon-regulator	Per	1185871	C→A	missense – Leu11Phe ; 223	3
cra	Catabolite repressor/ activator	Cyt	85321	1 bp deletion	Frameshift starting at AA 270 and truncates at AA 319; 334	18
creA	Right origin binding (rob) protein	Cyt	4625592	C→T	silent – Val85Val ; 157	18
rpoB	RNA polymerase ß subunit	Cyt	4174281	A→C	missense - Thr1037Pro ; 1342	8,22

Table 11.3 Continued…

rpoC	RNA polymerase ß' subunit	Cyt	4176359	T→G	missense - Leu361Arg ; 1407	2, 29
rpoC	RNA polymerase ß' subunit	Cyt	4175921	18 bp deletion	Frameshift starting AA 211 and stops at AA 230; 1407	18
rpoC	RNA polymerase ß' subunit	Cyt	4177637	C→T	missense – Ala787Val; 1407	21
rpoC	RNA polymerase ß' subunit	Cyt	4178500	C→T	missense – Arg1075Cys ; 1407	19, 20, 21, 23

Notes: Cellular Location; Cyt- Cytoplasm, Per- Periplasm, IM- Inner Membrane, Uk- Unknown

Table 11.4 Summary of mutations in genes encoding non-regulatory functional proteins

Gene	Gene Description	Cellular Location	Genomic Coordinate	Nucleotide Change/ Mutation Before → After	Effect of Nucleotide change; Length of Reference Amino Acid Sequence	Strain
acrB	Part of the acrAB-tolC multi-drug efflux complex	IM	478518	C→A	missense – Val448Leu ; 1049	3
acrB	Part of the acrAB-tolC multi-drug efflux complex	IM	478724	G→A	missense – Thr379Ile ; 1049	19
acrB	Part of the acrAB-tolC multi-drug efflux complex	IM	477159	T→C	missense – Val901Ile ; 1049	21
yohJ	Membrane protein	IM	2224428	T→G	missense – Leu109Arg; 132	18
dnaK	Molecular chaperone (HSP70)	Cyt	13292	T→G	missense – Val377Gly ; 638	18
groL	Chaperonin groEL (HSP60)	Cyt	4361677	C→T	missense – Pro279Leu ; 548	22

Table 11.4 Continued…

ilvN	Acetolactate synthase isozyme 1 small subunit – Activity regulator	Cyt	3844331	C→T	missense – Cys41Tyr ; 96	2, 3, 29
ygbK	putative 3-oxo-tetronate kinase	Uk	2856574	C→A	missense – Ala294Glu ; 388	2, 29
icd	Isocitrate dehydrogenas	Cyt	1,191,772	Deletion of the last 58 bp of the gene	Frameshift Starting AA 398	3
ymfD	Uncharacterized protein/ part of the prophage e14	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfE	Uncharacterized protein	IM	1,191,772	15,096 bp deletion	Deletion of entire gene	3
lit	Cell death peptidase/T4 exclusion	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
intE	Prophage integrase	Cyt	1,191,772	15,096 bp deletion	Deletion of entire gene	3
xisE	excisionase	Cyt	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfI	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
xisE	excisionase	Cyt	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfJ	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
cohE	Prophage respressor	Cyt	1,191,772	15,096 bp deletion	Deletion of entire gene	3
croE	Prophage transcriptional regulatory protein	Cyt	1,191,772	15,096 bp deletion	Deletion of entire gene	3

Table 11.4 Continued…

ymfL	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfM	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
oweE	pseudogene	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
aaaE	pseudogene	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfR	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
beeE	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
jayE	Putative protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
ymfQ	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
stfP	Uncharacterized protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
tfaP	tail fiber assembly protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
tfaE	tail fiber assembly protein	Uk	1,191,772	15,096 bp deletion	Deletion of entire gene	3
yhdE	a nucleotide pyrophosphatase	Cyt	3,391,684	Indels	Uk	3
mscK	mechano sensitive channel protein	IM	481, 992	Indels	Uk	5

Table 11.4 Continued…

ompT	outer membrane protein T/ surface membrane protease	OM	581,089	Indels	Uk	2, 6, 7
clsA	cardiolipin synthase A	OM	1,301,561	1 bp deletion	Frameshift starting AA 448 and truncates to AA 465	18
psuT	putative pseudouridine transporter	IM	2,250,368	8136 bp deletion	Deletion of first 446 bp of the gene	18
psuG	pseudouridine-5'-phosphate glycosidase	Uk	2,250,368	8136 bp deletion	Deletion of entire gene	18
psuK	putative pseudouridine kinase	Uk	2,250,368	8136 bp deletion	Deletion of entire gene	18
fruA	PTS system fructose-specific EIIB'BC component	IM	2,250,368	8136 bp deletion	Deletion of entire gene	18
fruB	sugar PTS diphosphoryl transfer protein	Cyt	2,250,368	8136 bp deletion	Deletion of entire gene	18
setB	sugar efflux transporter B	IM	2,250,368	8136 bp deletion	Deletion of AA start site	18
ompX	outer membrane protein X	OM	845,906	Indels	Uk	18
opgH	Glucans biosynthesis glucosyl-transferase	IM	1,106,602	G→C	Arg95Pro	18
atpI	ATP synthase protein I	IM	3,915,800	Indels	Uk	18
cpxA	sensor histidine kinase	IM	4,094,374	G→T	Pro177Glu	18
yieL	putative xylanase	Uk	3,893,318	Indels	Uk	19
mioC	FMN binding protein	Cyt	3,919,269	Indels	Uk	19
yhhJ	inner membrane transport permease	IM	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21

Table 11.4 Continued…

rbbA	ribosome- bound ATPase	IM	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiI	uncharacterized protein	IM	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiJ	uncharacterized protein	Uk	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiL	uncharacterized protein	Uk	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiM	inner membrane protein	IM	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiN	uncharacterized protein	Cyt	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
yhiN	uncharacterized protein	Cyt	3,630,122	1027 bp deletion	Deletion of the intergenic region and first 651 bp of the gene	20
pitA	phosphate transporter	IM	3, 618, 969	15,894 bp deletion	Deletion of entire gene	21
pitA	phosphate transporter	IM	3,630,122	1027 bp deletion	Deletion of the intergenic region and first 149 bp	20
lpxM	Lipid A biosynthesis myristoyl-transferase	IM	1,933,628	18 bp deletion	Removal of AA 267 to 272 without changing the succeeding sequence ; 323	2,29
pepA	Cytosol amino peptidase	Cyt	4,475,543	1 bp deletion	Change in AA sequence starting residue 76 and truncates at AA 99; 503	29

Notes: Cellular Location; Cyt- Cytoplasm, Per- Periplasm, IM- Inner Membrane, OM- Outer Membrane, Uk- Unknown

Table 11.5 Summary of mutations in non-coding regions

Gene	Gene Description	Cellular Location	Genomic Coordinate	Nucleotide Change/ Mutation Before → After	Effect of Nucleotide change; Length of Reference Amino Acid Sequence	Strain
Uk	+ 230 *appY* and +19 *ompT*	Uk	580116	G→T	Uk	5
Uk	- 271 *lrp* and - 274 *trxB*	Uk	927777	C→T	Uk	18
Uk	+ 45 *cohE* and -120 *ymfJ*	Uk	1197659	C→A	Uk	22
Uk	+ 4 *asmA* and + 270 *yegH*	Uk	2133236	T→A	Uk	23
Uk	-115 *atpI* and + 502 *rsmG*	Uk	3915915	T→G	Uk	23

Notes: Cellular Location; Cyt- Cytoplasm, Per- Periplasm, IM- Inner Membrane, Uk- Unknown

11.5.1 DNA sequence of affected genes

11.5.1.1 *acrR*

11.5.1.1.1 *acrR*-WT

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
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TGGCATTTTAAAGACAAGTCGGATTTGTTCAGTGAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAG
CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCA
CGGTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTCGGAGAAATGGCT
GTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGTATAGAACAAACGTTAAAACATTGTATT
GAAGCGAAAATGTTGCCTGCGGATTTAATGACGCGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCT
GATGGAAAACTGGCTCTTTGCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCCATCTTACT
GGAGATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAA

11.5.1.1.2 *acrR*-RNM-2

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TGGCATTTTAAAGACAAGTCGGATTTGTTCAGTGAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAG
CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGGAAGGTGCGAATAAGCGGGGAAATTCTT
CTCGGCTGA

11.5.1.1.3 *acrR*-RNM-3

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCAGCTGGCGTTACGCGCGGTGCAATCTAC
TGGCATTTTAAAGACAAGTCGGATTTGTTCAGTGAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAG
CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGGAAGGTGCGAATAAGCGGGGAAATTCTT
CTCGGCTGA

11.5.1.1.4 *acrR*-RNM-5

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC

ACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCAGCTGGCGTTACGCGCGGTGCAATCTAC
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CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCA
CGGTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTCGGAGAAATGGCT
GTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGTATAGAACAAACGTTAAAACATTGTATT
GAAGCGAAAATGTTGCCTGCGGATTTAATGACGCGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCT
GATGGAAAACTGGCTCTTTGCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCCATCTTACT
GGAGATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAAGGAAGGTGCGAATAAGCGGGGAAATTCTT
CTCGGCTGACTCAGTCATTTCATTTCTTCATGTTTGAGCCGATTTTTTCTCCCGTAAATGCCTTGAATCAGCCTAT
TTAG

11.5.1.1.5 *acrR*-RNM-6

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCAGCTGGCGTTACGCGCGGTGCAATCTAC
TGGCATTTTAAAGACAAGTCGGATTTGTTCAGTGAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAG
CTTGAGGGTGATGCTGCCAACTTACTGATTTAG

11.5.1.1.6 *acrR*-RNM-7

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCAGCTGGCGTTACGCGCGGTGCAATCTAC
TGGCATTTTAAAGACAAGTCGGATTTGTTCAGTGAGATCTGGGAACTGTCAGAATCCAATATTGGTGAACTAGAG
CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGGAAGGTGCGAACAAGTCCCTGA

11.5.1.1.7 *acrR*-RNM-18

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
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CTTGAGTATCAGGCAAAATTCCCTGGCGATCCACTCTCAGTATTAAGAGAGATATTAATTCATGTTCTTGAATCCA
CGGTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTCGGAGAAATGGCT
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GAAGCGAAAATGTTGCCTGCGGATTTAATGACGCGTCGCGCAGCAATTATTATGCGCGGCTATATTTCCGGCCT
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GAGATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAACCCTGAATCTGACTCCAGGATTTT
TCCTGGACATTTTCGTCGTTGCTATTCTGGTTCACTGCGTCGTGATATTCTTGCGGTTTGA

11.5.1.1.8 *acrR*-RNM-8

CAAGTTATAAACCCATTGCTGCGTTTATATTATCGTCGTGCTATGGTACATACATTCACAAATGTATGTAAATCTAA
CGCCTGTAAATTCACGAACATATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGA
TGTGGCTCTACGTCTTTTCTCACAGCAGGGGGTATCATCCACCTCGCTGGGCGAGATTGCAAAAGCAGCTGGCG
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CTTGAATCCACGGTGACAGAAGAACGGCGTCGATTATTGATGGAGATTATATTCCACAAATGCGAATTTGTCGGA
GAAATGGCTGTTGTGCAACAGGCACAACGTAATCTCTGTCTGGAAAGTTATGACCGTATAGAACAAACGTTAAAA
CATTGTATTGAAGCGAAAATGTTGCCTGCGGATTTAATGACGCGTCGCGCAGCAATTATTATGCGCGGCTATATT
TCCGGCCTGATGGAAAACTGGCTCTTTGCCCCGCAATCTTTTGATCTTAAAAAAGAAGCCCGCGATTACGTTGCC
ATCTTACTGGAGATGTATCTCCTGTGCCCCACGCTTCGTAATCCTGCCACTAACGAATAA

11.5.1.1.9 *acrR*-RNM-19

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCCATAAGCGCTAA

11.5.1.1.10 *acrR*-RNM-20

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCCATAAGCGCTAA

11.5.1.1.11 *acrR*-RNM-21

ATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGTCTTTTCTC
ACAGCAGGGGGTATCATCCCATAAGCGCTAA

11.5.1.1.12 *acrR*-RNM-22

CAAGTTATAAACCCATTGCTGCGTTTATATTATCGTCGTGCTATGGTACATACATTCACAAATGTATGTAAATCTAA
CGCCTGTAAATTCACGAACATATGGCACGAAAAACCAAACAAGAAGCGCAAGAAACGCGCCAACACATCCTCGA
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CATTGTATTGAAGCGAAAATGTTGCCTGCGGATTTAATGACGCGTCGCGCAGCAATTATTATGCGCGGCTATATT
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11.5.1.1.12 *acrR*-RNM-23

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AAGGTGCGAATAAGCGGGGAAATTCTTCTCGGCTGA

11.5.1.1.13 *acrR*-RNM-29

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CTCGGCTGA

11.5.1.2.1 *rob*-WT

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11.5.1.2.2 *rob*-RNM-2

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CAGGCGTAGTGCGACCGCCGATTTCGACAAACGACGAGCACGAATATACGCGCCAATAGCATGGCCAGTGACA
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11.5.1.2.3 *rob*-RNM-3

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CAGGCGTAGTGCGACCGCCGATTTCGACAAACGACGAGCACGAATATACGCGCCAATAGCATGGCCAGTGACA
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11.5.1.2.4 *rob*-RNM-7

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11.5.1.2.5 *rob*-RNM-18

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CAGGCGTAGTGCGACCGCCGATTTCGACAAACGACGAGCACGAATATACGCGCCAATAGCATGGCCAGTGACA
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11.5.1.2.6 *rob*-RNM-8

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11.5.1.2.7 *rob*-RNM-22

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11.5.1.3.1 *soxR*-WT

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AACGTGATGTGTTGCGATATGTTGCAATTATCAAAATTGCTCAGCGTATTGGCATTCCGCTGGCGACCATTGGTG
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AGAAGAGTTGGATCGGCGCATTCATACCTTAGTGGCGCTGCGTGACGAACTGGACGGATGTATTGGTTGTGGCT
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GCTGGAAGATGAACAAAACTAA

11.5.1.3.2 *soxR*-RNM-5

ATGGAAAAGAAATTACCCCGCATTAAAGCGCTGCTAACCCCCGGCGAAGTGGCGAAACGCAGCGGTGTGGCGG
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AACGTGATGTGTTGCGATATGTTGCAATTATCAAAATTGCTCAGCGTATTGGCATTCCGCTGGCGACCATTGGTG
AAGCGTTTGGCGTGTTGCCCGAAGGGCATACGTTAAGTGCGAAAGAGTGGAAACAGCTTTCGTCCCAATGGCG
AGAAGAGTTGGATCGGCGCATTCATACCTTAGTGGCGCTGCGTGACGAACTGGACGGATGTATTGGTTGTGGCT
GCCTTTCGCGCAGTGATTGCCCGTTGCGTAACCCGGGCGACCGCTAAGGAGAAGAAGGTACCGGCGCACGCTT
GCTGGAAGATGAACAAAACTAA

11.5.1.3.3 *soxR*-RNM-6

ATGGAAAAGAAATTACCCCGCATTAAAGCGCTGCTAACCCCCGGCGAAGTGGCGAAACGCAGCGGTGTGGCGG
TATCGGCGCTGCATTTCTATGAAAGTAAAGGGTTGATTACCAGTATCCGTAACAGCGGCAATCAGCGGCGATATA
AACGTGATGTGTTGCGATATGTTGCAATTATCAAAATTGCTCAGCGTATTGGCATTCCGCTGGCGACCATTGGTG
AAGCGTTTGGCGTGTTGCCCGAAGGGCATACGTTAAGTGCGAAAGAGTGGAAACAGCTTTCGTCCCAATGGCG
AGAAGAGTTGGATCGGCGCATTCATACCTTAGTGGCGCTGCGTGACGAACTGGACGGATGTATTGGTTGTGGCT
GCCTTTCGCGCAGTGATTGCCCGTTGCGTAACCCGGGCGACCGCTTAGGAGAAGAAGGTACCGGCCGCTTGCT
GGAAGATGAACAAAACTAA

11.5.1.3.4 *soxR*-RNM-23

ATGGAAAAGAAATTACCCCGCATTAAAGCGCTGCTAACCCCCGGCGAAGTGGCGAAACTCAGCGGTGTGGCGG
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AACGTGATGTGTTGCGATATGTTGCAATTATCAAAATTGCTCAGCGTATTGGCATTCCGCTGGCGACCATTGGTG
AAGCGTTTGGCGTGTTGCCCGAAGGGCATACGTTAAGTGCGAAAGAGTGGAAACAGCTTTCGTCCCAATGGCG
AGAAGAGTTGGATCGGCGCATTCATACCTTAGTGGCGCTGCGTGACGAACTGGACGGATGTATTGGTTGTGGCT
GCCTTTCGCGCAGTGATTGCCCGTTGCGTAACCCGGGCGACCGCTTAGGAGAAGAAGGTACCGGCGCACGCTT
GCTGGAAGATGAACAAAACTAA

11.5.1.4.1 *marR*-WT

GTGAAAAGTACCAGCGATCTGTTCAATGAAATTATTCCATTGGGTCGCTTAATCCATATGGTTAATCAGAAGAAAG
ATCGCCTGCTTAACGAGTATCTGTCTCCGCTGGATATTACCGCGGCACAGTTTAAGGTGCTCTGCTCTATCCGCT
GCGCGGCGTGTATTACTCCGGTTGAACTGAAAAAGGTATTGTCGGTCGACCTGGGAGCACTGACCCGTATGCT
GGATCGCCTGGTCTGTAAAGGCTGGGTGGAAAGGTTGCCGAACCCGAATGACAAGCGCGGCGTACTGGTAAAA
CTTACCACCGGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTGCACCAAGAATTAAC
AAAAAACCTGACGGCGGACGAAGTGGCAACACTTGAGTATTTGCTTAAGAAAGTCCTGCCG

11.5.1.4.2 *marR*-RNM-19

GTGAAAAGTACCAGCGATCTGTTCAATGAAATTATTCCATTGGGTCGCTTAATCCATATGGTTAATCAGAAGAAAG
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GCGCGGCGTGTATTACTCCGGTTGAACTGAAAAAGGTATTGTCGGTCGACCTGGGAGCACTGACCCGTATGCT
GGATCGCCTGGTCTGTAAAGGCTGGGGGGGAAAGGTTGCCGAACCCGAATGACAAGCGCGGCGTACTGGTAAAA
CTTACCACCGGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTGCACCAAGAATTAAC
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11.5.1.4.3 *marR*-RNM-20

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GCGCGGCGTGTATTACTCCGGTTGAACTGAAAAAGGTATTGTCGGTCGACCTGGGAGCACTGACCCGTATGCT
GGATCGCCTGGTCTGTAAAGGCTGGGGGGGAAAGGTTGCCGAACCCGAATGACAAGCGCGGCGTACTGGTAAAA
CTTACCACCGGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTGCACCAAGAATTAAC
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11.5.1.4.4 *marR*-RNM-21

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GGATCGCCTGGTCTGTAAAGGCTGGGGGGGAAAGGTTGCCGAACCCGAATGACAAGCGCGGCGTACTGGTAAAA
CTTACCACCGGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTGCACCAAGAATTAAC
AAAAAACCTGACGGCGGACGAAGTGGCAACACTTGAGTATTTGCTTAAGAAAGTCCTGCCG

11.5.1.5.1 *ompR*-WT

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ACCCGATGCCGATCATTATGGTGACGGCGAAAGGGGAAGAAGTGGACCGTATCGTAGGCCTGGAGATTGGCGC
TGACGACTACATTCCAAAACCGTTTAACCCGCGTGAACTGCTGGCCCGTATCCGTGCGGTGCTGCGTCGTCAGG
CGAACGAACTGCCAGGCGCACCGTCACAGGAAGAGGCGGTAATTGCTTTCGGTAAGTTCAAACTTAACCTCGGT
ACGCGCGAAATGTTCCGCGAAGACGAGCCGATGCCGCTCACCAGCGGTGAGTTTGCGGTACTGAAGGCACTGG
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GAACGCTCCATCGACGTGCAGATTTCGCGTCTGCGCCGCATGGTGGAAGAAGATCCAGCGCATCCGCGTTACA
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11.5.1.5.2 *ompR*-RNM-19

ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGAGCCTGCGTGCGCTGCTGGAACGTTATCTCAC
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ACCCGATGCCGATCATTATGGTGACGGCGAAAGGGGAAGAAGTGGACCGTATCGTAGGCCTGGAGATTGGCGC
TGACGACTACATTCCAAAACCGTTTAACCCGCGTGAACTGCTGGCCCGTATCCGTGCGGTGCTGCGTCGTCAGG
CGAACGAACTGCCAGGCGCACCGTCACAGGAAGAGGCGGTAATTGCTTTCGGTAAGTTCAAACTTAACCTCGGT
ACGCGCGAAATGTTCCGCGAAGACGAGCCGATGCCGCTCACCAGCGGTGAGTTTGCGGTACTGAAGGCACTGG
TCAGCCATCCGCGTGAGCCGCTCTCCCGCGATAAGCTGATGAACCTTGCCCGTGGTCGTGAATATTCCGCAATG
GAACGCTCCATCGACGTGCAGATTTCGCGTCTGCGCCGCATGGTGGAAGAAGATCCAGCGCATCCGCGTTACA
TTCAGACCGTCTGGGGTCTGGGCTACGTCTTTGTACCGGACGGCTCTAAAGCATGA

11.5.1.5.3 *ompR*-RNM-20

ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGAGCCTGCGTGCGCTGCTGGAACGTTATCTCAC
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ACCCGATGCCGATCATTATGGTGACGGCGAAAGGGGAAGAAGTGGACCGTATCGTAGGCCTGGAGATTGGCGC
TGACGACTACATTCCAAAACCGTTTAACCCGCGTGAACTGCTGGCCCGTATCCGTGCGGTGCTGCGTCGTCAGG
CGAACGAACTGCCAGGCGCACCGTCACAGGAAGAGGCGGTAATTGCTTTCGGTAAGTTCAAACTTAACCTCGGT
ACGCGCGAAATGTTCCGCGAAGACGAGCCGATGCCGCTCACCAGCGGTGAGTTTGCGGTACTGAAGGCACTGG
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GAACGCTCCATCGACGTGCAGATTTCGCGTCTGCGCCGCATGGTGGAAGAAGATCCAGCGCATCCGCGTTACA
TTCAGACCGTCTGGGGTCTGGGCTACGTCTTTGTACCGGACGGCTCTAAAGCATGA

11.5.1.5.4 *ompR*-RNM-21

ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGAGCCTGCGTGCGCTGCTGGAACGTTATCTCAC
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ACCCGATGCCGATCATTATGGTGACGGCGAAAGGGGAAGAAGTGGACCGTATCGTAGGCCTGGAGATTGGCGC
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CGAACGAACTGCCAGGCGCACCGTCACAGGAAGAGGCGGTAATTGCTTTCGGTAAGTTCAAACTTAACCTCGGT
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11.5.1.6.1 *phoP*-WT

ATGCGCGTACTGGTTGTTGAAGACAATGCGTTGTTACGTCACCACCTTAAAGTTCAGATTCAGGATGCTGGTCAT
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CTAAACCGTTTCATATTGAAGAGGTGATGGCGCGAATGCAGGCATTAATGCGGCGTAATAGCGGTCTGGCTTCA
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AAAAATTCAGGCACAATATCCCCAAGAAGTGATTACCACCGTTCGCGGCCAGGGCTATCTGTTCGAATTGCGCT
GA

11.5.1.6.2 *phoP*-RNM-3

ATGCGCGTACTGGTTGTTGAAGACAATGCGTTTTTACGTCACCACCTTAAAGTTCAGATTCAGGATGCTGGTCAT
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CTGACCGCGTTCGAATACACTATTATGGAAACGTTGATACGCAATAATGGCAAAGTGGTCAGCAAAGATTCGTTA
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GA

11.5.1.7.1 *cra*-WT

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AACGCCGTGGCAGCTGGGCTTCGTGCTGGACGCACACGTTCTATTGGTCTTGTGATCCCCGATCTGGAGAACA
CCAGCTATACCCGCATCGCTAACTATCTTGAACGCCAGGCGCGGCAACGGGGTTATCAACTGCTGATTGCCTGC
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GTTTGCGTTGTTGCAAGGAGTGATGGATGTCACGCTGCGTCGCGACGGCAAACTGCCTTCTGACCTGGCAATTG
CCACCTTTGGCGATAACGAACTGCTCGACTTCTTACAGTGTCCGGTGCTGGCAGTGGCTCAACGTCACCGCGAT
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11.5.1.7.2 *cra*-RNM-18

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AACGCCGTGGCAGCTGGGCTTCGTGCTGGACGCACACGTTCTATTGGTCTTGTGATCCCCGATCTGGAGAACA
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TCGACGTCGTTGCCTCCTGAGCATCCTTTTTATCAACGCTGGGCTAACGACCCGTTCCCGATTGTCGCGCTGGA
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GAGTTACGTAAGTTTCCCGCCGAGACGGTGCTTTATCTTGGTGCGCTACCGGAGCTTTCTGTCAGCTTCCTGCG
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GTTTGCGTTGTTGCAAGGAGTGATGGATGTCACGCTGCGTCGCGACGGCAAACTGCCTTCTGACCTGGCATTGC
CACCTTTGGCGATAACGAACTGCTCGACTTCTTACAGTGTCCGGTGCTGGCAGTGGCTCAACGTCACCGCGATG
TCGCAGAGCGTGTGCTGGAGATTGTCCTGGCAAGCCTGGACGAACCGCGTAAGCCAAAACCTGGTTTAACGCG
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11.5.1.8.1 *creA*-WT

ATGAAATACAAGCATTTGATCCTGTCTTTAAGCCTGATAATGCTGGGGCCATTGGCTCATGCAGAAGAGATTGGT
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AAAAATGTCACCTGTTATGTGAGCCGGGCGAAAACCGGTGGTATTAAAGGGGGATTGGGTCTGGCGGAAGATA
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CAGGGCGAGGTAGTATTCAAAAAACGCACGTCCCTGGTCTTTAAGTCGTTACAGGTCGTGCGCTTTTATGATGC
CAAACGCAACGCGCTCGCTTATCTGGCTTACTCCGACAAAGTTGTAGAAGGTTCGCCGAAAAACGCGATTAGCG
CGGTTCCTGTCATGCCGTGGCGGCAATAA

11.5.1.8.2 *creA*-RNM-18

ATGAAATACAAGCATTTGATCCTGTCTTTAAGCCTGATAATGCTGGGGCCATTGGCTCATGCAGAAGAGATTGGT
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CAGGGCGAGGTAGTATTCAAAAAACGCACGTCCCTGGTCTTTAAGTCGTTACAGGTCGTGCGCTTTTATGATGC
CAAACGCAACGCGCTCGCTTATCTGGCTTACTCCGACAAAGTTGTAGAAGGTTCGCCGAAAAACGCGATTAGCG
CGGTTCCTGTCATGCCGTGGCGGCAATAA

11.5.1.9.1 *rpoB*-WT

ATGGTTTACTCCTATACCGAGAAAAAACGTATTCGTAAGGATTTTGGTAAACGTCCACAAGTTCTGGATGTACCTT
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11.5.1.9.2 *rpoB*-RNM-8

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CTGCTTTCCGTTCCGTATTCCCGATTCAGAGCTACAGCGGTAATTCCGAGCTGCAATACGTCAGCTACCGCCTT
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GCGTCTGGTGATCTATGAGCGCGAAGCGCCGGAAGGCACCGTAAAAGACATTAAAGAACAAGAAGTCTACATG
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11.5.1.9.3 *rpoB*-RNM-22

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11.5.1.10.1 *rpoC*-WT

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11.5.1.10.2 *rpoC*-RNM-2

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11.5.1.10.3 *rpoC*-RNM-18

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11.5.1.10.4 *rpoC*-RNM-19

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11.5.1.10.5 *rpoC*-RNM-20

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11.5.1.10.6 *rpoC*-RNM-21

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11.5.1.11.2 *acrB*-RNM-3

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11.5.1.12.2 *yohJ*-RNM-18

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11.5.1.13.1 *dnaK*-WT

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11.5.1.13.2 *dnaK*-RNM-18

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11.5.1.14.1 *groL*-WT

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11.5.1.13.2 *groL*-RNM-22

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11.5.1.15.1 *ilvN*-WT

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11.5.1.15.2 *ilvN*-RNM-2

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11.5.1.15.3 *ilvN*-RNM-3

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11.5.1.16.1 *ygbK*-WT

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11.5.1.16.2 *ygbK*-RNM-2

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11.5.1.17.1 *clsA*-WT

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11.5.1.17.2 *clsA*-RNM-18

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11.5.1.18.1 *opgH*-WT

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11.5.1.18.2 *opgH*-RNM-18

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CTCGGTAAAGGCGCGTCTGGAACAAGCCTGGCCAGATTCACTTGCTGATGGACAGTTAATTAAAGACGACGAAG
GGCGCGATCAGCTGAAGGCGATGCCAGAAGCAAAACGCTCCTCGATGTTTCCCGACCCGTGGCCTACCAACCC
GGTAGGCCGTTTCTGGGATCGCCTGCGTGGACGCGATGTCACGCCGCGCTATCTGGCTCGTTTGACCAAAGAA
GAGCAGGAGAGCGAGCAAAAGTGGCGTACCGTCGGTACCATCCGCCGTTACATTCTGTTGATCCTGACGCTCG
CGCAAACTGTCGTCGCGACCTGGTATATGAAGACCATTCTTCCTTATCAGGGTTGGGCGCTGATTAATCCTATGG
ATATGGTTGGTCAGGATTTGTGGGTTTCCTTTATGCAGCTTCTGCCTTATATGCTGCAAACCGGTATCCTGATCC
TCTTTGCGGTACTGTTCTGTTGGGTGTCCGCCGGATTCTGGACGGCGTTAATGGGCTTCCTGCAACTGCTTATT
GGTCGCGATAAATACAGTATATCTGCGTCAACAGTTGGCGATGAACCATTAAACCCGGAGCATCGCACGGCGTT
GATCATGCCTATCTGTAACGAAGACGTGAACCGTGTTTTTGCTGGCCTGCGTGCAACGTGGGAATCAGTAAAAG
CCACCGGGAATGCCAAACACTTTGATGTCTACATTCTTAGTGACAGTTATAACCCGGATATCTGCGTCGCAGAGC
AAAAAGCCTGGATGGAGCTTATCGCTGAAGTCGGTGGCGAAGGTCAGATTTTCTATCGCCGCCGCCGTCGCCG
CGTGAAGCGTAAAAGCGGTAATATCGATGACTTCTGCCGTCGCTGGGGCAGCCAGTACAGCTACATGGTGGTG
CTGGATGCTGACTCGGTAATGACCGGTGATTGTTTGTGCGGGCTGGTGCGCCTGATGGAAGCCAACCCGAACG
CCGGGATCATTCAGTCGTCGCCGAAAGCGTCCGGTATGGATACGCTGTATGCGCGCTGTCAGCAGTTCGCGAC
CCGCGTGTATGGGCCACTGTTTACAGCCGGTTTGCACTTCTGGCAACTTGGCGAGTCGCACTACTGGGGACATA
ACGCGATTATCCGCGTGAAACCGTTTATCGAGCACTGCGCACTGGCTCCGCTGCCGGGCGAAGGTTCCTTTGC
CGGTTCAATCCTGTCACATGACTTCGTGGAAGCGGCGTTGATGCGCCGTGCAGGTTGGGGGGTCTGGATTGCT
TACGATCTCCCGGGTTCTTATGAAGAATTGCCGCCTAACTTGCTTGATGAGCTAAAACGTGACCGCCGATGGTG
CCACGGTAACCTGATGAACTTCCGTCTGTTCCTGGTGAAGGGTATGCACCCGGTTCACCGTGCGGTGTTCCTGA
CGGGCGTGATGTCTTATCTCTCCGCTCCGCTGTGGTTTATGTTCCTCGCGCTCTCTACTGCATTGCAGGTAGTG
CATGCGTTGACCGAACCGCAATACTTCCTGCAACCACGGCAGTTGTTCCCAGTGTGGCCGCAGTGGCGTCCTG
AGCTGGCGATTGCACTTTTTGCTTCGACCATGGTGCTGTTGTTCCTGCCGAAGTTATTGAGCATTTTGCTTATCT
GGTGCAAAGGAACGAAAGAATACGGCGGCTTCTGGCGCGTTACATTATCGTTGCTGCTGGAAGTGCTTTTTTCC
GTGCTGCTGGCTCCGGTACGCATGCTGTTCCATACGGTCTTCGTTGTCAGCGCGTTCCTTGGCTGGGAAGTGGT
GTGGAATTCACCGCAGCGTGATGATGACTCCACTTCCTGGGGTGAAGCGTTCAAACGCCACGGCTCACAGCTG
CTGTTAGGGTTAGTGTGGGCTGTTGGGATGGCGTGGCTGGATCTGCGTTTCCTGTTCTGGCTGGCACCGATTGT
CTTCTCGTTGATCCTGTCACCGTTTGTTTCGGTGATTTCCAGCCGTGCCACCGTTGGTCTGCGCACCAAACGCT
GGAAACTGTTCCTGATCCCGGAAGAGTATTCGCCGCCGCAGGTGCTGGTTGATACCGATCGGTTCCTTGAGATG
AATCGTCAACGCTCCCTTGATGATGGCTTTATGCACGCAGTGTTTAACCCGTCATTTAACGCTCTGGCAACCGCA
ATGGCGACCGCGCGTCACCGCGCCAGTAAGGTGCTGGAAATCGCCCGTGACCGCCACGTTGAACAGGCGCTG
AACGAGACGCCAGAGAAGCTGAATCGCGATCGTCGCCTGGTGCTGCTAAGCGATCCGGTGACGATGGCCCGTC
TGCATTTCCGTGTCTGGAATTCCCCGGAGAGATATTCTTCATGGGTGAGTTATTACGAAGGGATAAAGCTCAATC
CACTGGCATTGCGTAAACCGGATGCGGCTTCGCAATAA

11.5.1.19.1 *pepA*-WT

ATGGAGTTTAGTGTAAAAAGCGGTAGCCCGGAGAAACAGCGGAGTGCCTGCATCGTCGTGGGCGTCTTCGAAC
CACGTCGCCTTTCTCCGATTGCAGAACAGCTCGATAAAATCAGCGATGGGTACATCAGCGCCCTGCTACGTCGG
GGCGAACTGGAAGGAAAACCGGGGCAGACATTGTTGCTGCACCATGTTCCGAATGTACTTTCCGAGCGAATTCT
CCTTATTGGTTGCGGCAAAGAACGTGAGCTGGATGAGCGTCAGTACAAGCAGGTTATTCAGAAAACCATTAATAC
GCTGAATGATACTGGCTCAATGGAAGCGGTCTGCTTTCTGACTGAGCTGCACGTTAAAGGCCGTAACAACTACT
GGAAAGTGCGTCAGGCTGTCGAGACGGCAAAAGAGACGCTCTACAGTTTCGATCAGCTGAAAACGAACAAGAG
CGAACCGCGTCGTCCGCTGCGTAAGATGGTGTTCAACGTGCCGACCCGCCGTGAACTGACCAGCGGTGAGCG
CGCGATCCAGCACGGTCTGGCGATTGCCGCCGGGATTAAAGCAGCAAAAGATCTCGGCAATATGCCGCCGAAT
ATCTGTAACGCCGCTTACCTCGCTTCACAAGCGCGCCAGCTGGCTGACAGCTACAGCAAGAATGTCATCACCCG
CGTTATCGGCGAACAGCAGATGAAAGAGCTGGGGATGCATTCCTATCTGGCGGTCGGTCAGGGTTCGCAAAAC
GAATCGCTGATGTCGGTGATTGAGTACAAAGGCAACGCGTCGGAAGATGCACGCCCAATCGTGCTGGTGGGTA
AAGGTTTAACCTTCGACTCCGGCGGTATCTCGATCAAGCCTTCAGAAGGCATGGATGAGATGAAGTACGATATG
TGCGGTGCGGCAGCGGTTTACGGCGTGATGCGGATGGTCGCGGAGCTACAACTGCCGATTAACGTTATCGGCG
TGTTGGCAGGCTGCGAAAACATGCCTGGCGGACGAGCCTATCGTCCGGGCGATGTGTTAACCACCATGTCCGG
TCAAACCGTTGAAGTGCTGAACACCGACGCTGAAGGCCGCCTGGTACTGTGCGACGTGTTAACTTACGTTGAGC
GTTTTGAGCCGGAAGCGGTGATTGACGTGGCGACGCTGACCGGTGCCTGCGTGATCGCGCTGGGTCATCATAT

TACTGGTCTGATGGCGAACCATAATCCGCTGGCCCATGAACTGATTGCCGCGTCTGAACAATCCGGTGACCGCG
CATGGCGCTTACCGCTGGGTGACGAGTATCAGGAACAACTGGAGTCCAATTTTGCCGATATGGCGAACATTGGC
GGTCGTCCTGGTGGGGCGATTACCGCAGGTTGCTTCCTGTCACGCTTTACCCGTAAGTACAACTGGGCGCACC
TGGATATCGCCGGTACCGCCTGGCGTTCTGGTAAAGCAAAAGGCGCCACCGGTCGTCCGGTAGCGTTGCTGGC
ACAGTTCCTGTTAAACCGCGCTGGGTTTAACGGCGAAGAGTAA

11.5.1.19.2 *pepA*-RNM-29

ATGGAGTTTAGTGTAAAAAGCGGTAGCCCGGAGAAACAGCGGAGTGCCTGCATCGTCGTGGGCGTCTTCGAAC
CACGTCGCCTTTCTCCGATTGCAGAACAGCTCGATAAAATCAGCGATGGGTACATCAGCGCCCTGCTACGTCGG
GGCGAACTGGAAGGAAAACCGGGGCAGACATTGTTGCTGCACCATGTTCCGAATGTACTTTCCGAGCGAATTCT
CCTTTTGGTTGCGGCAAAGAACGTGAGCTGGATGAGCGTCAGTACAAGCAGGTTATTCAGAAAACCATTAATAC
GCTGAATGATACTGGCTCAATGGAAGCGGTCTGCTTTCTGACTGAGCTGCACGTTAAAGGCCGTAACAACTACT
GGAAAGTGCGTCAGGCTGTCGAGACGGCAAAAGAGACGCTCTACAGTTTCGATCAGCTGAAAACGAACAAGAG
CGAACCGCGTCGTCCGCTGCGTAAGATGGTGTTCAACGTGCCGACCCGCCGTGAACTGACCAGCGGTGAGCG
CGCGATCCAGCACGGTCTGGCGATTGCCGCCGGGATTAAAGCAGCAAAAGATCTCGGCAATATGCCGCCGAAT
ATCTGTAACGCCGCTTACCTCGCTTCACAAGCGCGCCAGCTGGCTGACAGCTACAGCAAGAATGTCATCACCCG
CGTTATCGGCGAACAGCAGATGAAAGAGCTGGGGATGCATTCCTATCTGGCGGTCGGTCAGGGTTCGCAAAAC
GAATCGCTGATGTCGGTGATTGAGTACAAAGGCAACGCGTCGGAAGATGCACGCCCAATCGTGCTGGTGGGTA
AAGGTTTAACCTTCGACTCCGGCGGTATCTCGATCAAGCCTTCAGAAGGCATGGATGAGATGAAGTACGATATG
TGCGGTGCGGCAGCGGTTTACGGCGTGATGCGGATGGTCGCGGAGCTACAACTGCCGATTAACGTTATCGGCG
TGTTGGCAGGCTGCGAAAACATGCCTGGCGGACGAGCCTATCGTCCGGGCGATGTGTTAACCACCATGTCCGG
TCAAACCGTTGAAGTGCTGAACACCGACGCTGAAGGCCGCCTGGTACTGTGCGACGTGTTAACTTACGTTGAGC
GTTTTGAGCCGGAAGCGGTGATTGACGTGGCGACGCTGACCGGTGCCTGCGTGATCGCGCTGGGTCATCATAT
TACTGGTCTGATGGCGAACCATAATCCGCTGGCCCATGAACTGATTGCCGCGTCTGAACAATCCGGTGACCGCG
CATGGCGCTTACCGCTGGGTGACGAGTATCAGGAACAACTGGAGTCCAATTTTGCCGATATGGCGAACATTGGC
GGTCGTCCTGGTGGGGCGATTACCGCAGGTTGCTTCCTGTCACGCTTTACCCGTAAGTACAACTGGGCGCACC
TGGATATCGCCGGTACCGCCTGGCGTTCTGGTAAAGCAAAAGGCGCCACCGGTCGTCCGGTAGCGTTGCTGGC
ACAGTTCCTGTTAAACCGCGCTGGGTTTAACGGCGAAGAGTAA

11.5.2 Amino acid sequence of affected proteins

11.5.2.1 AcrR

11.5.2.1.1 AcrR-WT

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLREILIHVLESTVTEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADL
MTRRAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCPTLRNPATNE

11.5.2.1.2 AcrR-RNM-2

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLRKVRISGEILLG

11.5.2.1.3 AcrR-RNM-3

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLRKVRISGEILLG

11.5.2.1.4 AcrR-RNM-5

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLREILIHVLESTVTEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADL
MTRRAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCPTLRNPATKEGANKRGNSSRLTQSFHFFMFE
PIFSPVNALNQPI

11.5.2.1.5 AcrR-RNM-6

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEGD
AANLLI

11.5.2.1.6 AcrR-RNM-7

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLRKVRTSP

11.5.2.1.7 AcrR-RNM-18

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLREILIHVLESTVTEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADL
MTRRAAIIMRGYISGLMENWLFAPQSFDLKKEPAITLPSYWRCISCAPRFVILPLTNNPESDSRIFPGHFRRCYSGSLR
RDILAV

11.5.2.1.8 AcrR-RNM-8

QVINPLLRLYYRRAMVHTFTNVCKSNACKFTNIWHEKPNKKRKKRANTSSMWLYVFSHSRGYHPPRWARLQKQLAL
RAVQSKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTVTEERRRLLMEIIFHKCEFVGEMAVVQ
QAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTRRAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCP
TLRNPATNE

11.5.2.1.9 AcrR-RNM-19

MARKTKQEAQETRQHILDVALRLFSQQGVSSHKR

11.5.2.1.10 AcrR-RNM-20

MARKTKQEAQETRQHILDVALRLFSQQGVSSHKR

11.5.2.1.11 AcrR-RNM-21

MARKTKQEAQETRQHILDVALRLFSQQGVSSHKR

11.5.2.1.12 AcrR-RNM-22

QVINPLLRLYYRRAMVHTFTNVCKSNACKFTNIWHEKPNKKRKKRANTSSMWLYVFSHSRGYHPPRWARLQKQLAL
RAVQSKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTVTEERRRLLMEIIFHKCEFVGEMAVVQ
QAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTRRAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCP
TLRNPATNE

11.5.2.1.12 AcrR-RNM-23

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELGKVRIS
GEILLG

11.5.2.1.13 AcrR-RNM-29

MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQ
AKFPGDPLSVLRKVRISGEILLG

11.5.2.2.1 Rob-WT

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTARPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRH
EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.2 Rob-RNM-2

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTARPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFHH

EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.3 Rob-RNM-3

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTARPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFHH
EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.4 Rob-RNM-7

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTVRPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRH
EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.5 Rob-RNM-18

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTTRPILDIALQY
RFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRHE
MRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGLGT
GVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.6 Rob-RNM-8

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTARPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFHH
EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.2.7 Rob-RNM-22

MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVALRLTARPILDIALQ
YRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRLGEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFHH
EMRYQFWHDFLGNAPTIPPVLYGLNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGL
GTGVQEFILTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR

11.5.2.3.1 SoxR-WT

MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGVLPE
GHTLSAKEWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGARLLEDEQN

11.5.2.3.2 SoxR-RNM-5

MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGVLPE
GHTLSAKEWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDR

11.5.2.3.3 SoxR-RNM-6

MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGVLPE
GHTLSAKEWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGRLLEDEQN

11.5.2.3.4 SoxR-RNM-23

MEKKLPRIKALLTPGEVAKLSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGVLPE
GHTLSAKEWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGARLLEDEQN

11.5.2.4.1 MarR-WT

MKSTSDLFNEIIPLGRLIHMVNQKKDRLLNEYLSPLDITAAQFKVLCSIRCAACITPVELKKVLSVDLGALTRMLDRLVCK
GWVERLPNPNDKRGVLVKLTTGGAAICEQCHQLVGQDLHQELTKNLTADEVATLEYLLKKVLP

11.5.2.4.2 MarR-RNM-19

MKSTSDLFNEIIPLGRLIHMVNQKKDRLLNEYLSPLDITAAQFKVLCSIRCAACITPVELKKVLSVDLGALTRMLDRLVCK
GWGERLPNPNDKRGVLVKLTTGGAAICEQCHQLVGQDLHQELTKNLTADEVATLEYLLKKVLP

11.5.2.4.3 MarR-RNM-20

MKSTSDLFNEIIPLGRLIHMVNQKKDRLLNEYLSPLDITAAQFKVLCSIRCAACITPVELKKVLSVDLGALTRMLDRLVCK
GWGERLPNPNDKRGVLVKLTTGGAAICEQCHQLVGQDLHQELTKNLTADEVATLEYLLKKVLP

11.5.2.4.4 MarR-RNM-21

MKSTSDLFNEIIPLGRLIHMVNQKKDRLLNEYLSPLDITAAQFKVLCSIRCAACITPVELKKVLSVDLGALTRMLDRLVCK
GWGERLPNPNDKRGVLVKLTTGGAAICEQCHQLVGQDLHQELTKNLTADEVATLEYLLKKVLP

11.5.2.5.1 OmpR-WT

MQENYKILVVDDDMRLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPGEDGLSICRRLRSQSNPM
PIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDE
PMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVP
DGSKA

11.5.2.5.2 OmpR-RNM-19

MQENYKILVVDDDMSLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPGEDGLSICRRLRSQSNPM
PIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDE
PMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVP
DGSKA

11.5.2.5.3 OmpR-RNM-20

MQENYKILVVDDDMSLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPGEDGLSICRRLRSQSNPM
PIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDE
PMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVP
DGSKA

11.5.2.5.4 OmpR-RNM-21

MQENYKILVVDDDMSLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPGEDGLSICRRLRSQSNPM
PIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDE
PMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVP
DGSKA

11.5.2.6.1 PhoP-WT

MRVLVVEDNALLRHHLKVQIQDAGHQVDDAEDAKEADYYLNEHIPDIAIVDLGLPDEDGLSLIRRWRSNDVSLPILVLT
ARESWQDKVEVLSAGADDYVTKPFHIEEVMARMQALMRRNSGLASQVISLPPFQVDLSRRELSINDEVIKLTAFEYTI
METLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRKKIQAQYPQEVITTVRGQGYLFELR

11.5.2.6.2 PhoP-RNM-3

MRVLVVEDNAPLRHHLKVQIQDAGHQVDDAEDAKEADYYLNEHIPDIAIVDLGLPDEDGLSLIRRWRSNDVSLPILVLT
ARESWQDKVEVLSAGADDYVTKPFHIEEVMARMQALMRRNSGLASQVISLPPFQVDLSRRELSINDEVIKLTAFEYTI
METLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRKKIQAQYPQEVITTVRGQGYLFELR

11.5.2.7.1 Cra-WT

VKLDEIARLAGVSRTTASYVINGKAKQYRVSDKTVEKVMAVVREHNYHPNAVAAGLRAGRTRSIGLVIPDLENTSYTRI
ANYLERQARQRGYQLLIACSEDQPDNEMRCIEHLLQRQVDAIIVSTSLPPEHPFYQRWANDPFPIVALDRALDREHFT
SVVGADQDDAEMLAEELRKFPAETVLYLGALPELSVSFLREQGFRTAWKDDPREVHFLYANSYEREAAAQLFEKWLE
THPMPQALFTTSFALLQGVMDVTLRRDGKLPSDLAIATFGDNELLDFLQCPVLAVAQRHRDVAERVLEIVLASLDEPR
KPKPGLTRIKRNLYRRGVLSRS

11.5.2.7.2 Cra-RNM-18

VKLDEIARLAGVSRTTASYVINGKAKQYRVSDKTVEKVMAVVREHNYHPNAVAAGLRAGRTRSIGLVIPDLENTSYTRI
ANYLERQARQRGYQLLIACSEDQPDNEMRCIEHLLQRQVDAIIVSTSLPPEHPFYQRWANDPFPIVALDRALDREHFT
SVVGADQDDAEMLAEELRKFPAETVLYLGALPELSVSFLREQGFRTAWKDDPREVHFLYANSYEREAAAQLFEKWLE
THPMPQALFTTSFALLQGVMDVTLRRDGKLPSDLALPPLAITNCSTSYSVRCWQWLNVTAMSQSVCWRLSWQAWT
NRVSQNLV

11.5.2.8.1 CreA-WT

MKYKHLILSLSLIMLGPLAHAEEIGSVDTVFKMIGPDHKIVVEAFDDPDVKNVTCYVSRAKTGGIKGGLGLAEDTSDAAI
SCQQVGPIELSDRIKNGKAQGEVVFKKRTSLVFKSLQVVRFYDAKRNALAYLAYSDKVVEGSPKNAISAVPVMPWRQ

11.5.2.8.2 CreA-RNM-18

MKYKHLILSLSLIMLGPLAHAEEIGSVDTVFKMIGPDHKIVVEAFDDPDVKNVTCYVSRAKTGGIKGGLGLAEDTSDAAI
SCQQVGPIELSDRIKNGKAQGEVVFKKRTSLVFKSLQVVRFYDAKRNALAYLAYSDKVVEGSPKNAISAVPVMPWRQ

11.5.2.9.1 RpoB-WT

MVYSYTEKKRIRKDFGKRPQVLDVPYLLSIQLDSFQKFIEQDPEGQYGLEAAFRSVFPIQSYSGNSELQYVSYRLGEP
VFDVQECQIRGVTYSAPLRVKLRLVIYEREAPEGTVKDIKEQEVYMGEIPLMTDNGTFVINGTERVIVSQLHRSPGVFF
DSDKGKTHSSGKVLYNARIIPYRGSWLDFEFDPKDNLFVRIDRRRKLPATIILRALNYTTEQILDLFFEKVIFEIRDNKLQ
MELVPERLRGETASFDIEANGKVYVEKGRRITARHIRQLEKDDVKLIEVPVEYIAGKVVAKDYIDESTGELICAANMELS
LDLLAKLSQSGHKRIETLFTNDLDHGPYISETLRVDPTNDRLSALVEIYRMMRPGEPPTREAAESLFENLFFSEDRYDL
SAVGRMKFNRSLLREEIEGSGILSKDDIIDVMKKLIDIRNGKGEVDDIDHLGNRRIRSVGEMAENQFRVGLVRVERAVK
ERLSLGDLDTLMPQDMINAKPISAAVKEFFGSSQLSQFMDQNNPLSEITHKRRISALGPGGLTRERAGFEVRDVHPTH
YGRVCPIETPEGPNIGLINSLSVYAQTNEYGFLETPYRKVTDGVVTDEIHYLSAIEEGNYVIAQANSNLDEEGHFVEDLV
TCRSKGESSLFSRDQVDYMDVSTQQVVSVGASLIPFLEHDDANRALMGANMQRQAVPTLRADKPLVGTGMERAVA
VDSGVTAVAKRGGVVQYVDASRIVIKVNEDEMYPGEAGIDIYNLTKYTRSNQNTCINQMPCVSLGEPVERGDVLADG
PSTDLGELALGQNMRVAFMPWNGYNFEDSILVSERVVQEDRFTTIHIQELACVSRDTKLGPEEITADIPNVGEAALSKL
DESGIVYIGAEVTGGDILVGKVTPKGETQLTPEEKLLRAIFGEKASDVKDSSLRVPNGVSGTVIDVQVFTRDGVEKDKR
ALEIEEMQLKQAKKDLSEELQILEAGLFSRIRAVLVAGGVEAEKLDKLPRDRWLELGLTDEEKQNQLEQLAEQYDELK
HEFEKKLEAKRRKITQGDDLAPGVLKIVKVYLAVKRRIQPGDKMAGRHGNKGVISKINPIEDMPYDENGTPVDIVLNPL
GVPSRMNIGQILETHLGMAAKGIGDKINAMLKQQQEVAKLREFIQRAYDLGADVRQKVDLSTFSDEEVMRLAENLRKG
MPIATPVFDGAKEAEIKELLKLGDLPTSGQIRLYDGRTGEQFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQ
QPLGGKAQFGGQRFGEMEVWALEAYGAAYTLQEMLTVKSDDVNGRTKMYKNIVDGNHQMEPGMPESFNVLLKEIR
SLGINIELEDE

11.5.2.9.2 RpoB-RNM-8

MVYSYTEKKRIRKDFGKRPQVLDVPYLLSIQLDSFQKFIEQDPEGQYGLEAAFRSVFPIQSYSGNSELQYVSYRLGEP
VFDVQECQIRGVTYSAPLRVKLRLVIYEREAPEGTVKDIKEQEVYMGEIPLMTDNGTFVINGTERVIVSQLHRSPGVFF
DSDKGKTHSSGKVLYNARIIPYRGSWLDFEFDPKDNLFVRIDRRRKLPATIILRALNYTTEQILDLFFEKVIFEIRDNKLQ
MELVPERLRGETASFDIEANGKVYVEKGRRITARHIRQLEKDDVKLIEVPVEYIAGKVVAKDYIDESTGELICAANMELS
LDLLAKLSQSGHKRIETLFTNDLDHGPYISETLRVDPTNDRLSALVEIYRMMRPGEPPTREAAESLFENLFFSEDRYDL
SAVGRMKFNRSLLREEIEGSGILSKDDIIDVMKKLIDIRNGKGEVDDIDHLGNRRIRSVGEMAENQFRVGLVRVERAVK
ERLSLGDLDTLMPQDMINAKPISAAVKEFFGSSQLSQFMDQNNPLSEITHKRRISALGPGGLTRERAGFEVRDVHPTH
YGRVCPIETPEGPNIGLINSLSVYAQTNEYGFLETPYRKVTDGVVTDEIHYLSAIEEGNYVIAQANSNLDEEGHFVEDLV
TCRSKGESSLFSRDQVDYMDVSTQQVVSVGASLIPFLEHDDANRALMGANMQRQAVPTLRADKPLVGTGMERAVA
VDSGVTAVAKRGGVVQYVDASRIVIKVNEDEMYPGEAGIDIYNLTKYTRSNQNTCINQMPCVSLGEPVERGDVLADG
PSTDLGELALGQNMRVAFMPWNGYNFEDSILVSERVVQEDRFTTIHIQELACVSRDTKLGPEEITADIPNVGEAALSKL
DESGIVYIGAEVTGGDILVGKVTPKGETQLTPEEKLLRAIFGEKASDVKDSSLRVPNGVSGTVIDVQVFTRDGVEKDKR
ALEIEEMQLKQAKKDLSEELQILEAGLFSRIRAVLVAGGVEAEKLDKLPRDRWLELGLTDEEKQNQLEQLAEQYDELK
HEFEKKLEAKRRKIPQGDDLAPGVLKIVKVYLAVKRRIQPGDKMAGRHGNKGVISKINPIEDMPYDENGTPVDIVLNPL

GVPSRMNIGQILETHLGMAAKGIGDKINAMLKQQQEVAKLREFIQRAYDLGADVRQKVDLSTFSDEEVMRLAENLRKG
MPIATPVFDGAKEAEIKELLKLGDLPTSGQIRLYDGRTGEQFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQ
QPLGGKAQFGGQRFGEMEVWALEAYGAAYTLQEMLTVKSDDVNGRTKMYKNIVDGNHQMEPGMPESFNVLLKEIR
SLGINIELEDE

11.5.2.9.3 RpoB-RNM-22

MVYSYTEKKRIRKDFGKRPQVLDVPYLLSIQLDSFQKFIEQDPEGQYGLEAAFRSVFPIQSYSGNSELQYVSYRLGEP
VFDVQECQIRGVTYSAPLRVKLRLVIYEREAPEGTVKDIKEQEVYMGEIPLMTDNGTFVINGTERVIVSQLHRSPGVFF
DSDKGKTHSSGKVLYNARIIPYRGSWLDFEFDPKDNLFVRIDRRRKLPATIILRALNYTTEQILDLFFEKVIFEIRDNKLQ
MELVPERLRGETASFDIEANGKVYVEKGRRITARHIRQLEKDDVKLIEVPVEYIAGKVVAKDYIDESTGELICAANMELS
LDLLAKLSQSGHKRIETLFTNDLDHGPYISETLRVDPTNDRLSALVEIYRMMRPGEPPTREAAESLFENLFFSEDRYDL
SAVGRMKFNRSLLREEIEGSGILSKDDIIDVMKKLIDIRNGKGEVDDIDHLGNRRIRSVGEMAENQFRVGLVRVERAVK
ERLSLGDLDTLMPQDMINAKPISAAVKEFFGSSQLSQFMDQNNPLSEITHKRRISALGPGGLTRERAGFEVRDVHPTH
YGRVCPIETPEGPNIGLINSLSVYAQTNEYGFLETPYRKVTDGVVTDEIHYLSAIEEGNYVIAQANSNLDEEGHFVEDLV
TCRSKGESSLFSRDQVDYMDVSTQQVVSVGASLIPFLEHDDANRALMGANMQRQAVPTLRADKPLVGTGMERAVA
VDSGVTAVAKRGGVVQYVDASRIVIKVNEDEMYPGEAGIDIYNLTKYTRSNQNTCINQMPCVSLGEPVERGDVLADG
PSTDLGELALGQNMRVAFMPWNGYNFEDSILVSERVVQEDRFTTIHIQELACVSRDTKLGPEEITADIPNVGEAALSKL
DESGIVYIGAEVTGGDILVGKVTPKGETQLTPEEKLLRAIFGEKASDVKDSSLRVPNGVSGTVIDVQVFTRDGVEKDKR
ALEIEEMQLKQAKKDLSEELQILEAGLFSRIRAVLVAGGVEAEKLDKLPRDRWLELGLTDEEKQNQLEQLAEQYDELK
HEFEKKLEAKRRKIPQGDDLAPGVLKIVKVYLAVKRRIQPGDKMAGRHGNKGVISKINPIEDMPYDENGTPVDIVLNPL
GVPSRMNIGQILETHLGMAAKGIGDKINAMLKQQQEVAKLREFIQRAYDLGADVRQKVDLSTFSDEEVMRLAENLRKG
MPIATPVFDGAKEAEIKELLKLGDLPTSGQIRLYDGRTGEQFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQ
QPLGGKAQFGGQRFGEMEVWALEAYGAAYTLQEMLTVKSDDVNGRTKMYKNIVDGNHQMEPGMPESFNVLLKEIR
SLGINIELEDE

11.5.2.10.1 RpoC-WT

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKA
AKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLE
AQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRIT
EYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAA
RSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAANDRVSKAMMDNLQTETVINRDGQ
EEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALKTA
NSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQW
CDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAA
ESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHT
MPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLRPALKIVDAQGNDVLIPGTDMPAQYFLPG
KAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGS
DPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQML
RKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.2 RpoC-RNM-2

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYRRLHQCGLPKKMALELFKPFIYGKLELRGLATTIK
AAKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTL
EAQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRI
TEYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYA
ARSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAANDRVSKAMMDNLQTETVINRDG
QEEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALK
TANSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQ
WCDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAA
AESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPH
TMPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLRPALKIVDAQGNDVLIPGTDMPAQYFLP
GKAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDG

SDPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQM
LRKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.3 RpoC-RNM-18

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRIKLLEAFVQSGNKPEWMILTVLPV
LPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAITGSNKRP
LKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKAAKKMVE
REEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLEAQLEAR
ALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRITEYEKDA
NGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAARSGASV
GIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAANDRVSKAMMDNLQTETVINRDGQEEKQVS
FNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALKTANSGYLT
RRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQWCDLLEE
NSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAAESSIQVK
NKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHTMPVITEV
SGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLRPALKIVDAQGNDVLIPGTDMPAQYFLPGKAIVQLE
DGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGSDPYEEMI
PKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQMLRKATIVNA
GSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGKRDELRGLK
ENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.4 RpoC-19

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKA
AKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLE
AQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRIT
EYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAA
RSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAANDRVSKAMMDNLQTETVINRDGQ
EEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALKTA
NSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQW
CDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAA
ESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHT
MPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLCPALKIVDAQGNDVLIPGTDMPAQYFLPG
KAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGS
DPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQML
RKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.5 RpoC-20

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKA
AKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLE
AQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRIT
EYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAA
RSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAANDRVSKAMMDNLQTETVINRDGQ
EEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALKTA
NSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQW
CDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAA
ESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHT
MPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLCPALKIVDAQGNDVLIPGTDMPAQYFLPG
KAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGS
DPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQML

RKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.5 RpoC-21

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKA
AKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLE
AQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRIT
EYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAA
RSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAAANDRVSKAMMDNLQTETVINRDGQ
EEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTVLKTA
NSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQW
CDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAA
ESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHT
MPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLCPALKIVDAQGNDVLIPGTDMPAQYFLPG
KAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGS
DPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQML
RKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.10.7 RpoC-23

VKDLLKFLKAQTKTEEFDAIKIALASPDMIRSWSFGEVKKPETINYRTFKPERDGLFCARIFGPVKDYECLCGKYKRLKH
RGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSLPSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQIL
TEEQYLDALEEFGDEFDAKMGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEWMI
LTVLPVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNNRLKRLLDLAAPDIIVRNEKRMLQEAVDALLDNGRRGRAIT
GSNKRPLKSLADMIKGKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALELFKPFIYGKLELRGLATTIKA
AKKMVEREEAVVWDILDEVIREHPVLLNRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGDQMAVHVPLTLE
AQLEARALMMSTNNILSPANGEPIIVPSQDVVLGLYYMTRDCVNAKGEGMVLTGPKEAERLYRSGLASLHARVKVRIT
EYEKDANGELVAKTSLKDTTVGRAILWMIVPKGLPYSIVNQALGKKAISKMLNTCYRILGLKPTVIFADQIMYTGFAYAA
RSGASVGIDDMVIPEKKHEIISEAEAEVAEIQEQFQSGLVTAGERYNKVIDIWAAAANDRVSKAMMDNLQTETVINRDGQ
EEKQVSFNSIYMMADSGARGSAAQIRQLAGMRGLMAKPDGSIIETPITANFREGLNVLQYFISTHGARKGLADTALKTA
NSGYLTRRLVDVAQDLVVTEDDCGTHEGIMMTPVIEGGDVKEPLRDRVLGRVTAEDVLKPGTADILVPRNTLLHEQW
CDLLEENSVDAVKVRSVVSCDTDFGVCAHCYGRDLARGHIINKGEAIGVIAAQSIGEPGTQLTMRTFHIGGAASRAAA
ESSIQVKNKGSIKLSNVKSVVNSSGKLVITSRNTELKLIDEFGRTKESYKVPYGAVLAKGDGEQVAGGETVANWDPHT
MPVITEVSGFVRFTDMIDGQTITRQTDELTGLSSLVVLDSAERTAGGKDLCPALKIVDAQGNDVLIPGTDMPAQYFLPG
KAIVQLEDGVQISSGDTLARIPQESGGTKDITGGLPRVADLFEARRPKEPAILAEISGIVSFGKETKGKRRLVITPVDGS
DPYEEMIPKWRQLNVFEGERVERGDVISDGPEAPHDILRLRGVHAVTRYIVNEVQDVYRLQGVKINDKHIEVIVRQML
RKATIVNAGSSDFLEGEQVEYSRVKIANRELEANGKVGATYSRDLLGITKASLATESFISAASFQETTRVLTEAAVAGK
RDELRGLKENVIVGRLIPAGTGYAYHQDRMRRRAAGEAPAAPQVTAEDASASLAELLNAGLGGSDNE

11.5.2.11.1 AcrB-WT

MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDTVTQVIEQNMNGIDNLMYMSSN
SDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQEVQQQGVSVEKSSSSFLMVVGVINTDGTMTQEDISDYVA
ANMKDAISRTSGVGDVQLFGSQYAMRIWMNPNELNKFQLTPVDVITAIKAQNAQVAAGQLGGTPPVKGQQLNASIIA
QTRLTSTEEFGKILLKVNQDGSRVLLRDVAKIELGGENYDIIAEFNGQPASGLGIKLATGANALDTAAAIRAELAKMEPF
FPSGLKIVYPYDTTPFVKISIHEVVKTLVEAIILVFLVMYLFLQNFRATLIPTIAVPVVLLGTFAVLAAFGFSINTLTMFGMV
LAIGLLVDDAIVVVENVERVMAEEGLPPKEATRKSMGQIQGALVGIAMVLSAVFVPMAFFGGSTGAIYRQFSITIVSAM
ALSVLVALILTPALCATMLKPIAKGDHGEGKKGFFGWFNRMFEKSTHHYTDSVGGILRSTGRYLVLYLIIVVGMAYLFV
RLPSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYYLTKEKNNVESVFAVNGFGFAGRGQNTGIAFVSLKD
WADRPGEENKVEAITMRATRAFSQIKDAMVFAFNLPAIVELGTATGFDFELIDQAGLGHEKLTQARNQLLAEAAKHPD
MLTSVRPNGLEDTPQFKIDIDQEKAQALGVSINDINTTLGAAWGGSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIGDW
YVRAADGQMVPFSAFSSSRWEYGSPRLERYNGLPSMEILGQAAPGKSTGEAMELMEQLASKLPTGVGYDWTGMSY
QERLSGNQAPSLYAISLIVVFLCLAALYESWSIPFSVMLVVPLGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIV
EFAKDLMDKEGKGLIEATLDAVRMRLRPILMTSLAFILGVMPLVISTGAGSGAQNAVGTGVMGGMVTATVLAIFFVPVF
FVVVRRRFSRKNEDIEHSHTVDHH

11.5.2.11.2 AcrB-RNM-3

MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDTVTQVIEQNMNGIDNLMYMSSN
SDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQEVQQQGVSVEKSSSSFLMVVGVINTDGTMTQEDISDYVA
ANMKDAISRTSGVGDVQLFGSQYAMRIWMNPNELNKFQLTPVDVITAIKAQNAQVAAGQLGGTPPVKGQQLNASIIA
QTRLTSTEEFGKILLKVNQDGSRVLLRDVAKIELGGENYDIIAEFNGQPASGLGIKLATGANALDTAAAIRAELAKMEPF
FPSGLKIVYPYDTTPFVKISIHEVVKTLVEAIILVFLVMYLFLQNFRATLIPTIAVPVVLLGTFAVLAAFGFSINTLTMFGMV
LAIGLLVDDAIVVVENVERVMAEEGLPPKEATRKSMGQIQGALVGIAMLLSAVFVPMAFFGGSTGAIYRQFSITIVSAMA
LSVLVALILTPALCATMLKPIAKGDHGEGKKGFFGWFNRMFEKSTHHYTDSVGGILRSTGRYLVLYLIIVVGMAYLFVRL
PSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYYLTKEKNNVESVFAVNGFGFAGRGQNTGIAFVSLKDWA
DRPGEENKVEAITMRATRAFSQIKDAMVFAFNLPAIVELGTATGFDFELIDQAGLGHEKLTQARNQLLAEAAKHPDMLT
SVRPNGLEDTPQFKIDIDQEKAQALGVSINDINTTLGAAWGGSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIGDWYV
RAADGQMVPFSAFSSSRWEYGSPRLERYNGLPSMEILGQAAPGKSTGEAMELMEQLASKLPTGVGYDWTGMSYQE
RLSGNQAPSLYAISLIVVFLCLAALYESWSIPFSVMLVVPLGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIVEF
AKDLMDKEGKGLIEATLDAVRMRLRPILMTSLAFILGVMPLVISTGAGSGAQNAVGTGVMGGMVTATVLAIFFVPVFFV
VVRRRFSRKNEDIEHSHTVDHH

11.5.2.11.3 AcrB-RNM-19

MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDTVTQVIEQNMNGIDNLMYMSSN
SDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQEVQQQGVSVEKSSSSFLMVVGVINTDGTMTQEDISDYVA
ANMKDAISRTSGVGDVQLFGSQYAMRIWMNPNELNKFQLTPVDVITAIKAQNAQVAAGQLGGTPPVKGQQLNASIIA
QTRLTSTEEFGKILLKVNQDGSRVLLRDVAKIELGGENYDIIAEFNGQPASGLGIKLATGANALDTAAAIRAELAKMEPF
FPSGLKIVYPYDTTPFVKISIHEVVKTLVEAIILVFLVMYLFLQNFRATLIPTIAVPVVLLGIFAVLAAFGFSINTLTMFGMVL
AIGLLVDDAIVVVENVERVMAEEGLPPKEATRKSMGQIQGALVGIAMVLSAVFVPMAFFGGSTGAIYRQFSITIVSAMA
LSVLVALILTPALCATMLKPIAKGDHGEGKKGFFGWFNRMFEKSTHHYTDSVGGILRSTGRYLVLYLIIVVGMAYLFVRL
PSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYYLTKEKNNVESVFAVNGFGFAGRGQNTGIAFVSLKDWA
DRPGEENKVEAITMRATRAFSQIKDAMVFAFNLPAIVELGTATGFDFELIDQAGLGHEKLTQARNQLLAEAAKHPDMLT
SVRPNGLEDTPQFKIDIDQEKAQALGVSINDINTTLGAAWGGSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIGDWYV
RAADGQMVPFSAFSSSRWEYGSPRLERYNGLPSMEILGQAAPGKSTGEAMELMEQLASKLPTGVGYDWTGMSYQE
RLSGNQAPSLYAISLIVVFLCLAALYESWSIPFSVMLVVPLGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIVEF
AKDLMDKEGKGLIEATLDAVRMRLRPILMTSLAFILGVMPLVISTGAGSGAQNAVGTGVMGGMVTATVLAIFFVPVFFV
VVRRRFSRKNEDIEHSHTVDHH

11.5.2.11.4 AcrB-21

MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDTVTQVIEQNMNGIDNLMYMSSN
SDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQEVQQQGVSVEKSSSSFLMVVGVINTDGTMTQEDISDYVA
ANMKDAISRTSGVGDVQLFGSQYAMRIWMNPNELNKFQLTPVDVITAIKAQNAQVAAGQLGGTPPVKGQQLNASIIA
QTRLTSTEEFGKILLKVNQDGSRVLLRDVAKIELGGENYDIIAEFNGQPASGLGIKLATGANALDTAAAIRAELAKMEPF
FPSGLKIVYPYDTTPFVKISIHEVVKTLVEAIILVFLVMYLFLQNFRATLIPTIAVPVVLLGTFAVLAAFGFSINTLTMFGMV
LAIGLLVDDAIVVVENVERVMAEEGLPPKEATRKSMGQIQGALVGIAMVLSAVFVPMAFFGGSTGAIYRQFSITIVSAM
ALSVLVALILTPALCATMLKPIAKGDHGEGKKGFFGWFNRMFEKSTHHYTDSVGGILRSTGRYLVLYLIIVVGMAYLFV
RLPSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYYLTKEKNNVESVFAVNGFGFAGRGQNTGIAFVSLKD
WADRPGEENKVEAITMRATRAFSQIKDAMVFAFNLPAIVELGTATGFDFELIDQAGLGHEKLTQARNQLLAEAAKHPD
MLTSVRPNGLEDTPQFKIDIDQEKAQALGVSINDINTTLGAAWGGSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIGDW
YVRAADGQMVPFSAFSSSRWEYGSPRLERYNGLPSMEILGQAAPGKSTGEAMELMEQLASKLPTGVGYDWTGMSY
QERLSGNQAPSLYAISLIVVFLCLAALYESWSIPFSIMLVVPLGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIV
EFAKDLMDKEGKGLIEATLDAVRMRLRPILMTSLAFILGVMPLVISTGAGSGAQNAVGTGVMGGMVTATVLAIFFVPVF
FVVVRRRFSRKNEDIEHSHTVDHH

11.5.2.12.1 YohJ-WT

MSKTLNIIWQYLRAFVLIYACLYAGIFIASLLPVTIPGSIIGMLILFVLLALQILPAKWVNPGCYVLIRYMALLFVPIGVGVMQ
YFDLLRAQFGPVVVSCAVSTLVVFLVVSWSSQLVHGERKVVGQKGSEE

11.5.2.12.2 YohJ-RNM-18

MSKTLNIIWQYLRAFVLIYACLYAGIFIASLLPVTIPGSIIGMLILFVLLALQILPAKWVNPGCYVLIRYMALLFVPIGVGVMQ
YFDLLRAQFGPVVVSCAVSTLVVFRVVSWSSQLVHGERKVVGQKGSEE

11.5.2.13.1 DnaK-WT

MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVTNPQNTLFAIKRLIGRRFQDE
EVQRDVSIMPFKIIAADNGDAWVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAG
RIAGLEVKRIINEPTAAALAYGLDKGTGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
VEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVTRAKLESLVEDLVNRSIEP
LKVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFFGKEPRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPL
SLGIETMGGVMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVTF
DIDADGILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRKQVEEAG
DKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLMEIAQQQHAQQQTAGADASANNAKDDDVVDAEF
EEVKDKK

11.5.2.13.2 DnaK-RNM-18

MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVTNPQNTLFAIKRLIGRRFQDE
EVQRDVSIMPFKIIAADNGDAWVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAG
RIAGLEVKRIINEPTAAALAYGLDKGTGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
VEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVTRAKLESLVEDLVNRSIEP
LKVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFFGKEPRKDVNPDEAVAIGAAGQGGVLTGDVKDVLLLDVTP
LSLGIETMGGVMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVT
FDIDADGILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRKQVEEA
GDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLMEIAQQQHAQQQTAGADASANNAKDDDVVDAE
FEEVKDKK

11.5.2.14.1 GroL-WT

MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKE
VASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSD
ETVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLP
VLEAVAKAGKPLLIIAEDVEGEALATLVVNTMRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGTVISEEIGMELEKATLE
DLGQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKA
RVEDALHATRAAVEEGVVAGGGVALIRVASKLADLRGQNEDQNVGIKVALRAMEAPLRQIVLNCGEEPSVVANTVKG
GDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVTDLPKNDAADLGAAGGMGGMGGMGG
MM

11.5.2.14.2 GroL-RNM-22

MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKE
VASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSD
ETVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLP
VLEAVAKAGKPLLIIAEDVEGEALATLVVNTMRGIVKVAAVKALGFGDRRKAMLQDIATLTGGTVISEEIGMELEKATLE
DLGQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKA
RVEDALHATRAAVEEGVVAGGGVALIRVASKLADLRGQNEDQNVGIKVALRAMEAPLRQIVLNCGEEPSVVANTVKG
GDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVTDLPKNDAADLGAAGGMGGMGGMGG
MM

11.5.2.15.1 IlvN-WT

MQNTTHDNVILELTVRNHPGVMTHVCGLFARRAFNVEGILCLPIQDSDKSHIWLLVNDDQRLEQMISQIDKLEDVVKV
QRNQSDPTMFNKIAVFFQ

11.5.2.15.2 IlvN-RNM-2

MQNTTHDNVILELTVRNHPGVMTHVCGLFARRAFNVEGILYLPIQDSDKSHIWLLVNDDQRLEQMISQIDKLEDVVKV
QRNQSDPTMFNKIAVFFQ

11.5.2.15.3 IlvN-RNM-3

MQNTTHDNVILELTVRNHPGVMTHVCGLFARRAFNVEGILYLPIQDSDKSHIWLLVNDDQRLEQMISQIDKLEDVVKV
QRNQSDPTMFNKIAVFFQ

11.5.2.16.1 YgbK-WT

MIKIGVIADDFTGATDIASFLVENGLPTVQINGVPTGKMPEAIDALVISLKTRSCPVVEATQQSLAALSWLQQQGCKQIY
FKYCSTFDSTAKGNIGPVTDALMDALDTPFTVFSPALPVNGRTVYQGYLFVMNQLLAESGMRHHPVNPMTDSYLPRL
VEAQSTGRCGVVSAHVFEQGVDAVRQELARLQQEGYRYAVLDALTEHHLEIQGEALRDAPLVTGGSGLAIGLARQW
AQENGNQARKAGRPLAGRGVVLSGSCSQMTNRQVAHYRQIAPAREVDVARCLSIETLAAYAHELAEWVLGQESVLA
PLVFATASTDALAAIQQQYGAQKASQAVETLFSQLAARLAAEGVTRFIVAGGETSGVVTQSLGIKGFHIGPTISPACRG

11.5.2.16.2 YgbK-RNM-2

MIKIGVIADDFTGATDIASFLVENGLPTVQINGVPTGKMPEAIDALVISLKTRSCPVVEATQQSLAALSWLQQQGCKQIY
FKYCSTFDSTAKGNIGPVTDALMDALDTPFTVFSPALPVNGRTVYQGYLFVMNQLLAESGMRHHPVNPMTDSYLPRL
VEAQSTGRCGVVSAHVFEQGVDAVRQELARLQQEGYRYAVLDALTEHHLEIQGEALRDAPLVTGGSGLAIGLARQW
AQENGNQARKAGRPLAGRGVVLSGSCSQMTNRQVAHYRQIAPAREVDVARCLSIETLAAYEHELAEWVLGQESVLA
PLVFATASTDALAAIQQQYGAQKASQAVETLFSQLAARLAAEGVTRFIVAGGETSGVVTQSLGIKGFHIGPTISPACRG

11.5.2.17.1 ClsA-WT

MTTVYTLVSWLAILGYWLLIAGVTLRILMKRRAVPSAMAWLLIIYILPLVGIIAYLAVGELHLGKRRAERARAMWPSTAK
WLNDLKACKHIFAEENSSVAAPLFKLCERRQGIAGVKGNQLQLMTESDDVMQALIRDIQLARHNIEMVFYIWQPGGMA
DQVAESLMAAARRGIHCRLMLDSAGSVAFFRSPWPELMRNAGIEVVEALKVNLMRVFLRRMDLRQHRKMIMIDNYIA
YTGSMNMVDPRYFKQDAGVGQWIDLMARMEGPIATAMGIIYSCDWEIETGKRILPPPPDVNIMPFEQASGHTIHTIAS
GPGFPEDLIHQALLTAAYSAREYLIMTTPYFVPSDDLLHAICTAAQRGVDVSIILPRKNDSMLVGWASRAFFTELLAAGV
KIYQFEGGLLHTKSVLVDGELSLVGTVNLDMRSLWLNFEITLAIDDKGFGADLAAVQDDYISRSRLLDARLWLKRPLW
QRVAERLFYFFSPLL

11.5.2.17.2 ClsA-RNM-18

MTTVYTLVSWLAILGYWLLIAGVTLRILMKRRAVPSAMAWLLIIYILPLVGIIAYLAVGELHLGKRRAERARAMWPSTAK
WLNDLKACKHIFAEENSSVAAPLFKLCERRQGIAGVKGNQLQLMTESDDVMQALIRDIQLARHNIEMVFYIWQPGGMA
DQVAESLMAAARRGIHCRLMLDSAGSVAFFRSPWPELMRNAGIEVVEALKVNLMRVFLRRMDLRQHRKMIMIDNYIA
YTGSMNMVDPRYFKQDAGVGQWIDLMARMEGPIATAMGIIYSCDWEIETGKRILPPPPDVNIMPFEQASGHTIHTIAS
GPGFPEDLIHQALLTAAYSAREYLIMTTPYFVPSDDLLHAICTAAQRGVDVSIILPRKNDSMLVGWASRAFFTELLAAGV
KIYQFEGGLLHTKSVLVDGELSLVGTVNLDMRSLWLNFEITLAIDDKGFGADLAPFRTIIFRVHVCSMPVYG

11.5.2.18.1 OpgH-WT

MNKTTEYIDAMPIAASEKAALPKTDIRAVHQALDAEHRTWAREDDSPQGSVKARLEQAWPDSLADGQLIKDDEGRDQ
LKAMPEAKRSSMFPDPWRTNPVGRFWDRLRGRDVTPRYLARLTKEEQESEQKWRTVGTIRRYILLILTLAQTVVATW
YMKTILPYQGWALINPMDMVGQDLWVSFMQLLPYMLQTGILILFAVLFCWVSAGFWTALMGFLQLLIGRDKYSISAST
VGDEPLNPEHRTALIMPICNEDVNRVFAGLRATWESVKATGNAKHFDVYILSDSYNPDICVAEQKAWMELIAEVGGEG
QIFYRRRRRRVKRKSGNIDDFCRRWGSQYSYMVVLDADSVMTGDCLCGLVRLMEANPNAGIIQSSPKASGMDTLYA
RCQQFATRVYGPLFTAGLHFWQLGESHYWGHNAIIRVKPFIEHCALAPLPGEGSFAGSILSHDFVEAALMRRAGWGV
WIAYDLPGSYEELPPNLLDELKRDRRWCHGNLMNFRLFLVKGMHPVHRAVFLTGVMSYLSAPLWFMFLALSTALQVV
HALTEPQYFLQPRQLFPVWPQWRPELAIALFASTMVLLFLPKLLSILLIWCKGTKEYGGFWRVTLSLLLEVLFSVLLAPV
RMLFHTVFVVSAFLGWEVVWNSPQRDDDSTSWGEAFKRHGSQLLLGLVWAVGMAWLDLRFLFWLAPIVFSLILSPF
VSVISSRATVGLRTKRWKLFLIPEEYSPPQVLVDTDRFLEMNRQRSLDDGFMHAVFNPSFNALATAMATARHRASKV
LEIARDRHVEQALNETPEKLNRDRRLVLLSDPVTMARLHFRVWNSPERYSSWVSYYEGIKLNPLALRKPDAASQ

11.5.2.18.2 OpgH-RNM-18

MNKTTEYIDAMPIAASEKAALPKTDIRAVHQALDAEHRTWAREDDSPQGSVKARLEQAWPDSLADGQLIKDDEGRDQ
LKAMPEAKRSSMFPDPWPTNPVGRFWDRLRGRDVTPRYLARLTKEEQESEQKWRTVGTIRRYILLILTLAQTVVATW
YMKTILPYQGWALINPMDMVGQDLWVSFMQLLPYMLQTGILILFAVLFCWVSAGFWTALMGFLQLLIGRDKYSISAST
VGDEPLNPEHRTALIMPICNEDVNRVFAGLRATWESVKATGNAKHFDVYILSDSYNPDICVAEQKAWMELIAEVGGEG
QIFYRRRRRRVKRKSGNIDDFCRRWGSQYSYMVVLDADSVMTGDCLCGLVRLMEANPNAGIIQSSPKASGMDTLYA
RCQQFATRVYGPLFTAGLHFWQLGESHYWGHNAIIRVKPFIEHCALAPLPGEGSFAGSILSHDFVEAALMRRAGWGV
WIAYDLPGSYEELPPNLLDELKRDRRWCHGNLMNFRLFLVKGMHPVHRAVFLTGVMSYLSAPLWFMFLALSTALQVV
HALTEPQYFLQPRQLFPVWPQWRPELAIALFASTMVLLFLPKLLSILLIWCKGTKEYGGFWRVTLSLLLEVLFSVLLAPV
RMLFHTVFVVSAFLGWEVVWNSPQRDDDSTSWGEAFKRHGSQLLLGLVWAVGMAWLDLRFLFWLAPIVFSLILSPF
VSVISSRATVGLRTKRWKLFLIPEEYSPPQVLVDTDRFLEMNRQRSLDDGFMHAVFNPSFNALATAMATARHRASKV
LEIARDRHVEQALNETPEKLNRDRRLVLLSDPVTMARLHFRVWNSPERYSSWVSYYEGIKLNPLALRKPDAASQ

11.5.2.19.1 PepA-WT

MEFSVKSGSPEKQRSACIVVGVFEPRRLSPIAEQLDKISDGYISALLRRGELEGKPGQTLLLHHVPNVLSERILLIGCGK
ERELDERQYKQVIQKTINTLNDTGSMEAVCFLTELHVKGRNNYWKVRQAVETAKETLYSFDQLKTNKSEPRRPLRKM
VFNVPTRRELTSGERAIQHGLAIAAGIKAAKDLGNMPPNICNAAYLASQARQLADSYSKNVITRVIGEQQMKELGMHS
YLAVGQGSQNESLMSVIEYKGNASEDARPIVLVGKGLTFDSGGISIKPSEGMDEMKYDMCGAAAVYGVMRMVAELQ
LPINVIGVLAGCENMPGGRAYRPGDVLTTMSGQTVEVLNTDAEGRLVLCDVLTYVERFEPEAVIDVATLTGACVIALG
HHITGLMANHNPLAHELIAASEQSGDRAWRLPLGDEYQEQLESNFADMANIGGRPGGAITAGCFLSRFTRKYNWAHL
DIAGTAWRSGKAKGATGRPVALLAQFLLNRAGFNGEE

11.5.2.19.2 PepA-RNM-29

MEFSVKSGSPEKQRSACIVVGVFEPRRLSPIAEQLDKISDGYISALLRRGELEGKPGQTLLLHHVPNVLSERILLLVAAK
NVSWMSVSTSRLFRKPLIR

11.6 RNA sequencing

Table 11.6 List of differentially up and down regulated genes for the BMA tolerant strains with respect to the parental strain

Strain	Genes
RNM-2	***inaA acrB acrA fumC ydcL ompR frvX oppF oppA envZ oppC yhcN oppB nfsA oppD yacH ybjH yhbW setB rimK mltF frvR dadA fhlA yadG ybjC mdtG chaC yadH plaP dppF ydeE lpxL yfeO proY sbp dppC iceT micF mnmH clcB mdaB yoeI yqeG dadX ybiJ nhoA acrR ydcO acrZ ybdO ycgZ frvB baeS shoB ymgC ymgA ariR yibT aaeA aslB insH1 tauA tauB gldA hisL yfcR gcvB asnA yjhI acrE hyi aaeX yibW citG symR torC hycB yqfE phnJ istR hycA owes*** *ompF gadC hdeA fliC ompT gadB hdeB gadA livJ fimA hdeD fimI tar mdtF yhjE rob fimD mdtE tap yhiD livK ybaT fliD motA narZ tsr motB flgK cheA livH yfdC glsA slp narU flgL yeeR yebV narY ycgR fimC dctR stpA cheR fimH ymdF cheW csgD narV flgN narW gadE cheY fimG ynaI yhdW fliA fimF flgM cheB yhjH cheZ fliS fliK fliZ flu yjcZ flgH fliI csgF yhdV flxA arrS fliT yhiM isrC csgE fliJ yeeS flhE ybfL ybfB fliQ hyaB hyaA ybfC ghoS rutE yibS yehA yicT rrsC yjbM yjiS rrsH yjbS rrsA rrlD agaS rrsE wcaH rrsD waaH agaB rrlH ymdE rrlCrrlA yqeK yghG*
RNM-5	***soxS fpr nhoA pfo fumC sodA acrR nfo acrA citG mdtG acrB yeiI poxB ompN nfsA ybjC fldA kdgT zwf inaA yhcN yjjW yjjI ygfZ yggX frvX yobH ydeE soxR ribA fldB rimK acnA frvR treF ybaL lipA uof idi pptA mltC ltaE mdlB yjiY yaaY ypeB yaiA mdlA fur ybaO ligA map eamA yncD ymgA ybdO acrZ ygaY ariR pgi micF*** *flu aspU isrC ompT arrS tar gadB gadA motB fliC eaeH gadC motA yhjH yhiD yeeR tap cheB rrsA ydeT hdeB gadE fimD hdeA cheW hdeD cheA fliD tsr cheR rrlC yehA fimC mdtE fimF fimI ycgR fliT rrsG fimG rrlA fliQ mdtF rrlH fliS cheZ rrsC cheY sieB fimA yjiC yeeS yicT rob rrsH cbeA flgK yibS fimH rrsD iraD cspH pgaC yiaB fliP fliR insD1 fliA flgL yhiM ygcW ybcK tnaB alaW dctR flhE fliJ asnW valW ttcC yhiL pagP flxA kbaZ flgI ynfN slp bglG yjcZ flgJ yneK yahM malK bdm flgH pspB yncI fliZ fliI gspC ves ydiL yfgH fliO pheP essQ ymdA flgG fliL lamB ttdB ybfG glsA casB sfmZ fimE yqiI ybaT ynaE*
RNM-18	***nirC nirD nirB insH1 narH yehC narG narJ narI acrR narK yhbU nikE yddA nikC yjjW nikD yhbV yehD nikB ydfA yjjI essD ydfD rzpD nikA fepE micF dmsB feoC ynfO yghG fdnG napG dicB rrrD fecR yghF fecA napH cysG yddB ydfB napD hybB napB napF yaiV napA hybA yehB insZ dmsC yhfL dmsA feoB yliF pinH nmpC yliE yjbE dicF yibW add yoeA fecI hypC hybO afuB ydfC yjjZ epd lrhA hypB entF hypD ynfK napC fecC insF1 pqqL ythA cirA yehL hisC yfbL focA ccmB ybdZ bssR nrfC adhE yehA ackA yecH hisD yihR fes ynfM nrfD ybcW fepB arpA pppA ccmA fdnH stpA ryhB yihN hisB entD hypE ybhH abrB fecB nrfB gldA hypA fecD sslE hcr fepG pta ttdR ycdT yiaY yhgA cysA ynjE cysW gpmM hisH ydfZ nrfA yihM cysU ygiL fepC nrdE hisG fumB glnQ ybdO acrB inaA mscK rbsA cysP grcA yqcE pflB insB1 nrdF acrA pepE yihL ydiE ydaG feoA pflA yfjZ hybC alsA yjeH lrp yihU insA hisA lysC ccmC glnP ybbD nrdI fecE ybfP fepA yagF ygeH mdtI ydiF yqgA ppiA ynaK fhuF fdhF eutS ybcV ghoT rbsD ryeA tonB gnsA eutQ ccmD yfcC hisF mdtJ pgk maa pheL afuC aspA ariR yncE rarA exbD sbp ybiY gfcA ygiC malT alx hha fdnI insJ frvX yhhZ yobD ftsK yaaX gfcD garD yihT ompW rnk ygcP fepD yibG frdB ykgE wcaG yjeT ycgX yghE ymgA cysM yfgG nfsA ycaL entA leuA yhcC entH hisI ccmE ybjC rnhB ratB gfcC nrdG nikR frdC leuC ykgH yfaT fhuB mdtH fhuD ychE fhuC yehP phnH yhdU ygcN cysJ ydeM yjaZ leuD alaT yjaA ybeF cpsB leuX glxR nrdH pphB lpxP aspV ymcE srlA ymgG cnu leuB blr yncI eno yciX yhfZ borD yfaA agrB intZ gfcB srlE ftnB ydhY insC1 frvR ygaZ yagE yjfI tsaA ydjX ygaQ lysA ycjV exbB yqeC cusF alsB srlB nrdD ygbE yhbS rbsR insK nanA cysC cbrA yigM ydjY yjbD yeaI cysD ymiA fnrS srkA dcuB pitB viaA frdA entB hybE yfeX yaaY mgtA ygcO ygiB ydiO ymgD yjeJ ybcK ccmF opgE idnD ynjC hybG fbaA hypF wcaI wcaC yghW renD ygeO yjgL ygaH uspC cysH cysI cho ais rpiB yfgF hybF ynjB ypdA yacC rpoD frwA ppdD dapE cdd yehM yjhE ygcW rhsE yafT fhuA fabB yafQ ldrD yoeG rspB ybeT mgrR tqsA yhhL sfmZ bglG chiP gspD yjiT yeeL yehT uhpB*** *gltT fruA fruK fliA ompX flgC flgB flgD setB rrlB fliF fliL flgF yjiS flgE tar fliN yahO fruB gadA narU yhjH fliH ybgS fliC ytjA fliZ codB insE1 flgG arrS yohP rclA ybaY yagU motB fliI gadC ykfK fliM fliP fliO gadB tap fliQ hdeD hdeA glsA osmY flgH slp phoH fliJ fliG narW flgK glcD yghA ymdF fliK ycaC yhbO fliE cheA cheB ompF pspC hdeB ycgR motA pspD yhjG flxA ycgB gadE narY fliD psuG pspB narZ livK proX pspG yiaG flgJ fimA flgIfliS sra ygaM ygdI glcE ggt upp livH blc mdtE ybaT prpR dps pspA ydeI yegS waaH otsB yciF glcG alaE astC cheY flgA ybdL flhB yaiY ybgA yahD osmC codA gcvB katE argA flu proW cheR ymgE narV yeaQ tyrP elaB uraA livM flgL yjdJ dctR glcF yfcG yciG metA metF yjdI cheW livF yebV yfdC glcB yodD hyaA yjbJ tsr fimI fimG gcvH patA yhhA psiF yraQ yhjY ugpB fimC fbaB poxB gabP argI proV ygaU osmE gcvT yeaH hchA csgC talA ppsA gcd mcbR livG otsA tktB ydeJ potF potH hyaB msyB ydaM puuD dppF bdm dppA yeaG astA potG fliT wza fimF osmF paoA yhiD artJ fimD livJ yciN ytfK yjdN amyA ybdK sodC dppD yedA yohF fliR yicS dkgA mdtF gcvP metR ecnB alaA adhP argC ydcK yedL paoB clsB yhiM flhE cheZ ybhP paoD mmuP yncG ybhQ yhcO yphA valW csiE yccJ uspB ugpA yeeR metN phr pspE yciE bolA ahr wrbA ybjP potI dosC argF yedP gabT pstS dppB hdhA aidB pyrI argB dppC pyrB treF flhA psiE ves yehY yahK yegR yegP yohC mtr fsr isrC nanM csgE yddH fadB mlrA aphA yobH ycjO mscS patD ybfL ygaY ugpE oppA rutB ycfJ csgD ybeL yqjD csgF ycgY copA lhgO fimH yjgH puuP ymdA efeU artI puuA ddpX ybhN yhhT ybfD csrC rutA yqjE yjbM curA treA efeO dtpB ybdR ridA metK aldB lysP rcsA hiuH putP yahM argD emrD csiD dosP ddlA yicT pphA ldtE psaA hycA yoaC yhhQ yajI paoC argT yicG yehX ydhS ydiL yqjG yhaO gabD ygiW yqjK ygdR cueO yhjD dtpA ydhQ yoaD yebQ ysaB glpD mhpR ypfG cfa argG yehW cbpA yqiJ sfmA yegJ yhjB valV ydiZ yqjC ddpA ypeC hycB fadA efeB kptA caiF flgN yjdC tam cspI yjfN ivy sfsA puuC ygfF arnC ydcX csgG ysgA bssS yfiL yjiR yjcZ carA arnB mhpA artQ puuR ydeE yagI flc gltP flgM yhcN yeiP guaC malM oppF glcA artP nhaA yjdP fadE sodB metI cysQ yccT yqjF gdhA ybhG grxA ycjM pyrL lamB crl yceA ybiO feaR elbB rhaR ynbA eco brnQ ydcV ansP yaiO sibA yniA pepD msrA oppD metJ yhaL amiC yahG ycaP rutE ybdH cbpM yjgR ppsR sstT carB qorA uspA glpA qseB astD nadA ypjA eamB yggE yeeS yidQ sbmC ycfT rhtA mliC yggL yecF oppC rob tnaB yhaH argH adeQ frmR amtB ydcS sdaA yceB dksA mscL oppB sdhD pnuC artM astB wcaE yjjU yebF aer metQ hokE ybaO yqeG slyX glpF yjbT acnA pgl csgA acnB ychH spy fadL uspG ynfN ydbC yjdM yhdW eutH yidL mdh sdhC soxS pstC yqiK btuE ydhC gstB rutF yphF phnD elaA yfcF tfaE yehE potD dbpA arnA tusA ydcJ rlmF ynjH yeaJ sgcB argE ynaI puuB wzzB phoE pinQ ldtA yaeR degQ ybiH dtpD yedR glpK yjgA folA ybaA hinT rutD nuoM azuC ltaE astE yncL pagP frmA phoB csrA actP hipB ydcF erpA aaeX bluF pdxK gltI gadW ydfK csiR ygeA yegL kefC ugpC yfeK yegD trmA yhjE nuoN puuE mcbA yccX yciH sdsR nanC yaeP kefF nac cspD hyaE hns yjjV pck ydcI hyaD yebW yodC ygiD galP pstA lpoB ybfC nuoJ nuoL glcC sdhB cadC yggN ybcY ybiU mglB nemA ybjS ydaW yohJ pdxJ trmI ybbY*
RNM-21	***ileY narI narH nirC narJ nirD bssR narG nrfD nrfB nrfA yiaY nrfC narK nirB gldA flu dmsB dmsC yehD yhdV feoC napB yehC yjjI frdD yeeS frdC marA yeeR yjjW frdB marB nrfE ansB frdA dmsA marR yhbU yhfL nikE ynfO nikC citG nikD yfcZ hypC hybA hypB napG yqeI hybO ccmB yhcN napC ynfF yhbV hypD hybB feoB nrfG nrfF hcr yecH ccmA napH omrA ynfG napA yqeJ isrC aspA ynfM abrB ccmD nikB ccmC hypA yfeH hypE cysG ynfK omrB napD fumB ycbJ asr yfcV ynfE uspG ymfR pepE fnrS ryeA napF raiA ccmE ydiH cydX glnQ nikA glnP cydB ldrD aegA focA ynjE grcA dcuB cspD cydA add ldrA ydcH glnH dhaM yoeA ccmF hybC rmf ydfZ uspE yhgA fruB dhaL dcuC yqgA yfbS yhbS pflB ompW rydB sokC mdtG glnD hisD ybgE acrZ uspF feoA hisG hisC fruK dsbE ydjX ynfH yhhI croE lysU fdhF katG pqqL csrC oppB oppA ydjY hsrA ftnB yhbT ccmH aaaE insH1 oppC eutK eutL narL nfsA nrdG insD1 yjjZ ycgV hisB dhaK sthA yjbR pflA ttdR ybjC yqfA fruA ymfL yecD yjbQ pliG rspB oppD ymfM nrdD ycfP yjiL ymfQ csrB yjiY ackA yehB oppF ampD hemB hisH fecA yjiM btd ompC ypfM ampE hyi ylcI hybF fecR zur rraA uspC ybiW fdnG dcuA ybfA hybG ygbE envY yeiQ hisA yhbW azuC dicF ravA hisF ybhB chiQ dinJ glpC ydjZ viaA yhhY hisI yciY rbsR pta inaA yddB eutR yjjP yfcC ycbK yoaF psiE ysaA beeE clpA glmY lrhA dkgB ydeA idi yqhD yeiG yecE glxR rhtC tatE iclR ssrA yjaZ fsr gpmM hybE yjcO eutB xisE ycbL glpA afuB hybD yjiX grxB yeiE tpx alkA ompN cbeA yagJ ymgF sbcB dinB clpS tabA ynjA panM glyV ychE talB eutC yieP murI nikR yibT ghoT mntS yfeX entF ebgR yafV ygiN ribA yqiJ uspA yaaX dinG ymfJ yjjB yfdP intA ydfO araJ yhhN yaaJ yafD yhcC rhsC fecI hcp yahB fdnI thrA yddE rpiA sohB ptsG ulaC yiaU tatD acrB yadH yjiA yffR nrdR yohO fdnH mnmC rihC hemC galK ampC baeR frsA yadG ynhF yidG nanR glyX hycG cspE dinQ tonB uvrC idnD hemD ybcW ybaP nadK mrp yccM ilvX arcB gmhB galT ydhR afuC umuD ribD narX pepT ydiK thrB araC allB gstA yifB yeeZ queG cbdX mobA mdtH soxS rimK ybdZ yhjA yacL mdaB dadX bglA yoeB fepC caiA idnO hdfR yjbD rraB fecB yfbV torT dgoA ttdB ygeH fepB aaeR yncE yfjK yjcE yagH yejH zupT prkB garD ygdH yhcB ydjA chrR tfaX ymcE yfdQ galM yohC cvrA ybaE ynjB galE dadA yidH yceB yffL fbp uidR thrC mtfA hsdR ycil hypF mokC pgpA mutH brnQ amn citT nfsB insQ rof fsaA yicR tisB gudD gcl yciZ ychN rob yjhC serS tdcF yjhY yciN rimL exuR ylbG aphA helD acrA yegH ryjA yjiG yoaH ybcL yidA ylaC ygeR ykgL uxaC nudK yecJ ratB frwA yeeO atoS fecC crp rlmB gnsA yefM uvrY zapA gpmA yaeP yffS yciT uxaA yfbT yfeD yeaO sdhE yjgX nadC chbG yieE ybbJ rcnR yafC yihD ryjB yfdY ymfI ybfF dcuR qseB mgsA ydfE torI yebG uxuR shoB uvrB fucR sppA yhaK ubiA qseC yfeC fepG sdiA yadI dinD yicN recA smrB dgoD*** *yhiM yhhJ yhiI yhiN rbbA csgF yhiJ fimC malK yhjH csgE fimI fimD fimA ompF fliA flgB lamB flgC malE motB fliF gadA flgD tar metF fliL hdeB yhiD motA fliN flgE hdeA gadB insE1 malM csgD ykfK fliQ fliP fliM flgF tap hdeD yhiL argB mdtE flil metA fimG malF fliZ gadC fimF yjdQ flgG metN ileT flgH fliC cheA fliD flhB flgJ fliH gadE argH flgI fliO flgK cheR ileX fliG yjbG metR carA yceQ yiiE malG carB wza metK fiu fliE ydeT emrK fliK glsA waaH cheB gdhA wzb ycgR flhD pspD uraA fadL yagI yafZ argD ycjM upp fliJ mdtF ybaT argI prpR rcsA yciF mcbR fliR yicT ilvC flhC dctR yjbF ldtC narU slp mmuP codB alaT yghG ycjU arrS serT tsr fimH metI metE ilvB puuB glcA pspC fliS proX uhpB mglA argQ argA puuP ndk arnC puuC osmF uhpA csgB osmY borD yehY insL1 mqo plaP yccT valW xanP yqjF*

Table 11.6 Continued…

	metB rttR ydaM yoeI ibpA paoA paoB flgA ecpR alx yohP yrhC yfhR ydeJ yfdX mdtJ tyrP puuE narZ cheY argG intG narW flgL yfjU ilvN narY ariR puuA yahO yhiS intS metC rfbB lysP yceK caiF ycaO argF wzc gmr proW csgG dnaJ pyrL ybiX yraQ cirA alaV ybdL mhpB arnB talA ugpB ansP groS tsgA yhaI ytjA ydeI fliT ggt yciE argC fimB sstT ygaY yiiX ves proV yeiP tktB uhpC wcaE ugpA rrsE potG ybiT yfdE flxA ybhC cheZ yjcH yciG cheW fadB hisM yjaA ybgS spy yciH yedN nanM actP serB ymgG codA yjeV ygcW dnaK fimE artJ ymgC chaA glcB gadX potH yahE metL trpT ycjN dosC flhE rfbD bhsA ecnA osmB yoeD hyfA purR yehX argZ glcG hycA psrO aroM lysZ yahM csgC ugpE rfbC lysY pspB argW thrW ygfI rrlB mzrA clsB thrT ykfM yfdV yjiC yahK narV topA hisQ proM yhbO hisJ yqjA katE wzxB tsx rdgC yodB pyrF yjcZ mglB frc yceJ dosP yhjE ybhN yhjR yjbH gltF yecT yfdC ygaM fucP puuD pphA flhA mglC emrY cspF fadE nanS metQ yqgB opgB ybfG htpG yehW cvpA potF lysQ curA glcE rsxB gpt bcsQ ivbL stpA yfcJ ycaM oxc rfbA mdtI tyrA yggI pheP folA rsxA ompG adeD patA hisP chiP ygeQ groL leuT ydiL thiP yghA thiB mhpA glcF yhjG fxsA ybhP recF cyaR rsmG yeaV higB psiF ycgX gabD ibpB ybdH ybfD mltF gntP ymgA pagP yahA ompT dgcZ ebgA ydjE rluB cmk malS glcD fadA nmpC insJ yegS yaiC arnD nepI rdlB yjeJ opgC lhgO glyT treA nadA ybiI aroF yedL yciX rlmN pstS yfgH atpI rclA hchA rpsU sfmA ymfA ffs hcaF ysaB aslB pspE ybfL gspC yjfZ gabT yfcG pyrD amyA yqjG dusC casB csgA metT yidD ecnB malT xylF ybjO paoC aldA rluC yojI opgD pncC artI yjgH ybaY gabP yhdU lysT ddpB yrhB dacC eptC leuW hydN msbA galP mnmG mhpR leuZ yqjD iscR blc rapA nadB artQ yihG ygiQ arnA fabA pcnB htrE xseA hslR nhaA ampG holC speA agrA uhpT ydhS tyrV ymgD artP yddH putP ubiX pheM greA maa otsA leuV yaiI nfuA metJ yghE lysW radA leuS mtlA yiaA yahD yaiA yqjE ydcX yqjK valT ybjE ymiA yidB ycgZ ydcP tyrU mppA metY rnpA rhtA csiD ftsN yoaC mtlR nemR osmC argU rlmL yciA yecR galS argY tnaB sdhD yfiL hscB ybbY ydaS mdtA metU glyU yifE ydjL thrU bglH hslV waaA ycbX poxB elaB yegQ dnaG speC mscK gluQ selC yhjY yceA ymdA yeiW mgrR sgcX acpT
RNM-22	***cusF cusB hycF citG dcuC citE yacH hycE citD micF yfcC yaiV hycB yjiC cusC ygcW hycD gldA yecH frvX yhbU yehC fnrS dcuB hycG fdhF ynfG ydhY inaA ynjE acrR fumB pepE dmsB ynfF dmsA hybA cusA dmsC yhbV hybB oppF hycA yjiM oppD hybO ynfK hycC oppC ygcU yehD aslB citF oppB aegA yqcE sfmC frvR setB ynfE acrE nikA yjjI nikC hypC nikE leuO nikB ybdO yjiL ompW hypB frdC yfdR hypD nrfA insF1 nikD cydA yfeO frdA cydB cydX frdB oppA yjjW hycH yfcZ hypA frdD focA nrfB pflB yibT aspA ydcL ykgR dtpA essD ybcW hybC ynfH yiaY bssR ackA rzpD ybgE nirB hypE glnQ feoC grcA citX cysU ttdR cysP nrfD ansB rrrD cysW acrA feoB yjfL yjiH ybjM csrC agrB ydaF acrB hybF hybE ydfJ fruA nrfC yqgA pta hcr ycbJ nirC istR ydjX mdtG abrB mltF ysaA yccM nirD yehL ygcE ybcV cysA dsrA ilvA hybD ptwF ybeT frvB glnP uxaB proY fryC mdtM yrbL torC ykgE yeeE ydeE citT ghoT insB1 ppdD ydjY ilvM hybG napF cysD psuK yjfI garD ybjH yoeG yeeD chaC hydN yfgF cysM mnmH ybjC hlyE fryB sbp deaD yfcR narI yjhE idnD feoA cysC napD ydhV ypdF cysJ clcB ilvD cysN yihR ilvX ccmA insC1 fhlA yfbS essQ rhsC leuE fruK ilvG ompN envZ sstT nfsA yjaA yqfE yfaT yhbS edd ycgV mdtH yjhF eutS yhcC torS cysI iceT ydaG glgS afuB ccmB nohD glnH pykA yecT astD ydfR yaiX torT yrdF yehB glnG yqeI ychE yhcG rdlB rydB insA yjjP dmsD yaaY hcp yjeN insZ pheL ompR yfcV cnu lrhA yrbN cadA nrfE adhE tauB symR yciW ydfZ sfmD brnQ rhmR ylbI yqeC yjhH mlaE dauA narJ hisI ybhI hycI dppC ydjZ astB cysH yijF rihB nrfF dppF hyfA yqeG gpmM pinQ ybaV pfkA yhfL yeaV eda sdiA yihT dppD sfmF lpxP ydhP yrdE mlaF yacC ybhD astE ybjL php napC yhfU dsbE ibsA tabA ydbD uidC yadG aaaE yfeX ygcP idnO yjiG ilvE ydjN yaaJ yadI yhbT srlB tdcF cspI yagK rimK tfaD preT mgrR yaiT pepT yadH yeeO yihV dgoT yjeJ ycaM yjhG yneF narQ ibsC ygbE rbfA icdC dcuA ydbA ytfA mlaD dadX yegK yhjA melB yhhY recX dcuD fryA yfdQ yecS torY truB ccmC*** *glcD glcE flu fiu tap phoH sucC gadA pyrI gadB flgD sucD ycgR yhjH puuE glcG ompF puuB flgC flgF tar flgE fliN flgB ytjA pyrB fliC gadC sucB glcF osmY acs fliA ybgS yciF yghA fliI fliL arrS hdeB flgG ybiX yhbO hdeA fliP katE fliQ glcB fliF fliK ycaC fliJ fliO puuC sucA fadB yfdC fliM fliZ patA yjcH cheB flgK flgH narU hchA ugpB yahO fliH motB flgI puuD cheA ymdF aldA puuA fadA fliG bdm ugpA ycgB wzb cheR cheY narW ompT codB prpR ybiI cheW ugpE fliD livJ wza mhpR narZ hdeD actP stpA dps narY glsA fliT flgJ ybaY fliE yhiD codA sdhB tsr fliS phnK aceA entB sodA talA tktB entE blc motA ybjP narV glcA ygaU yhjE gabP yfcG entH ybgA fliR flgL ymgE icd otsB entA rclA cirA mdtE ddpX yhjG otsA ysgA ygdI entC pspD ivy yohP cheZ mcbR pliG ybaT wzc zinT yciG ridA yiaG ydaM rrsA yedP pspC isrC sdhA yegP osmE yegS flxA osmC mdh ybeL aidB elaB puuP ggt ydeI fadE gadE yeaQ flhB ybhP yjdI aceK dkgA rrsC clsB pspA yciE yodD gltA pspB curA betA rrsG csgF slp yebV adhP ydhS ybdK yeeR aceB sdhD livF flgA pstS ybdZ msyB nrdI pspG nrdH treA cyoE sodC yahK livH livG yeaG livM yhiM yceI spy nrdE agaS rrlA insH1 yohF ybhG yhjD metE tam yaiY livK rrlC yhcO rcsA acnB psiF yddA rrlH amyA gcvP yddH ldtE osmB osmF csiD carB ugpC csgE yibI potF pyrL ybhB nmpC bioB ecnB rrlD potH poxB yhjY entF nuoM yqjD yhhA yeaH rrsH ybhN uspB mglA yebF yqjE nuoK upp mdtF betB rrsD yjdN yjdJ ahr waaH ydcK hcaR mglC yqjK nuoN sodB paoA fes fimA ygiW cyoD ydeJ cstA betI yggE fumA ymdA dctR puuR carA flhE bioD nuoL nuoJ lldD sra ybdR ytfK pntB potG ygaM potI gcvH fepA yffQ znuA hdhA ynfD uraA tpx mglB pspE pyrC yehY acnA folX bioC nuoI cfa yncG loiP yjbF yqaE lhgO fbaB grxB csgD mqo ndk paoB fimG yajI yphA yhhJ yjbJ fic can mliC ydeT bioF hslJ wcaF ybiO yigI yqjG folE lpd yddB gabT ykgM nuoH ybhF borD ybiU yjgH pntA pnuC ybhR rbbA mntH btuE ddpA yqjC ldtC efeO gltF fadH yfiL bolA aldB efeB wrbA mcrC gmd phr cbpA frmR yegR phnD yeaR yhfG nrdF gpmA ydiU ydcF yciH ygdR frmA yjbH csiE yebW yjiR yceA arnC yheV flgN hisJ ybhS mhpA ycfJ nuoG mlrA dosP mscS speD flhA lysP fimI degP yjdC grxA fimF yehX cpxP rob entD nadA kgtP yhhT nanM trpD pstB gabD gltD yifK crl gnd yjgR paoC ppsA gcvT wbbK yobH dosC mscL ybdD pgl yncE yccJ hmp yncL amn tyrV yidQ yehW ydhC glpD ygfI fimH asnC yfeK thiH yibF trpC cbpM hiuH yhcH yohC ugpQ thiS pstA wbbJ trxC fimC pfkB csgC yibH ysaB wbbL fpr rbsC luxS arnB yeeS pyrF yoaD ydiZ wbbI pstC yifE tufB***
RNM-23	***narI dcuC soxS kdpF narJ nirC narH yfcC citE nirD nrfD yecH yehD yehC yhbU citG gldA pfo dmsC cusF yhbV ynjE cusB dmsB fpr nirB napB nikE pepE yjjW ymfR nrfC yjjI nrfB frdC ompN edd nrfE hypD hypC nikD dmsA nikC frdD tauA sbp nrfA napC oppD ccmB oppC frdB hypB oppF tauB nrfG cusC hypE oppB ccmA narG dtpA hybB frdA nrfF napH napG nikB yeiI ynfF ynfG kdgT yfcZ hypA feoC nikA narK nhoA aegA pflB mdtG citF nfo hybA yjiM fnrS hisI dsbE cydX insH1 acrA ynfK hisF cydB fumB ackA yiaY hybO hybC yhfL ccmC ccmF tauC cysP yaiV cydA acrB citX ybgE eda dcuB ysaA hisA ccmH focA ccmD oppA ccmE cysD ynfE yjaA napA yciW napF yjiL cysU feoB napD ompW glnQ fumC hisH inaA pta hcr cysG bssR abrB yjiC fldA cdh uxaB ydaF hybF acrE ydeE cysW insZ fldB hisB proY ybjC hybE gpmM cusA ribA grcA ppdD ypeB yqgA insF1 rrsB cysN hybG yhcN gntU ynfH aspA ydjX hisC glnP yehB lipA brnQ nfsA hybD zwf yhcC agrB cysC cysJ fruA ampC fdhF istR ydjY insC1 ygfZ fdnG ligA cbl nrdG hisD metR yfeX yrdF tauD sstT tfaX cysA yccM yhbS dsrA ghoT ybcW yeeE afuB torC proK ylbI ibsC ttdR adhE setB deaD feoA isrC ygcN essD garD ycbJ frvX acrR dcyD rhsB pykA ansB yecS ygcW yjeM fdnH yjdQ yecC ygbE mdlB purA fruK ybjM eptB yfbS ydjZ pepT ygcP hisG yjfI fliY yhgA yhbE glnH yadN rdlB rzpD tabA mdlA glpX ssuA dppC recX yaaY yrbN yhbT endA rrrD nikR yobH nrdD yjiH cbeA ilvA hycF obgE atpI ydfZ yjcB asnU ydjN cysM citT yaaJ ynjC gntK map ybeT ycgV rimK ybiW insA uxaA ygaY leuX hcp nsrR yfgF rhsC eno iaaA yjeN mutT dppB yfaT dppD cysI yehL mdtM rlmB uof cadB dppF lpxP tfaD ygcO fdnI tsgA yeeD ilvD eco ddlA yihV ybdO*** *gadB glcE glcD gadA gadC hdeA hdeB tap tar ycgR ybgS narU patA ycaC yhjH acs fliC glcF ytjA cheA fliJ fadB hchA fliE yhiD motB fliL arrS motA hdeD puuE fliA fliF yciF katE fliO sdhB puuB flgB yhbO fliN glcG phoH cheR aldA sucC mglA pyrI yjcH yghA osmY yciG fliK fliI sucD flgC glcB ycgB yahO narZ pyrB sucA narW flgK ymdF sdhA flgD fliR puuC narY yohP sucB fliQ glsA flgE fliD fadA fliM fliP gabP prpR mglB csgF fliZ dps yjdN ybaT tktB flgG gadE ydeI fimG mhpR mdtE flgF elaB tsr actP ygaU ompT talA aceA mcbR rclA fliS cheW sdhD fliH otsB fliG yfcG cheB yedP ggt ygaM ddpX glcA otsA ymgE yiaG aldB cheY malK flgH fbaB yodD flxA csiD yhjG flgL fimA slp blc stpA yegP fimD ybaY yohF puuD puuA sdhC fadE ybgA ygjJ narV aidB clsB wzb ysgA aceK flgI fimC yebV dctR fliT codB osmE csgE ygdI yahK yfdC fimI yeaQ flgJ nmpC sra aceB codA yjdJ amyA fadH uspB yeaG fiu fimF yeaH mglC ybhP psiF yjdI potI lhgO gltA ydhS ldtE mdtF arnC mdh pyrL osmC yedN yhjY ybeL tam rutA ydcK rutE cheZ gabT puuP yhiM yddH csiE yqjE yciE wzc ridA gabD ydaM treA yhjD ybhN betA hdhA mlrA arnB yqjK fimH potH hcaR phnI yqjD ybiX yncG uraA ugpB livJ ydeJ patD metE potG ppsA pstS ddpA potF pyrC csgD ytfK phnK adhP ydcK yibH lamB ecnB yjgH ycjO caiF dkgA osmF yjbJ curA flhB upp carB paoA malF ahr ydcV yegS sodC ugpA icd yhcO nanM malE yehY ydeR yghX sufA feaR yphA fumA glgS paoB bfr agaS msyB dosP yohC carA ybjP dosC yqaE yqjG wrbA ybiI ydiT ugpE ybfC acnB flgA yehW ddpB yceI paoC ndk pliG cbpM yqjC betB ybhB bolA ychH cbpA ivy ybhG gcvH csgG puuR cyoE fic yhhT sufB ybdR folX ycgG yehE argT yebF lldD thrC ydcT yebW yceQ tnaA flhA glcC psrO chaB yccJ rob mcbA ydcS sufC yhhA efeB ynaI malG malM gcvP yjgR yeaR ygiW ldtA yhhJ cfa rbsC pstC btuE efeO dctA pstA yjcC ppsR fdoH ymdA yfdE entH ybhF cstA pstB yheV entB betI yncL tufB ydiZ eutT yegR ybiO ydeO spy ydcI rbsA xylF sufD tnaC ybhS bioB yqeF flgN yfeK flhE entA cyoD paaA yeiE phr yehX ugpC gadW csgC yhdV yhfG thrB folE ytfE lsrA pnuC nuoN yjcZ yhcA ycaP yoaG nuoM nanS pphA mhpA lpd rutF dinI holE flgM treF glxK flhC yibA paoD sodB marB entC ybjO gcvT yhjR flhD dacC lldR ydbJ gstA ydcU ybhR yceA nupC yccT entE bioC rihA ydcJ paaK nhaA grxA grxB ybfD ydbC yciH pntB yhaH yjfY rbsD emrK yhcH insL1 degP nuoJ ynjA nuoL mscS bioF hisJ rclR dmlR glpD melR btuD rutB phnL yhbW mcrC bcsQ yibF yjiT ftnA bioD sgbH tpx lsrD paaJ malT thrA nanK nuoK pgaB yfiL yfdV yfcH lsrC rbbA eutK crl yggE pyrF evgS sufS yoaD pntA eutD proX yagG hmp gnd gtrS yidQ borD yddW nadA lsrK paaZ sufE***

Notes: Differentially upregulated genes correspond to genes with logFC ≥ 1.0 and pvalue <0.05, while diffferntially downregulated genes correspond to genes with logFC ≥ 1.0 and p-value <0.05. Text in bold italic were differentially upregulated genes, while the text in regular italics were differentially downregulated genes.

Table 11.7 List of differentially up and down regulated genes after BMA addition

Strain	Genes
E. coli BW25113	***aaaE aaeX abrB acnA acrA acrB acrD acrR acrZ add adhE adiC adiY afuB afuC ahpF ais alkA alx amiD ampD ampE amtB anmK ansB appA araC araF araG araH arcB arfA arfB argT ariR arnB aroF aroL arsB arsR ascB aslA aslB asnA asnC asr astA astB astC astD astE atoA atoD atoE azoR baeR baeS basR bdm betB betI bfr bglA bglJ bhsA blc bolA bsmA bssR bssS btuR cadB caiE cbdX cbeA cbl cbpM cbrC ccmA ccmB ccmC ccmD ccmE ccmH cdd chaA chaB chaC chbA chbC chbF chbG chbR chpB chpS citG citT clcA clpA clpB clpP clpS clpX clsA clsB clsC cobU cof copA cpsB cpsG cpxP cra csiD csiE cspD cspI csrB csrC curA cusF cutC cydA cydB cydX cysQ cysZ dacC dcrB dcuA dcuB dcuC dcuS dedA degP degQ deoA deoB deoC deoD dgcZ dgoA dgoD dgoK dgoR dgoT dhaK dhaL dicF dinB dinJ dinQ dkgA dksA dmlA dmsB dmsC dnaJ dnaK dps dsbA dsbE dsdC dsdX dxr ecnB ecpA ecpR eda edd elaB elbB envY envZ epd eptA erpA eutB eutK eutQ eutR exuR fabR fadE fadM fdrA fdx fetA fetB fhlA fic fimE fnrS focA frdC frdD frsA fruA fruB fruK frvB frvR frvX frwA frwB frwD fryC fsaA ftnA ftnB ftsA ftsH ftsI ftsZ fucU fumC fxsA gadW gadY galE galK galP galR galU garR gcl gcvA ghxQ gldA glgS glk glmY glmZ glnA glnG glnH glnK glnP glnQ glnS gloA glpA glpB glpC glpD glpF glpK glpQ glpT gltI gltP gmd gmhB gntR gntX grcA greB groL grpE grxA grxB gshB gstA gudD hchA hdhA hemB hemL hflX hfq hha hicA hicB higA higB hipA hipB hlyE hmp hns hofM hokB hokD hpf hscA hslJ hslO hslR hslU hslV hspQ htpG htpX hyaA hyaB hyaC hybB hycI hyfC hyfD hyfE hyfF iap ibpA ibpB ibsA ibsB ibsD ibsE iceT idi ihfA ihfB ileS ileY ilvC inaA insH1 insL1 insM intA intF intQ iraD iscA iscR iscS iscU iscX ispA ivy kbaY kdgR kdpA kdpB kdpF kefB kefC kefF kefG kptA lacY ldcA ldcC ldhA ldrA ldrD ldtA ldtB ldtC ldtD ldtE leuO lfhA lipB loiP lolA lon lpoA lpp lpxC lpxL lrhA lspA lsrK lysR maa macB mall malX malX malY manA marA marB marC marR matP mcbA mdaB mdlA mdlB mdtA mdtB mdtC mdtG mdtM melB metA metB metJ metN metR mfd mgrB mgrR mgsA mgtA mgtL miaA micF mlaB mlaC mlaD mlaE mlaF mlc mliC mmuM mmuP mngR mnmH mntS mobA mocA modF moeA mokB mokC mpaA mpl mqsA mqsR mrcB mrp mscK msrA mutH mutM nac nanR napC narH narI narJ narP narV ndh nemA nemR nfo nfsA nfuA nhoA nirC nirD nlpD nlpE norR norV norW nrdD nrdR nrfD nupG nusB ogrK ompR ompW ompX omrA omrB opgB oppA oppF osmB osmC osmE osmY otsA otsB oweE pagP panB panE paoA paoB paoC paoD pepB pepE pepN pepP pepT pflA pflB pgaD pgi pgk pgpA pgpC pgrR pgsA pheA pheL phnC phoB phoH phoP phoQ phoR phoU pmrD pmrR polB preT prlC prlF proP prpB psiE pspA pspB pspC pspD pspE pspG pstA pstB pstC pstS ptwF qmcA qorA qorB qseB qseC queE raiA rapA rarA rayT rbbA rcnA rcnR rcsB rcsC rcsD recN recT relB relE rem rhsA rhsB rhsC rhsJ rhsO ribD ribE rihB rimJ rimK rlmD rlmE rluA rmf rnpB rnr rof rpoD rpoH rpoS rppH rraA rraB rrfA rrlA rrlC rrlD rrlG rrlH rrsA rrsC rrsD rrsG rrsH rseA rsmH rsmJ rspB rssB rstA rstB rtcA rtcB rtcR rutA rutB rutC rutD rutE rutF rutG ryeA ryfA ryjA safA sbmC sdaA sdhE secA selD serX sfmD sfsA sgrR shoB sieB sixA slt slyB smf smg sodB sodC soxR soxS speG spy sra srkA sseA ssrA ssrS stfP tabA tadA tatA tatE tauA tauB tauC tauD tesB tfaD tfaP tfaR tfaS thiL thrA tisB tomB torC torR torT tqsA treB treF treR trpL trpR truA trxC tus tusB tusE tyrA tyrR uacT ubiA ubiC ubiF ucpA udp ugd uhpT umuD ung uspA uspB uspC uspD uspE uspF uspG uvrB uxuR ves waaH wcaA wcaB wcaC wcaD wcaE wcaF wcaG wcaH wcaI wcaJ wcaM wcaN wecH wrbA wza wzb wzc xapB xapR xerD xylB xylE xylF yaaX yabI yacH yacL yadC yadG yadH yadL yadM yaeH yaeP yaeR yafC yafD yafE yafN yafS yafU yafV yagA yagF yagG yagH yagJ yagM yahA yahN yaiA yaiE yail yaiO yaiX yaiY yajI yajL yajO ybaO ybaP ybaY ybbA ybbJ ybbN ybbP ybcL ybcM ybcW ybdF ybdG ybdH ybdJ ybdK ybdL ybdO ybdR ybeD ybeL ybeX ybeY ybeZ ybfA ybfB ybfC ybfD ybfL ybfQ ybgE ybgS ybhD ybhF ybhG ybhL ybhM ybhN ybhP ybhQ ybhR ybhS ybiB ybiH ybiJ ybiO ybiW ybjC ybjD ybjH ybjP ybjQ ybjX ycaN ycaP ycbJ yccA yccJ yceB yceG yceH yceM ycfJ ycfQ ycfT ycgB ycgY ychH ychN yciB yciC yciE yciF yciG yciK yciM yciN yciS yciY ycjF ycjN ycjX ydaN ydcF ydcH ydcK ydcL ydcO ydcY ydeA ydeI ydeJ ydeP ydeQ ydfZ ydgA ydgD ydgU ydhB ydhL ydhQ ydhR ydhS ydhU ydiH ydiK ydiM ydiP ydjA ydjM yeaG yeaH yeaI yeaM yeaO yeaQ yeaY yebE yebO yebS yebW yecA yecD yecH yecN yedI yedP yedR yedY yeeO yeeX yeeY yeeZ yefM yegH yegP yegR yegS yehD yeiG yfbK yfbL yfbR yfbS yfbU yfbV yfcO yfcP yfcQ yfcR yfcV yfcZ yfdC yfdE yfdL yfdT yfdY yfeN yfeO yffB yffL yffR yfgG yfgO yfhH yfhM yfiL yfjJ yfjX yfjZ ygaC ygaM ygaP ygaU ygaV ygbA ygbE ygbI ygdI ygdR ygeW ygeY ygfB ygfZ yggE yghA ygiB ygiD ygiN ygiV ygiW ygjP yhaH yhaK yhaL yhaV yhbO yhbQ yhbS yhbW yhcN yhdN yhdV yhfG yhgE yhhA yhhJ yhhN yhhS yhhT yhhW yhhX yhhY yhiI yhjA yhjD yhjE yhjX yiaO yiaU yiaY yibA yibH yibI yibT yicH yicI yidE yidH yidL yifL yihI yihO yihQ yiiM yiiS yijD yjbB yjbD yjbE yjbJ yjbQ yjbR yjcB yjcE yjcO yjdJ yjdN yjdP yjfN yjfP yjfY yjhG yjhH yjhI yjiC yjjB ykfB ykfG ykgA ykgE ykgL ykgQ ykgT ykiA ylil ymdB ymdF ymfJ ymfM ymgE ynaJ yncG yncJ yneM ynfB ynfD ynfE ynfF ynfG ynfH ynfL ynfM ynhF ynjH yoaF yoaI yobB yodD yoeB yohC yohF yohJ yohK yohP ypdI ypdK ypeC ypfG ypfH ypfM ypfN yphA yqaE yqcA yqeB yqeC yqeH yqfA yqfB yqgA yqhC yqhD yqhG yqhH yqiJ yqiK yqjA yqjC yqjD yqjE yqjG yqjK yraK yraR yrbL yrdA yrdD yrfF ysaB ytfB ytfH ytfJ ytfK ytjA ytjC zapB zitB zntA zntR zraP zraR zupT zur zwf*** *aas aat accA accB accC aceA aceB aceK acnB acs actP adeD adeP adiA adk aer agaS agrA alaC alaE alaS alaT alaV alaW alaX alr alsB alsR ampC ampG amyA ansA ansP apaH apbE aphA appY apt argA argB argC argD argE argF argG argH argI argO argS argU argV argW argX argY argZ aroA aroC aroG aroH arpA arrS artI artJ artM artP artQ asnS asnT asnU asnV asnW aspC atoC atpA atpC atpD atpF atpG atpH avtA azuC bamA bamB bcp bcr bcsA bcsB bcsQ bcsZ bdcR bioA bioB bioC bioD bioF birA bisC blr bluF borD btuB caiA caiC can carA carB cdh cheA cheB cheR chiP cirA cmk cmoA cmoB cnu coaA coaD cobB cobC codA codB corA cpxA csgD csgE csgF csgG cspA cspB cspC cspF cspG cspH cstA cusS cvpA cyaR cyaY cyoA cyoB cyoC cyoD cyoE cysA cysC cysD cysH cysI cysJ cysK cysM cysN cysP cysS cysT cysU cysW dacA dacB dam dapD dbpA dcm dctA dctR ddpD deaD degS der dinF dinI djlC dnaA dnaB dnaE dnaN dnaX dppA dppB dppC dppD dppF dsbG dsrA dtpD dusA dusB dusC dut ebgA ebgR ecnA efeB efeO efeU efp emtA entA entB entC entD entE entF entH entS envC epmB eptC era essD ettA evgS exbB exbD eyeA fabA fabF fabG fabH fadA fadB fadL fdnG feaR fecA fecB fecC fecD fecE fecI fecR fepA fepB fepC fepD fepE fepG fes fhuA fhuB fhuC fhuD fhuE fhuF fimB fimC fimD fimF fimG fimH fimI fis fiu fklB flgA flgB flgC flgD flgE flgF flgG flgH flgI flgJ flgK flgL flgN flhA flhB flhC flhD flhE fliA fliD fliE fliF fliG fliH fliI fliJ fliK fliL fliM fliN fliO fliP fliQ fliR fliS fliT fliZ flu folB folC folD folE folK frmB frmR frr frwC ftsP fumA fusA gabP gadA gadB gadE galS gatA gatR gcvB gcvH gcvP gcvT gdhA gfcA gfcB gfcC gfcD ggt ghxP glcB glcE glcF glcG glf glmS glmU glnU glnV glnW glpG glpX glrR glsA glsB gltB gltD gltF gltI gltJ gltP gltQ gltV gltW gltX gluQ glyA glyT glyU glyW glyY gnsA gnsB gntK gntP gntU gph gpp gpsA gpt grxC gsiD gsk gspD gspE gspF gspG gspH gspI gspJ gspK gspM gtrA gtrB gtrS guaA guaB guaC gyrA hcaB hcaC hcaE hcaF hcaR hcaT hda hdeA hdeD hemA hflD hisA hisM hisP hisR hisS hofC holD holE hsdM hsdS hupB hycB hypD hypF hypT iadA icd icdC iclR idnT ileX ilvH ilvI infA infB insA insB1 insG insJ insK insZ intG intS intZ ispB ispD ispE ispF kbaZ kup lacI lamB lepA lepB leuE leuT leuU leuV leuW leuX leuZ ligT lit livF livG livJ livK livM lomR lplT lpxD lpxH lpxK lpxT lrp lysA lysP lysQ lysS lysT lysV lysW lysY lysZ malF malK malM malO malQ malT mcrA mcrC mdh mdtE mdtF mdtI mdtJ mdtL mdtP menC menD menE menH mepA mepH mepM mepS metE metG metH metT metU metV metW metY metZ mglA mglB mglC mhpD miaB mioC mipA mltA mltB mltC mltD mnmA mnmE mnmG mntH mntP moaA modA modC motA motB mqo mrcA mrdA mrdB mreB mreC mreD msrC murC murJ murQ murR mutS mutY napA napD napF napG napH ndk nei nikA nikB nikC nikR nlpI nmpC nrdA nrdB nrdE nrdF nrdI nth nudC nudJ nuoA nuoB nuoC nuoE nuoF nuoG nupC nusA nusG obgE ogt ompF ompG ompT opgC opgE paaF pabB panF parC parE pcnB pdxA pdxY peaD pepA pheM pheP pheV phnK phnO phnP phr pitA plaP plsC plsX pncA pncC pnp pntA pntB potA potB potC ppa ppdA ppdB ppiB ppiC pppA pqqL prc prfB prfC priA priB prmA prmB prmC proB proL proM proQ proS proV proW proX prpR prs psrD psrO pssA psuG pth ptsH purB purC purD purE purF purH purK purL purM purN purR purT purU puuA puuB puuC puuD puuE puuP puuR pyrB pyrC pyrD pyrE pyrF pyrH pyrI pyrL queA queD radA rbsA rbsC rbsD rbsK rbsR rclA rclC rdgC rdlD recB recC recD recG recO recQ recR renD rep rfbA rfbB rfbC rfbD rffG rffH rhlE rho rhsH rhtA ribB ridA rihA rihC rimI rimM rimO rimP rlmA rlmC rlmG rlmH rlmI rlmJ rlmL rlmN rluB rluC rluE rnb rnc rnd rne rng rnpA rnt rpe rph rpiB rplA rplB rplC rplD rplE rplF rplI rplJ rplK rplL rplM rplN rplO rplP rplQ rplR rplS rplT rplU rplV rplW rplX rplY rpmA rpmB rpmC rpmD rpmE rpmF rpmG rpmH rpmI rpmJ rpoA rpoB rpoC rpsA rpsB rpsC rpsD rpsE rpsF rpsG rpsH rpsI rpsJ rpsK rpsL rpsM rpsN rpsO rpsP rpsQ rpsR rpsS rpsT rpsU rrD rsd rseP rsfS rsmA rsmB rsmC rsmF rsmG rspA rspR rssA rsxA rsxB rsxC rsxD rsxE rsxG rtn rttR ruvC ryhB sbcC sbcD scpA sdaB sdaC sdhA sdhB sdhC sdhD sdiA secD secE secF secG secY selC serB serC serT serU serV serW sfsB skp slmA slyD sodA speC speD speE spoT srmB sroH sthA sucA sucB sucC sucD suhB tap tff tgt thiB thiC thiD thiE thiF thiG thiH thiI thiK thiM thiP thiQ thiS thrT thrU thrW thyA tig tktA tnaA tnaB tnaC tolQ tolR tonB trkG trmA trmD trmH trmI trmL trmN trpS truB truC truD trxB tsaA tsaB tsaD tsf tsgA tsx ttcC ttdR tufA tufB tusC tusD typA tyrP tyrT tyrU tyrV ubiX udk ugpA ugpB ugpC ugpE uhpA uhpB uhpC ulaB ulaE umpG upp uraA uup uxuA uxuB valT valV valW valX valY valZ viaA vsr waaA waaC waaL waaS wbbH wbbI wbbJ wbbK wbbL wcaK wcaL wecA wecB wecC wecD wecF wecG wzxB wzxE wzyE xanP xerC xseA xseB xthA yaaA yacC yadS yaeF yaeI yafK yafT yafZ yagI yagL yagU yahK yahM yaiS yajR ybaN ybaT ybbC ybdZ ybfE ybfP ybgC ybgF ybgQ ybhC ybhK ybiR ybiT ybiU ybiX ybjE ybjS ybjT ycaD ycaL ycaO ycaQ yccF yccS yccT yceA yceD yceO ycfZ ycgG ycgH ycgL ycgR ycgX ychF yciA yciH yciI yciT yciW yciX ycjW ydaY ydbD ydbJ ydcD ydcI ydcP ydcT ydcU ydcV yddA yddB yddG yddW ydfG ydfO ydfU ydgC ydgH ydgI ydgK ydhC ydhP ydiE ydiF ydiJ ydiL ydiU ydiY ydjH ydjI ydjJ ydjK ydjL ydjN ydjX ydjY yeaL yeaP yeaR yebC yebF yecF yecJ yecT yedE yedL yedN yeeD yeeE yeeN yeeR yeeS yegD yegQ yegU yegV yehH*

Table 11.7 Continued…

	yehT yehU yeiB yeiH yeiP yeiQ yeiR yeiW yejE yejF yejG yejK yfaD yfaE yfaT yfaZ yfcA yfcF yfcJ yfdF yfeA yffN yfgM yfhG yfhL yfhR yfiP yfjQ yfjU yfjV yfjW ygaH ygbL ygcB ygcN ygcO ygcP ygcU ygdB ygdG ygeK ygeO ygeQ ygfF yggI yggM yggU yghE yghF yghG ygiQ ygiS ygjQ yhaC yhbE yhbP yhbU yhbV yhbY yhcA yhcC yhcD yhdE yhdJ yhdP yhdT yhdU yheO yheS yheT yhfW yhfX yhfY yhfZ yhgF yhiD yhiN yhiS yhjH yhjV yiaD yiaJ yibN yibQ yibS yicC yicG yicT yidC yidD yidQ yidX yieH yifE yifK yigB yigL yihG yihU yihW yiiQ yijE yjaA yjaZ yjbL yjbM yjcH yjcZ yjdQ yjeO yjfZ yjgH yjgL yjgN yjhD yjhX yjhZ yjiG yjiH yjiK yjiR yjjG yjjZ ykfA ykfF ykfL ykgH ykgM ykgO ylbF ylcI yliE ymcE ymfA ymfD ymiA ymjA ynaE ynaK ynbC ynbD ynjD ynjI yoaA yoaB yoaE yoaJ yoaK yobD yobF yoeI yojI ypjF yqaA yqcC yqeF yqeG yqeJ yqgB yqgC yqiA yqiG yqiH yqiI yqjH yraJ yraQ yrbN yrdE ytfL zapE zinT znuA znuB znuC
RNM-2	***ydgU ibpB asr spy ibpA pspA pspC pspD pspB raiA yjiY ybfA ynfM ldhA yhdV bssR pspE ymgC narG grcA uspG nirB ariR pspG ymgA yhjX bhsA yneM narI tqsA fruB rutA ybiH ybhG yecH ybgS ypfM ycgZ yohJ ompW yohK dmsA glnK ymdF adiY glpA rmf glnH bssS zraP gldA ynfK ynfE rrsC aroF putA cydX gadC yhhA narV yehD rrsH osmB yfcZ gadB yjjI rrlH nirD yccJ yibT ychH yegP hdeA fruK csgF ygdI ynfD rrlC ycaC hdeB ilvC ypeC csrC ycfJ sra rrlA rrsA osmC gadA rrsD yohC soxS narW cydA rrsG hcr yjfN wrbA rutE uspF yciG hyaB dcuC ndh rutF rpoS glgS frdA ycbJ narY rrlD feoB dps aldB frdC nirC hlyE omrB frdD grxA feoA frdB psiE fnrS narH clpB narZ ptwF glpD udp qmcA aceF hdhA katE deoC rutB mgtL csgG csgB eutG dcuB yfiL dmsB ygiW yaiY pflB mliC cdd adhP mcbA aceE hokD glpF focA ggt sodB yjbJ yhbU cydB adhE qorA yebE rhsA ytjA ybeD yeaG ynfB fumB narK hyaA fbaB hspQ loiP glnP yagG ybhF yqaE slp dmsC pfkA ydeI ldtE uspE yagH hybO cusF phnH fruA uspB csgA yhcN pepT relE uspA gabD yeaH ybhL tktB gntK patD ygaU oppA ymgE edd uspD paoB gabT paoC dmlA ydhY katG ahr yhbO ydfZ mdtM narJ yaaX yhjE yiaG yfdY mlaC yahO hybB gabP yfeO narU csrB slyB yhaK relB yeaQ yfbS glxR cusC rclA yahG amtB dhaK yhbT hchA ompX gstA ilvX deoA yafC yaeH yahK osmY paoD qorB cspD ftnB phoP ansB tyrA glk yqhD paoA ytfK yeaO leuD treF clpA dacC hdeD ybjP ecpA elbB ackA hybA leuC yfjJ omrA mgtA ygaM eutJ yjfY yhbS deoB osmE hyaC dcrB tabA pfkB cbdX ygdR yjdJ ybhS ybiB talA ybgE ydiZ rutD yhbV ssrA yniA ynfF ycgB lipA ylil yjiX ybaT chrR tomb eutE ydcF ryjA glsA garR yiiS allE ydeP yihQ zntR sbmC mscS gpmM ldtC nuoK csgE cbpM ivy rraA aegA yccA yghU yphA zwf acuI cfa fxsA add nac yciE dkgA deoD pgl pgpC leuA cbpA eda degQ yciF hypB yoaC feoC glpK pgk cpxP ydcH sseA nrfA agp eno dhaL grxB ybbJ pepE bglA yohF higA ycjX pstS yjhH yodD ybhR yghA sgbH ecnB dinJ ybbA zntA dicF tpiA ldtD mdtA eutM yhbW slt hypC higB oppD yecD ldtA tisB rpoH oppF glmZ ushA yffB norV pykF degP ybeL elaB yjhG htpX lolA yfdQ nuoI ogrK ryfA yjbR yfbU yceI poxB frwA gudD yccM ydgA nuoL sfmA ycfP yhiI yqgA yqjD chaA smg galU gstB mgrB pepN yjhC ycjF rof ynjE glmY eutD nuoJ ycjQ ybcW curA lacA uhpT crr msrA pykA pfo gltI yajL cpsG glpC psiF sstT tdcF nuoH yfbT ydiH yajO pta xylB paaI yffR btuE lsrF mqsA patA sodC yaeP leuB ydhR dnaK yqjG ycgM nuoM trpR yahF yahD dppA lhgO metR mqsR yjdN hycI aroL nuoG hycG araH ybjQ ynhF ftnA pepP yodC nlpD insH1 yjhF fucU tdh pheL yfdV rhmA hpf pflA hipB amyA preT ynfG gshB maeB luxS*** *asnV ykfF cspH pawZ yjdQ insE1 tyrV ydaQ yehK sokA alaX metV lysV fliF cspF argW valY yjjZ argZ argY fliA flgB fliM leuZ entD argU argQ carA yqeJ cirA valX uraA glyU metT fliO rttR fiu fes cysT fecI lysQ codB yhdU fliL metU leuT argV gltV leuW leuX ycfZ glyW alaW alaV ynfN lysT yfhR lysY yafF serV pheV lysW tyrT ybiX fliQ carB flil valT entC fliG gltT ydiE fecR zinT fliH fliP ileX entH valW serU argC glnW ymcE valZ flgC argX proM metW fliN hisR gcvB yjbL alaT bglG ynbA glnU fliK lysZ asnU pyrB pyrI gcvT metY serT leuV flgJ efeU rpsT flgF fepA insA lysP pyrD flgD gltW cspG ycgX dsrA yddA entE arpA argA yjbF yahM flgE fhuF yliE argH yhcA agrB artJ fliR yeiW argD ykgM yedL argB fliJ glyY entA fepE yjeV flhE nmpC argF valV glcD yagI asnW yjbG opgE codA yhjH proK insJ yicT yhdJ yceO ubiX yibW nadB cspA ygfF malK fecA suhB nrdI argI flhB serW cvpA hda ffs selC renD essQ metE ynaE flgH rnpA fliE pyrE entS ybcI metF rpsU flgI yecT yidD thrW ydaY fliZ entB insK fepD ybeT tnaC gfcB yfhL azuC gfcC yggI queD purF nadA yecJ argG yqjH ynjI yoeI ppdD ybdZ leuQ mglA artP ryhB rcsA ymfD opgC isrC yeiB sfmZ tyrU yghF rrfD glyX cspB ymiA metZ peaD dusB pyrF holE istR ygeQ yijF flgG insC1 yeeL yjhD mltF yicG insD1 ybfP znuA rluC yghT intS yagN fhuE gltU efeB ybaN entF yojI ampG flgA iclR glcE yidX lamB fepB glnL essD caiF gcvH ttcC ydfK yciH ygcB ykfJ galS fepG artM yceA cdh gcvP ygiZ nrdE plaP yjaA gspJ bfd fepC symR ybgC hisP yafP fecE ybfE ygjI fecC lysC hisL yddK coaA ybcK waaA rsxA yliF ygjQ ypdK hycA rrrQ nrdH dctR fliT glnX yfaT yneG upp tyrP znuB rbsA yedV fliD dtpD ybjE mltD ydfU mglC pcnB thrT asnT fhuD cdaR ynaK ykgO yihG yciX rrlG ydaG wza trkG purK fhuC ycfT flhC ndk cysC glyT yhaO ompF uhpB xisE mtr pyrC yfjV leuP yeaP hisM yafT trmN yghG yobH rluB yqeF znuC glyV rpmH elfD ydiL purN emrY ydfR recQ mglB gtrA yafO ycjW holD endA rsxB pppA insI1 yebB yqiI yddB artQ yggM thrU yciW ydiY uhpC emrK yjbI yaeI yacC hyfA ygaC rlmA yjfI rhmR yhbE sanA cobC ygcN fecD yeiS ppdA yehU folK efeO fhuB dusC yfaH yedN sbp gpt rsxC trpT sdhC mreD gnsA queA ydjN exbB exbD purM gfcA rtn flhD icdC tusA fau glnV yqiG gluQ ymfE insB1 pyrH gatR yecR ymfA secG yeeE yjgZ glnG yraJ aspT tdk infA hisQ borD yjiR trmH yadS yidP ybjS tonB ydgK rsmG yjbH ydcD nrdF trmL purT rspR sthA yqgB yncI sfmF fis fimI kdgT motB pheP malM bioA tnaB mdtL yiiQ yjaZ clcB appY prs flhA dinI elaD chiP cedA gspF yibQ yehH rhaR yfeA ydbD sdaB mioC gspD yadD rlmH yaeF mnmA folA pinQ yjfJ waaL motA yifN rimI agaI yncD yqeI ftsB rimM rybB lpxP yraQ yncE yfiP rpmB yohO rluE yjhR yghE yhjV yqiH dnaB cysM ybjO rpsP ycdU ybcY gspE yajR pinR plsX cusS rsxD ybgQ leuU bioC iap proL dnaG tilS tolQ ybiR hflD fecB lpxH trmA ypjC yfjM yehT csgC yjeO pabB dinF cysJ ybeQ sdiA yegK yeeD ansP epmB cytR mmuP pncB ispU metA mrdA intZ priA rsfS chiX yshB yedW thiP rbsD yaaU recG folE tsaD yhiN rdlC cmk ydgI lipB bcsQ rlmG gspM flgK gspC rlmC emrE ydjE ygjV thiQ nth ygfI ymjA metL udk aspV yjiT rplU fliS gpp ttcA potA rseC rsmF ydiM gspG gspH yoaA malF ypjF rep hscB ispD emtA hofO rbsR mreC ydhK cysW rlmF ryjB sibC hcaT rfaH yecF rph fimC degS yhcD vsr amiC purH yfcA ybeF rcnA rnhB aat btuF hcaR sibD insO pyrL malE rlmN modC alsR ydeM yheO mepS gnsB rplY ycaQ ygiQ speE tgt*
RNM-5	***ibpB yhjX asr ibpA ydgU pspA ynfM yibT pspB pspC pspD spy pspG adiY raiA rutA pspE ybgS glpA ldhA narZ narU narV ybfA ybhG uspG narW narY yohK rutB bhsA ybiH yohJ ygdI tqsA bssR ypeC yhhA ydeI yacH bdm yjbJ rmf ftnA gadC ydhY glpD ycfJ ariR yneM bssS yaiY yegP osmB sieB ypfM gadB fruB gadA hyaB preT ynfD rrsA csrC clpB yohC uspF ytjA ymdF yghA yeaG ymgC osmC dps rutC glnK yhbO ychH rrlC grcA yccJ ymgA rutF yahO ymgE ygaU aroF rrsH putA hdeB rrlH mliC rrsG hdhA eutQ yeaH yfiL ycbJ hchA osmY oppA gabP glgS ldtE hdeA rrlA wrbA ydcF nrfA oxyS yiaG rutD yjdJ rrsD slp loiP ndh uspB rrsC dadX mscS rutE ycaC yibI mcbA yfeO yajI trpE hspQ hyaA sra qmcA yohP bfr ybhF oppD yagG uspD nac paoC sodC yqaE hyaC hdeD ybaT cpxP deoC pagP yagH yfcZ glnH katE ybiJ yciG hslJ oppC yjfN rrlD uspE oppF yhdV ggt amtB paoB fruK csgG paoD yjiY rpoS gabD ybeD csgF ytfK yqjD astD znTA ybdR ycgB phoH ybhL tdh ykgT ybaY ybbJ dadA kbl zntR osmE csgB patA yihQ glpB gabT paoA yaeH dtpB ybhR qorA oppB ahr ygiW yqjG eutP psiE zraP gldA aldB tktB ygaM mocA hyaD yeaQ fbaB yibH pgpC eco adhE hokD preA ygdR ybhS ybjP mlaC deoA cbpM yohF dacC yqeC hlyE nanM yodD talA ycgZ glsA uspA dkgA ecnB cdd ybiB ygaV ycjX astC rclA allE ydeJ yaaX relE udp grxB yjiC agp treR hybO glmZ yphA pfkA trpL ompX dmlA grxA csgA yfdY mgtL ldtA phoP relB csgE astA ycjF cbpA yjdP sbmC elaB yeaO micF cspD sodB cfa eutT yeaR slyB glxR ynfB yqjE ygaP yoaG yedP astB ivy yfdQ galU sgbH glmY ykgE yccA eutN yahK clpA yjfY ybiO yegS insH1 yqjK ybjQ sseA yebE yajO patD phnL yihP yniA pgaC yiiS deoB add yfbS csrB csiR dnaK aceF otsB edd yhiI ydiZ bolA eutD ldcC adhP ibsB yhdN chaA curA yeaI ldtC yjdN ssrA iceT ybaA ftnB rraA ybcW yhjG dosC yhbS glk ihfB ydiH ldtD ilvA degQ yibA araH ptwF dcrB ydgA moaB soxR yjdI nanC astE htpX yqeB gstA eutK psiF insB1 lolA mdtF fnrS yafC ydhS yoaC lsrB rstA ypfH marA ibsD glpC hycF lsrD chrR ybdK yhcN manA blc cydX gltP omrA yahG shoB fxsA ygeW ylil eutM yghU yfdC gstB glpK fruA pepT galP dosP yeeX fic aceE otsA mscL leuO yegJ cpsG eno yhiM fucU elbB omrB yihD dhaL yfhM tomB cysQ yciE hyaE csgD adiC rutG ycgM rhsA norV recT pfkB glgC ybiC rbbA ydcL mgrB rof thrB ydhR higA zitB norR yhaK msyB ytfT luxS dhaK eutG yeeY yacL yffR yhfG ynhF tisB htpG clsB qorB hycI phnM lhgO ryjA csiE dgoR tpiA yciF arnA acuI ysgA puuB tyrA dicF lysU fhlA norW yqiK bsmA dinQ pgl ushA dgoK lpp amyA copA eutL ycgV uhpT iraD yegH ybeL yjhI yhbT yhhJ gadE hyi narL yfbT yajL eutE mdtE kduI puuC rob ybcM ilvD ihfA glpF gudD dinJ hslV rnr glxK yaeP yfbU ybhN mlaB yjfF rnpB hipB garD lsrG ybhP kduD ycbX ybbN yahF yhbW thiE ompR gltI ilvC trpR selD yccU lldD uspC garR dcuB yccM thiG chbR ydcV rpoH msrA kptA ugpC yqjC bglA yqiJ ydiR fsr yjbQ ubiF hyaF pheL ydcO rydB ilvE yphC leuD aaeX aidB yodC betB fadM moeA eutC ssuD crr ydcN mqsR gadY ydiO alsC yqhD epd yfbK deoD treF ymdB eda treA yjbR yffB mqsA arnD amiD pdxJ ydhQ marR ryeA ypdE ykgQ btuE yagP puuE sgcB yqfA smg slt yhjD arnB ybbA tabA fetA ypdF aroL pykF cueR ybeZ nemA yhhX nlpE ypjA pgk higB yeiG proP ahpF potF mdtA yahD ygiN glgA melA maeB nuoK idnO ydhU mlaF rtcB aslA hinT ydcS yehR leuC hpf yaeR nlpD ycfL ydeP thiF yhcH rtcR cynS ygeV yhjY gltS lpoB ravA potE pepP arnT uvrB ygbM gor gadW ryfA moaC thiS yrbL sfmA thiC pstS dcyD clcA yehE gpmA xanQ sgcC dld macB narX araG ydcY frsA mdtC yoaD ydiP cbdB ybjD cusF casB yjgH mgsA yjbE ydcH yjjB moaD eutR yphF yecA ydaT ytfE hmp eutB intF ansB yccX nupG ydjA sgbU rraB csrA yqcA phoU mdtM intA ycfP alsB lsrF yaiO potD glnP yihO yehW ybhB ybdF ydiQ yjgR yggL gloA yggE lsrK fetB ydcT*** *cspH carA yjdQ uraA lysV tyrV ileV pyrB yicT fes efeU fliF valY fiu fecI flgB carB cirA metT pyrD purE metU leuW entC fecR yjjZ codB argY cspF pyrI argQ entD purK argZ ydiE xanP suhB yddA leuZ fhuF malK flgC argU asnU entH argV fepA leuT alaV serU yghF entS purM yidD valT efeO cysT glnU alaX plaP zinT metV rpsT fepB yoeI lysW fliN entE lysZ fepE metW lysT kdgT valX fliM yjaA ybiX flgD serV rnpA proM fhuE purF pyrC ybdZ fliA fliL entA yojI flgF fepC lysY yncE ymcE flhD flgE flil efeB lysQ argI hisR cvpA glnW yfhR gltT fliH nmpC rpsU valW leuV entB valZ yqjH bioB dusB argC fliG queD ynbA istR ydiY upp entF nadB rttR fliP bioF fhuD agrB leuX tyrT purN gltV fecA adeP peaD ghxP rluC purT argD fliE opgC gspJ gltW argA yjbF yhiS ndk ileX fepG metY ybaN fliO flhC yadV insA lysP fliR cspA pinE codA artJ opgE purH pcnB queA cspG bioC yeiW metF ubiX pyrE yhdJ fepD rluB*

Table 11.7 Continued…

	folK yciH yagI argF uhpB fis essD znuA metE bioA glyW ycfZ ygeK fliK argB bioD serT valV ompF flgJ flhB argX trmN yggI intS mltF ybjE yjhZ glyY galS rph pyrF glyU fliJ yncD lpxT yobH ampG rpmH yqgB ydjN nadA argH alaW lamB fhuB ryhB nrdE gpt tyrU fhuC flgH mglB yihG dsrA mdtL yecT bfd fliQ ppdA recQ borD prs rimO apt exbD uhpC proV purB purR rpmB rlmG ykgO dtpD mltC asnV ygiQ yhbE flgI metZ artP nrdF pncB ynaK waaA yjbG flgG insJ ybfE pabB ydcD rimM thrT proL prfC hsdS sdhC holD yhhZ yfhL thiI ybgC yqeF tonB dusC yecJ leuU argG gltU gtrA yneG yfjV ybfP mreC yraQ yegK ecnA gltF ligT flgA rnb yneE tsaB yliE rpmG yeeE gnsB mglA rpsP yiaD yceA yhcA yafF yegD mltA serB nrdI rsxC ydiL yjgZ ycaO coaA mltD infA cysJ rpoA uhpA fecE rplU gcvT yfaT mrcA rsmG rplQ hda rdgC glyT tsx yeeD dnaB rpsJ yfiP dinI rlmA mepS mrdA yidC yedL potA rplY rlmC rplC ydfU yeaP rhlE mnmA lysA insK metN tmL ykgM gtrS thiP glcD ygeN yacC secG tsaD gatR ygaH hsdM tsgA yfdF mnmG rplD yhjV typA rpsF yiiQ fecC rsxB gtrB gluQ yibQ eptC yeiR cmk yhjH pheP thrU potB mioC avtA flhE hyfA argS epmB rpsD glyA mreD ypjF lpxH pheV cspB yddB ymfD ygaQ rlmN hflD rluE tff trmD endA rsmC priB rimI ygfF tyrP speE rplW rbsD yahM rplB rpsK leuQ rihA tgt yaaI tolQ yqgC rpmA ybhA yadS rzpQ ppdD yehT xseB hcaT cdaR ymfA serW gfcA arpA ynjI ansP yfcJ mdtO mdtJ ffs yeiP yoaA zapE yijF fliZ yaeI ydcX eptB rlmL gsk cysC stpA livG pppA cysM ychF yliF rcsA waaL ebgA malE fpr caiT fecD glnV cyaR fecB rlmI priA mepM rpsO renD rsmF yafK rpsB selC slmA holE yciA nhoA pyrH yciW rtn pth livF alsR leuP mutT yecF rpsM yieH asnS yeiB mglC ydcP rcnA yajR cytR yjhD plsX emtA uof iclR rbsA yhdT ygaY rpmE yhiN mcrC ftsB tolR yjeO ynfN evgS bcp sbcD ispU hisP znuC secY glvG thiB rep rsxA glnX map fadL rimP tsaA yjfM accC cusS glnL lpxP ycaL gnsA yhaJ rlmH dctA rplJ yfcL fldB cysD sdaB thrW mutY malM rsxD rpsA yfjM rpsN cusR cdh ydbD ycdZ artM ilvB ribA amiC cysI adk rseC recG ygaZ speD proW aroA rplE tdk ybhC yigM dinF rsfS fabA malF yoaK murJ yceD yiiX fabF ybdO ilvN lacI znuB rhaS nth yfeA tusA thiQ sdiA asnT fldA yggW glsB rplN folB dbpA folC caiF yjbH yedA ycgX can tig ybgP livH fur dcm tmpR trpT ycgH ridA rpsL yohO glcE tusC ttcA trmA efp alr yjaZ yhbY rplX glpG ivbL intZ ybjN ycaD yidX gpp yijE hscB envC dacB yegQ flk nudJ yjjG folA rnt flhA yhfZ yehU miaB motA yhgF accB ygeQ panD ydcR livM cdsA ispD flgK udk cysH wecA ycfT tusD gntP rpsH pqqL trmH rnc tnaA yejM ygdG ansA ybgO dusA ampH obgE menA atpF ybeF yheS lpxK insF1 ampC dnaG truB perR ratB vsr yeiI fliD cyaY spoT yaeF atpH serC rnd proQ yccS tatD aroM fliS rpsG yneJ rplS ybgQ pldA wecB cobC atpB ybiT ycjW rhaR parE yraJ coaD yjaH gfcB ynaE emrY parC topA gspM ispE ompT rho rhaT alaS ribB prfB ibaG pitA aspT hemF pdxY yjhR speC setB secE rfbB fadD artQ dppB ydiU ybiR purU ymjA lepA rspR rpmJ ychQ rihC cysG yjgL yehH yfcA yiaJ yqiA ydhK yhfA lipB yicC waaC srmB nudG ydgI ygdB frr recF rnlB hcaE
RNM-18	***yhjX pspC pspB pspD pspA ibpB pspG pspE ibpA asr yjiY ldhA yibT ychH ybhG glgS yjiS ypfM ydgU yhhA uspG bssR raiA ypeC ybiH ybfA mliC yohJ yaiY clpB uspF ycaC pliG sodB sra ycfJ uspA ygdI yohK omrA uspB osmC yegP hdhA ydcH ybhQ bdm ycbJ bhsA zraP rmf yniA ydcF ynfM qorA loiP yccJ yiaG hyaB ykgR osmB hspQ preT cspD ompW slp ydfZ gadE wrbA ybhF yneM nanA ymdF yahN omrB dps ynfD adiY osmE mlaC gldA yffR ygdR rpoS ydhR treF yfiL ygaU uspE spy hyaA ybjP bssS ybhL htpG ycjX yhcN udp yhbO csrC frwA yghA ariR tqsA yajI qmcA yeaO ynfG rhsA ybhS yohC dcrB yjiX dnaK ynfE ilvC gntK yhiI ybgS yafC glnH ryeA yaeH cbpM agp aroF hokD gpr glpA dhaL yodD aaeX rutA ybeD yfdY hslV clpA glnK csrB yahO hdeA ygiW ldtE psaA yhdV uspD hchA mcbA grxA ycjF cbpA ytfK ybhR galP deoC yibA phoP yeaG hcr yhbW dkgA ymgC rraA nfsA edd hslU ycgB nanT phoH lgoR yeeX fucU atoA ybbJ tabA artJ fumC ivy rutF ybjQ mqsA preA grxB mlaB yfeO hdeB fruK glsA yihQ ybjC ybaY relE amtB rutB pptA ybdK galU yqcA dhaK yqaE yagH msrA zntR yjdP yfdT yqiJ shoB higA gadA csgA ttdB gudD eda groL elbB cdd eutL ydeI ycfP mdh dmlA yibI yqhD ymgE yeaQ ibsE marA dcuC insH1 ygeY eutK glgC pfo ydcL ygeV treR ygaM yciE ssrA yacL rnpB glmZ dgoR yjbR yfbS nuoK hipB gadC ybeL ysaA ompC pck lpxL yhjE yqjI yciF iraD yjfN matP yehD mgsA trpE eutR caiF dmsA ydaE yagG yhbS ybbN chrR ynfF serX glpB rbbA pstS slyB glk yjdJ fnrS deoB pheL acnA relB fxsA osmY degP erpA gltA yffQ ftnA mqsR yecH metQ grcA fbaB ogrK livK yihS yaiO curA frmA yajL ymgA yajQ dcuB dicF yhhJ eutH deoD ytjA yhhN yeaH talA yfdQ yahG nlpE sibA degQ zwf yedP tktB cydX sseA dkgB bolA glmY ansB narW ybaE qorB yggE gltI marB eutC phoU panE leuD cysQ yphA higB eutB gstA yceH deoA kptA yciK menI yidF glxR ybeZ ydfI araH katE eutG fadE ypdE pgi psiE msyB ybiB potD ubiA ibsB yihU ydiR hcp tdcC abrB icd ushA rlmE rihB argI ssuD gshB adhE gadB ydcY idi iraP csgB fsaB nac garR ompR ydiO ryfA glpF recT rpoH ulaG fsr yciO ldtD artI metC nuoL dinQ glpD dmsC adhP yjjB yjbQ rutC sdhB yhbT putA yiiS ydaC prlC yceI dmsB yhaK rutD mscS gabT dacC astC ahpF ucpA ydiT xylB yeiG nuoJ hfq rraB miaA araC hemB hslJ hyaC ynjH yihP trpA ecpA flu ahpC gabD pfkA dgoK narU blc ubiF lpxC ptwF pntB yihX yahK sodC rpiB trpL elaB ldtA dhaM trpD lolA ygiN yfcZ yihT lsrG rof pgpC hdeD yjiA narY cheY pepB sgbH gatY aceF katG yagE yhaH cheB yhiM nuoI nlpD hyi mdaB fliS argT manY smg kbaY yeaC ydbC yoaC hmp nuoH pntA sfsA cfa pqiC yqgA maeB narV hycI livJ ydiQ zitB ydjA manZ yjcE ybaT oppA fucO ihfA nanE pgl nfuA tyrA tpiA narZ yciN gntT glnP frlB narP ypfH frwB sfmA sgbE mlaA cydA rrsG ttdA nuoM amiD ompT lpp wcaE uxaC ynfH yihO bglA yccM mdtA rrsC zntA paoD fimG yfbV allB rtcB yciG cyoC pepN garL phnD yibH poxB leuC hisJ yagJ nupG pfkB eno iceT ydiZ ndh ssrS kduD dgoA selD ydeN trpB yaiE mhpT ompX eutM glpC srlD moaB xdhC bsmA rimK zapB hyaF gstB cydB rhmD gpmA arsC aldB dcp hlyE mdtE dsbA tpx gcvH yeaY fbaA yaeP hycD yqeH luxS ycjO patD acrB ybiW yajO xylE rrsA ypdF yjfY ftsZ sucD allE ydhQ yqjD glcB rseA sdsR nuoN ilvX yqjG yjcS glgA yohP fucK ahr intF ydcO clpP rtcR sbmC tisB norR ydiP pagP rrsD ggt cheW ydeP lsrF yfhM hpf glpK ybeY abgA pstB ampE ompA baeS rrsH tauC rrlH srlA livH psiF ytfJ rimJ mlaD hybO ybgE yjhC csiE crp ykgE aes cpsG zupT yhdN manX yadH rbsB yjhY gloA exuR ttdT galE eutQ yciS manA fetB mlc dksA ftsH yegR pepP tauD eutP gltD copA ybcH rpiA cyoD pflB glgX fucP secA aroP mdtG gabP lrp rcsB idnO ygaP ytfB mscL rclR galR ynaJ frdD yfaP cbdX pka pstA fimA ylil ybdF hipA yqeB yfbT fliY ykfB sgcE recE pyrI fruA dppF hyaD rem yqjK slt aroL macA frmB rrlC mtlD yacH yhaL yfdC ygaV yieP soxS yffL yffB ymdB metE lldD rnr hemL yccX yecD frsA fetA yhjB rpoD nagE clpS dedA yphD lgoD fumB ydgD chbR yfgO ynhF yrfF fryC aceE rrlA pstC yebV pgk potH ssuA eamB glcG menB uhpT guaD yegS ygeW ydgA fucI gltP arfA yihD hybB kefG ybaO metK yghU smf elfA eutD groS gadY yggR mntS ycjM yahI moeA acnB yceJ ibsD yfbU acuI tehB ycjN speB dppA srlE yafV fixB yffP ycjU agaV ynfK otsB yifE rutE yadL ptsG ychN yfeN yhjA nlpA frmR ftsA kefB yhjY dsdA gatC yhcB nrdB oppF caiD yqhC nfrA rbsD argB sgcB mrp gltB fimF asnC malP yggL yhjD yffS dld rspB frdC mlaE rbfA hybC ykgG ryjA ydhS otsA acs tdh sgcA yccA truA hisD sgrR ybcM hiuH torI hyaE xylA ybiJ nanM abgB ynfB mlaF yqeC gltS uspC proP yceB iscU yqjE yeaD ypjA ybbA rhsO paaZ frdB sucB cycA agaA eco iscA potE glnE aslA yjhG ulaB nfsB ysaB malI yqiK gntU nfo fumA yoaB chaB intA lpd lsrK yfbR yeaK yagA ssuE alsK gatD casE ygcW orn yceM cynS yjfP yicH ybcL ilvM lpoB hns hsrA pepT hldD meIA ydiH pnuC flxA yfbK fucA zraS cusF ydhC potF ttdR mprA crr iscS ghxQ ytfE csrA rclA tolC cueR dtpB ridA ydhF ybhJ ydfE ecnB ycjQ yebE cpdB yiiM yhcH phoB ftsQ sdhA livF ygfM macB allS paoB atoS ilvN yphC torZ glgP dgoD thiG gloB ulaA chbA cheR tag metI tfaP puuC ilvG mokB php csgG xdhB eutE ybiC hsdR ykgF aldA adiC ygjR gor yjjI lysU ybdM dsbE yjdM yphF flgL pheA lsrB yraR astA ybjD xapR hybA uvrB cbdA trpT gatZ feoA nadE tyrR galK cysA ydhB glcD dinJ chpS ygfZ dcuR ppsR atoB lrhA araG pgaD phnN ygbM dcyD cbdB bax ccmH rayT nuoG atpD livM argG yqfA dnaJ tdcF pdxJ yihY btuR*** *yhdU yjdQ fecR cspH alaT argY purE fecI cspF fepE insA napF yjjZ napD pawZ argZ leuX lysQ argQ lysZ lysV yddA purM fliE yghF purK yeiW yicT argV yghG hisR xanP lysY pyrD leuT ymcE metT fhuF yghE fes argX alaV efeU rttR cirA fecA ompF arpA napG proM rpsT peaD cspA rnpA ydiE folK insJ argW tyrV cspG ubiX entD argU yidD purN carA mdtJ azuC ymiA ecnA mdtI asnU ythA pcnB cspB rluC coaA hyfA dusB lysT yeaP rpsU gltV ydiY yecT opgE yoeI alaX ybfP rluE yigG serV napH napA yfhR suhB yibQ lpxP cysT valY ycgX queD valT fepB ppdD adeP ybaN entS gfcB yihG motA fiu insK mltD metU leuW tsaB ryhB ynaE leuZ gtrA mnmA metY queA intS glyU recQ ycfZ yoaA yhbE ynfO ynfN rph lysW yneE gnsA holD ydfK metV entC rim yggI gpt nrdE ygiQ flhC rimO ampG nrdI flhD gltW asnV yciX cmtB pinR ndk yijF apt rsxB yqiG fecC narK essQ wcaG glyY mreD artP rrlE ghxP epmB acrS rimI tyrT cysG fhuE glyW rhlE typA mdtL yhdJ rlmH mioC renD malK dnaB rluB iclR ybfE fhuD yneG cusS ybdZ dbpA mreC serU ynjI vsr yfiP bioF rrfD insZ yjhD yfgH waaA yjaA ybeT trmL yeiR rlmG yfhL tonB metW opgC lpxH valX gfcA galS fdnG yraI yfjV infA ycjW yfcJ dcm lpxT udk yqcC fecD fliP valZ yojI insF1 entH tsaA rsxC dacB rsfS yncI dusA yqeJ ybcI glyX rpsP arpB ymjA bluF cobC ydaG yafT yeiB ansA mnmG rsxA truC rtn yedN efeB ydfD napB pth rlmC pncB yjjG yiiQ aspV cyaY efeO ygeH trmN yacC yadS thiI hscC agaC intZ tsaD prs ycaL hda potA serB glsB yafK cmk ileX sdiA wzb gltU yliE cdh znuA agrB yhbY mltF secG avtA ligT fabA mepS tyrU yedV rplD tatD menF lacI hemA yciA ycjW yaeI ybcK rnc ais yoeA rsmG ygeO gluQ ybgC rpsJ priA uhpB yoaK djlB insI1 yhbV prmC lysA fhuC mltA rlmN yajR yhjV ypjF gpp rplC yjbG yjaH mcrA pinH wcaH gtrB gsk dsrA rpsA ydiU cytR nudJ yecJ rsmF rspR wcaB psuT envC tilS rimP essD gfcC leuU lolC rnt rzpD rplW rplU rlmA gspD plaP exbD ybeF rpmB nirC ybiX yaiS bglG accB rnb cvpA yniD rlmI gspC mnmC yqgB ydfU tolR tdk ybcN lpxK purF emrK rnd yfjU proL fecE bioC pheP yjfK yegQ fis yegK pdhR ispD pyrE ydfA ygeQ dnaX era narJ dinF ybiT nudE zapE evgS lolD yhdX bcsQ phnH serT entE ydhP yafP yliF yeaI rnhB ygdG glpG pbpG ycaO yijE ydfC fhuB mrdA selC cysS dnaA cspI yraJ folC proV tolQ rho tatC nikA ynaK pyrC cysJ scpA trmH gnsB wecB pabB glnU rep ampC accC fepC waaC sbcD ydcD fimI nudC rseC pppA gfcD ycbZ lipB ybcY yahB mcrC tsgA ybjE ebgA prfA psrO tusD glpE ybhK acpT yohO wcaC xseB ddpX yijO yegD nrdF nrdH msbA dusC wecC yihM radA ydbD argS prfB cusR yhfL rlmL rcnA mepM yggW btuF yfcA mltB yhdW plsY hcaT ftsB asnW ybiR rsmA yoaE yjbH flgB plsX yhcD yhdE yahL yehT ygdQ pabA tusC rrrQ fabB fecB ftsN hsdS yqeF lpxB tmcA hflD yihL rnhA fau purR ymfA yfhG pinQ yehH holA entF thrT sbp fimC rfbB nikC yidZ tmD purU gspG rssA alaW yibN yjgN fdhD xerC yjhZ hypT narH nadD proQ ydgI metZ wecA fdnH flgM ychF appY prc yhdT ychJ ygjQ ycdZ ynjC apaH bioB icdC yghS wzc yjjQ murR yhbU adk nikD rcsF fepA yfcC metF ykgH alr barA rpmH ppdA sanA yjeO yheS secE yjgZ flgJ prfC dnaG recO smtA glyV ygcN yagI asnS folD yehY yigB ydcP rpsK ydeM agaI amiC yhcG ebgR trg aroA pphB yjaZ mrcA yqaB uvrC phnO narI rplB elfD yieH uhpC ybdO cbrB nlpI yaeF yfcF zapC fepG yfdF rpsN ogt yciT*

Table 11.7 Continued…

	purH arcZ degS obgE ytjC yghO ydjN bcp ycgG ydgK ygeI nikB ubiG ydcR rbn tmpR gspE lpxM yceA yjfI mutY yncD pdxY recG mltC yrdE endA ygaH coaD lysP mglA tsaC yhhZ metB xseA wcaL gpsA efp parE rsmC yihN ypjD nikE pheV cydD yjeT ydfB kdgT entA mreB rpoA gltX yneK rplN rplQ bioA hsdM aroE rpmA rpmG serC rplX yqaA dnaE yafU glyT yfgF yejM wcaM glnW cysD slmA glrR ygcO ycbL yicC sfsB yfjD yjhR waaL ispU bcr znuC nikR cmoB menD mukF ycgH spoT ispB djlC mepA znuB lepA dosP nadK bcsA yfgG thrW ygbE sfmZ ydcZ sppA acpH flil phr kch glrK yifK murB can insB1 yjhE ygaZ insL1 gspF yjbI yigM yfjW yidX yciZ wzzB deaD thiP yahE nupC argC yadD valW yibB elaD ymgG cmoA ratB yjbF ribF shiA dut rhlB viaA yaaA uvrY yheO miaB cynR ycbK ftsP bdcR yfeA pncA folA rdgC feaR rihA yidC rybB thiK yjeN cusC menE yeaJ rpsD ybeM gspL exbB yibG yhcC nth yggU emtA yeaV dnaN eptC frr ydaY secD ygcP ybdN nsrR yqgF yfaT thiM ycaD ygjV ybbD moaA panF fabF murJ rpsM ybiI intG yneJ minC ycaQ gspM pdxA dicA thrU ynjE yrbN ibsC rbsR ybhC ydfR mntH fliG yrdB dfp rsxD yjfZ nohD emrE phnP thiQ ampH ynjB wzzE uvrD rarD insD1 ppiA hpt panD fhuA nfi ydhI aer ybfB msrC gspK bamD folE holE gmd sugE fadL yhgA fliM rhsE rnlB ppdC birA emrA mdtP ydgC casA pyrH nei yagN ugd ubiJ epmC alsR yciH yddE clcB pheM yhcA maa ydcI narX ychQ rluF nemR potB yiiG csdA sdhC sufB allA ydfV rplJ yjjJ yeiL yfiH yoaJ yheT yidA pldA yehA yfjM yfcL aas ygeN nusG apaG ymgI emrY cyaR yajD yhfG ylcI yghD rplE gapC rpsS yccT ydiM ubiB argK trkG proS pbpC gcvR malF flgI yehU aaaD tmk hcaF nadC queF nrfE rhtA yhaJ rdlD mntR wcaI cpxA rpsH yigA dnaQ yihF yedW ydbL yiiX yqeI npr pdxH cdaR speF ettA btuD mntP hisS ycgN ybjO pssA rseB ycfH atpI yhjK ydjY yjjX chiP yccF insQ dapA yagB dacD glpX deoR yehL rhtC ubiE fepD rplV ppdB yfiN ycbX hisP hscB ycdT hyfB holB yccS ydeT yjaG dinI umpH acrR tcdA ydcJ fadR yidI yhjJ lolE ugpA yafQ ispF ybiV ylaB ydhK yncE yiaY srkA ydbJ yhjR ecpB pitB hydN rdgB yghW bfd pabC mppA yiaD eptB yohD recX asnA insO ydgJ pyrF yicL cdsA cysM pldB flhB waaF yhiD rffG yeeW mog ygdB parC lysS rsuA lpxD flgN puuD hyfR abgR yoaG ynjD alx gmr yddB ytfL ychO lptD bamC ybeU adiA waaQ prmB ydiV cysE yjcC dinG dacA mlrA yidQ hcaC alaS yeaR murA yeaL yejL insG recB hcaE plsC yddW cyoA elfC yqiA aspA lamB djlA tgt sufD fliF rfbD yqeG yebZ yiaV ybgF ydjO mukE yfgD tatB yehM pitA truB yjgL ruvB ybjT dsbG ribB yjiK emrB racR ygiM recF cbrA serW nusA yggS ybfG ykgS yehI dctR yecM xylG yjhX apbE yhgF ybhA ybjJ folB sslE asnT yqjH scpC argO sufA epmA yfaA yicR rnlA yafL yaiT ytfF ypfJ pgaA yehB gyrA ibaG yfcI yecE mutS yqiI cyaA nmpC yfjH treC ykgM ratA hrpB yegW lafU ygfF nudF yhfK yeiS queG dsbB yebB rpsL thiB
RNM-21	***ibpA pspD ibpB pspC bhsA pspB ymdF pspA yibT cadB yacH glgS hdeB trpT micF hchA gadA pspG gadB hdeD ariR pspE hdeA yciE gadC ldhA yciF ypeC yciG ytfK aspT ybgS ychH katE aroF yfeO hdhA ilvC sra treF ftnA yfiL yahK ydcL yegP ydfZ osmB ybhG tktB yiaG yghA ygiW puuB ybfA ptwF mcbA slp mdtF ytjA fumC ycfJ ybiH elaB dnaK ycaC yaiY poxB talA clpB kilR aaeX psiF frvX pliG yeaQ cpsG ygdI ymgE marB csrC yqfG iceT mliC yfeN curA ynfM groL osmY marA rrlD yphA osmC ybjP ibsB ycjU yeaY ydeI yhcN aspU rmf tyrA yagG ygeG yqaE bfr adhP ydcF yadC msyB dps gabD adhE mdtE puuE lldD yagH htpG yhhA wrbA gadE fruB yccJ ybaT osmE fxsA yfdS rclA csrB ygaU ynfD zraP yahO yoaC ymgA rrlC yebV higB preT csgB yjhI gudD spy trpE mtlD rem yffR aldB glmZ ybhF yehE ypfM ahr puuC ydaQ baeS rrlH hokD ybcH yodB ihfB narV yfdQ sodB yjdJ yfjJ ybiJ yihQ yncL moaB fbaB metK hslJ ybjC ggt eutH loiP cbpM groS opgB mdaB ompT putA phnD yfjZ rpoS dicF eutG ecnB ymgC hyaC fryC ssrA narU rrlA degP yhjX narZ artI sfmA potH qorA pfkA ompW narW sgbH uspB metI yhcO pstS narY ydcY yajQ symR yibA pgi malM malG oweE nfsA ynfE yifE yqjD deoB cbpA gabT mlaC ivy ydgU yhbX yqjK ulaG ygaM inaA iraD yegS yfdX yrhC ybhS yhbO deoD yhiI erpA mhpB bssR adiY grxA tauA ybhL dgoT rrsH kdpA ylbI trpD ydhR yeaD ssuD hyaB cspD glsA argB patD torC yhaI ryjA yqeH deoC glmY ahpF kdpB agaA uspG yfdT fruK rrsA yjfY ypjJ yaiO yohJ yadL garR pheL fadE paoC mdtC rrsC ydfD borD ygdR yahG ydaC paoB yibI paoD yfdV potF dcp rpoH pqiC yqjG aceF metQ nanM ydjN yahN tauC rhsA yehW acnA potE ybaA ghxQ insL1 omrA eutP metC grcA dnaJ gltS alsB ttdT raiA nfsB ibsD sieB dkgB yjhB intF hmp yoeH menI ldtE cdd ydcO mgtA rrlG tyrP argH yeaO yjgR acrB tfaP uspA galU yjbJ ydfE nuoK dkgA ylil yahI malP frc wzxB yohK mdtG rimK atoD rnpB udp eamB hspQ yadK yegR yniA yihP ypfH moaE ybhQ bdm cadA hyfD yoeD fucU glxR ybhR rclC hycD mlaB slyB treB leuD bssS yhaH oxyS mtlA ykgT nanT hyi yfcR ybeD gloA yhcH yfdY yjhC ydhY eutR ryfA norR ybeL ycjN hyfE relE thiE yneM rfbC glcA cbdB uspD araC alsC gntK mdtB yeeE ycel tdcC nanA sapC thiG potG racC proC hemB pfo wcaJ acs csgF cpsB potI frvB yoaI yghU ybjQ ybhB yibH hlyE sdhB amyA pptA frmA pgaD yohC yjbE yafZ gabP qorB aroL ushA ykgJ emrY gltD kefC eutB fimG ycjM ybiU ompX cysU moaD pagP yajL gadY leuC yqjE elbB lsrG ypfG norV secA oxc ydiF chbC ydiO rhsB nnr ydcC panE yfgO gltB dsbA acuI nfrA yfiM rfbA fumA yajI omrB stpA mgsA mdh etp ynfG paaI pntA pstA garL dmlA ompN moaC kdpD sodC yhjG yffL wzxC hyaE yceH lpxC ydfI thiC flu shoB yehX chbR pgl mgtL fhlA nuoL ycjQ cbdA xylE pykF cysK mlaA yjcS frvR ynfF azoR ydiP aslB ompR ybgD thiS metR zwf ypdF yffO ybiO acrA eutC hslV isrC lolA lldR pfkB ydjI nupX yeeX cbdX yffQ mscS mdtA hpf yqgA ycjY ymfJ dsdA ycgJ iraP yagE uspF lgoD yihU caiF nlpE kdpC pstC otsA dacC treR ydiN aceE atpI menB waaB araH acrZ atoE cynS thiF fucI pyrI nuoJ ygeY yceJ ybdR yggL metN ybcL dedA sgcB ydhS yjdN manA tdcD ydfO panB ydeP ogrK ltaE agaW yaaX yoaB trpR glcB spf ynhF nuoH nuoN yccM pntB eutM eda glgC nanE gltF zapB clcB mtlR hyaD ldtA yodD eutE ybiB ihfA yhaL dtpD yeeR rtcR yhjB frlB yeeS appA pauD puuP yadM ydeJ ybhN eutK ybaY gor eutD pheA ygiN mqsA gshB ugpE eutA rpiA dsdX agp truA sgcE waaO sbmC rluA xylB ycaM ryeA ybjH nfuA msrA sixA relB lsrF bolA xanQ allE mglC nuoM metA malF recT nuoI ybdO stfP yhaK ydjJ lsrD xylA tsaE ydcX yggE glk lpxL rspB eutL hcp dcyD patA hyaF wbbI higA waaR yeaR yadH tisB metL ycaK xdhC yfdK csiE hcaD ssuC ypeB yjhG torZ sapB chiX yfcS ybjM lhgO hycC soxR gmr cfa cheY yihS eutJ ahpC pyrB malX ugpB adiC waaS araG ygiV yagA lpp ycjV treA yccX gstB yciO nhoA yhjE wbbH rseA ycjF dxr slyX rhsO yhbW actP yaeH tauB tolB sdsR yihD aroH ilvN kduD hyaA yggP yhgE ydiZ tdh rbsB degQ yieL setC marR mrdB yhhT yfjQ chrR hyfI grpE ybhM ycjP yjhH ygfZ ispE ypjA recE yfaZ yjeI fbaA ubiF yicI rof yddG ygbM ypaA aidB yobB csgA allB paaZ cysQ nuoG yagF fimE glnE yjeV yecD ridA mprA tabA prlC torR lsrC ybiW icdC ycjR tolA yphD ydaS qmcA ssuA ugpC mqsR dmsA yfdE yihO thrU pepB ldrB yceK thiD paaY eptA gapA pgiA hslU slt ypdK dinQ yfcP katG abgA clsB hyfF ldcC preA yffP pqiB ymfL ttdB rlmE argD idi pgaC gudX ygaV crr pgpC etk ybcM casE yahF ubiA ilvB hslO lpd yphC ybbJ rpoD pflB waaZ yedP sibA norW melA yjhY*** *asnU fes yjjZ ybdZ alaE yhdU fecI fecR entH entE entC fhuF insD1 ymcE sokE ydiE entA fepA asnV cspH ynfN entF entB fepC gspI pawZ cspA ilvX yncH glyY fepB cspG ppdD bluff narI napF ilvG iclR narJ pitA efeU ynaE nrfG fhuE fiu leuX fepG nrdE nrfF mdtJ ydfK yacC entD cspI yahB rimM proV azuC insA alaX cspF napD rttR entS ebgR nrdI fecA yncE argY hyfA fepD cspB rhlE argU fhuD yecJ ybaN napG xisD rnhA tonB nrfE argV napH yeiW rnpA gtrA sdiA napB yciZ yibQ ygeO ilvM napA lpxP rluE rpsT pinQ amtB glyX yfiP yeaP fhuC flgN yciT glnQ proM argX yidD efeO purE yoaA ybfE gcvT exbD feaR ecnA rlmH nrfD ygdG glnK glyW cysG agaC yegK rpsP epmB fhuA glyV rsfS yqjH ygcN nudE yigB rspR ydcI xerC ygeH mepS tatD gspM dnaX btuF coaA ynjI rluF cirA flgM suhB efeB ddpX yhcG yfeH essD uvrC folE serU tatC hemA cysT argZ yhjV ompF rph glcC nadC yqgF mltD yidA mioC ydiU argC yhaJ ydcD typA fepE yibG flgB nrdF ansB mdtL yjiO narH xseA ydiY yhbY gpp gnsA fimI yoeA yeiE priB dusB yicC infA hisR argQ ybcI trmL zapC dinG alaW phr yddE serV crfC potA gltT rlmG queD rpsF ycbK wcaF glnL rdlC yniD hemD waaC prmC nrdH rimP rplN ydgC ymjA rob uvrY fhuB yibF holD yqfE rihA yegW yqgE yafO leuT yihG gspK rlmC purU gltU rhlB yicR znuC yidX dusA yrbN lysV yhjR nrfC yejG leuZ tyrV rimI yedW ansA sthA lysY rplX insF1 ygaQ aroG prfA wecB kch trmD uidC ycbZ msrC gspC envC yfhL rpsJ queA rssA queG yqeI ygeN nadK aaaD yhbU yggI rrrD ycbL lexA ispD pgrR murB yraJ ybgC exbB panM elfD lacI yaeQ glcD mntH yccT ubiE nusB nudK pinR ybfP yahM pcnB glpE artP umpH yeiL pyrD bamD rpsK sokC metT cynR rbsR arpA yedA glyU rplD narK rnd yafE yqeF lysQ yjhZ yfeC rpoA rpsD ychJ yhdJ yafT mnmA leuW yqaB yjcZ chiQ rplQ rsgA intZ yrdA hemC rpmB spoT metU rluC ypjD pgpB folK glnG znuA yhdW dapF yeiB dnaB aroK ydfU pldB yjaH rluB lolC yjfI recR hisA hscC yidZ glnW ligT crl rpsU yddA queF yigA thrA rplC rrrQ yebZ exoX proQ lysZ yggS ygdQ yggU cbl ydfR yfhR ybiX rsxA insI1 yqaA bcsQ yijF ymiA rmuC waaF ratB yjhR pphB nadB puuD ygfF cmk yfeD trmN insQ yihL aspV rsxC gtrB fdhD cyaY lysT yhfA dapA apt yjfJ yneE nlpI valY glnP pncB ubiB plsY seqA ogt udk rseC setB exoD yjjX yajD tesA lacA yafK rtn rsmA ybeF glsB pheV rpsN fdnG lysW secG smtA insB1 ybiR deaD tmpR tusD ychO ybgA recQ ydbL ilvL modE tatB rdlD fecC yjfK hypD ydgK lpxH ydcZ cusR trmH yijE hda fimC yeaE glnU prfB yggW nudJ ebgA gapC yjbM frlR ribF elfC rplE mreD purN rnhB yfhG minC pbpG lpxM hisF yicS ascF yjiL dtpA bfd carA cstA ychQ ybiV mutY adk yadD ydaG ynaK rcnA lpxB fliT hisG nirC yneG waaA shiA sspB yqiH gcvR fau bcsE ribE ydhI frr yaeI hemN sdhC valT rhtC hisH nikR bcp dnaA cedA yohO ygcP renD yhcD hybD rpsL yigL yjhD ftsB amiC yedL rcsF zapE dbpA ypdA tfaX yecM viaA yiiG yjjW fucR yedV pdhR rplJ recX yjiK torT alsR menF lipB yecT perR pabC avtA alkA hpt yheO ygeR thiI oppB speD fadR argP sfmZ yhfK hemX yihA ispB ubiJ tusC yfiE metY nohQ prs yjiR galS ybbO mngR yjiA ynfL yqeJ mobA uxaB ycdZ lpxK abgR cytR dsrA yobA yjtD yafS bamC yigM nikE yhcC ycjW mreC yjaG yfjM mntR yfcF gpsA fliM fliH rsxB dfp yafN pspF ygcO cvrA dcm yaiL rpsR mobB ycaQ rsmB cdh hisS gspD smpB rnc ytfF ppdB fecB djlB yibL yggD xapR ygiQ gatR ratA wcaD nagC srlR ygiC rpsM yibN secE rlmA dicA uof ytjB ampC ynfC glpG asnT nudI hemH tgt cyaA phnO rarD ybaP accB ycgG wcaM yfjL asnA yjaZ yadS yhdE yhbV rho fadI yegD cmtA ais sbcB cmoA mhpR stfQ ruvB dcuS gsk csdA ykgM rsmC ubiX wecC caiT hsrA yehS insJ gmhB sbcD ampD fliF yicL polB cho caiA ycdY yjjG fdhF mnaT yhcB thrB mdtH yrdE valZ rpmG xseB afuB dinD tsaB fliI yjiM truB tsaC kdgT yafP sspA agaR ilvD mhpC narX msbA yfiN yjjJ leuE ackA aroE yfjV tyrT yggC yejH atpH cnu ycgX insN tmk murR nikD crp pepE fecE ygfK lrp insE1 yecR ynjE sfmD dinF ybiT gltV ychF basR rdgB yaaY hypC yqfA yqiG ycdX rimO truC hybA deoR hisB murI yfiH gltJ umpG ydaY ilvY ypdC ynbE psrD mltC ruvA ppk tsaD flgJ ycbX wecA rbsK xylG priA pdxA yheS puuR znuB lysR holA hisL dacB frdA gpt yihF ybaL hofB ycgR dam nanR ymgF yebK fur dnaC ypeA nudF mukF hybC folD yfjD pmrD ispF ppiD tsaA ydbD hybB yjgX thrC yieH ftnB yojI yfcU yobH metV hypE yebB evgA mpaA fdoG ppdC*

Table 11.7 Continued…

	thrL trg aphA rimL hisI cobC gltX nadD wzzB glyQ srkA ytfP fabR dgt opgE recG ytjC slyD yiiQ birA eyeA folP gluQ cysB syd ycaR pnp hdfR yjiG yfgF soxS modB acpH speE yiaF hflX mhpD dnaQ
RNM-22	***yhjX asr ybgS pspA pspD pspC ydgU spy pspB ibpB ibpA ygdI glpA osmB pspE yhhA yhbO ybhG yhdV yghA raiA ynfD pspG narG yaiY glpB ymdF ycfJ osmY ydeI bdm ynfM ypeC osmC glpC ytjA loiP mliC dps yegP yajI yfiL omrA rutA ybiH ycaC uspG osmE ybhF ypfM ldhA yciF tqsA ivy yeaG ybaY yjbJ fruB ybfA ydcF bhsA pliG ymgE ychH glnK aroF ariR ygdR clpB hchA ytfK ygaU ykfH yodD ilvC yohC sra ybjP glnH ldtE yqaE yciG uspB paoA uhpT katE wrbA galU hdhA yedP waaH yebE bssR glpD ycgB yibT ycjX yccJ ygiW treF yneM yjfN rmf ybeD pstS astC sodC yeaH fruK dcrB paoB bssS puuB sucD rutB hslJ ybdK yahO wzb ycjF yiaG yfdC tyrA omrB rpoS adhP tktB zraP fxsA sodB narH ldtD ybhS wcaA puuE yjiY glmZ yohK cpxP dnaK yeaQ ydeJ ompX hspQ ldtC amtB yhcN galP htpG ycjM yciE mlaC adiY mcbA yagE pgpC ygaM dacC wzc yegS talA acs sucB yohP lolA ybdR ybhL narK nuoK sucC phoP hslV yeaO insH1 yibI zntR qmcA puuC phoH yqjD yohJ elaB trpE ybeL cdd wcaF gabP wcaB yggE qorA fbaB narV ybiC ecnB glk yjdP uspF uspE degQ curA nuoL yagH ydhS patA preT gntK blc nirD treR betB yqjE otsB betI gabT gltI ydcH yohF mlaB dgoR clpA glpF ygaV prlC ybbN pagP nuoJ gabD yafC glgS ygaP ggt yagG yqjK ymgA yaaX slt grxB degP wza htpX rutC gadC uspA soxS narU mdtA ybfD yhjD mgtL yjdJ hslU sseA yceI narW dmlA ynfB ybfC ybbJ paoC ybbA hemL sodA bolA dkgA trpD higA ldtA rraA asnA yccA glnP pyrI ves nuoI eutL ybhB psiF nuoM nac ahr cysQ trpL slyB aldB ybfL fruA yqjG nlpE gstA otsA ybfQ eutK deoB afuB dhaL yniA yaeH rpoH ptwF rof norR ybiI dhaK yidF lldD fadE nuoN astA sucA yjfY nuoH hokD pgl manA glmY yhdN tomB ydgA udp relE norV yhjE ydiZ narY aldA glpK miaA wcaE ybhR rclA clsB pfo ydhR yjbE yaiA yebO yaeP eutM aaeX trpR pfkB yahK ybeZ ycbJ deoD hcr rutD yjbF yqjC cfa rutE ylI yjdN ybiB ybhP csiE yffR asnC yagF eutH higB yphA yhjY rhsO rcsB nfuA yfdY csrB lon putA lpxC grxA mdh ypfH mgrB mscS deoA yagA aceF ymgC ftsZ deoC yjdI eutC ybjQ eutR eutB bglA dgoK pepB yibH narZ yjbG cspD ihfA selD gadB nirB afuC aroL yacL mgtA ybbP ybcO ftnB yhhN poxB icd fimE sfmA galE lhgO ryjA glcG msyB cbpM yecD tpx yrfF relB frwA groL cpsG rutF hycA psiE kilR chaA ydcO pstC yhbW ldtB uspD ypfG ycjN gpmA yhcO hdeA dedA ypdE yceH dicF yfbR gshB eutG shoB sdaA rrlH yfcZ speG arfA dksA rrsH rbbA pyrB yajL narJ xylB zitB yciK uvrB paoD cbpA gadA yhjG qorB nlpD ascB frmA nuoG csgA galT betA rrsG ydgD glnQ yhaK ryeA gudD yqjF grpE rssB hdeB rrlC yegR groS sfsA araF hfq hycC ssuD gntT yajQ ymgD phoU ecpA fadA ssrA erpA phoB pepP yffB psaA ypdF rrsC grcA yfcV yciN aceA rrlD cyoD hupA zwf moaB argT rhsA ubiF rpoD dxr tatE moeA fiu yqcA mqsA sbmC glcB yhbS csrC gmd ybhQ dinJ yjcH livJ ymgG ucpA melR cyoE pstA acnA wcaN gpr clpP ridA rrsA ldcC actP osmF truA ftsA araC chaB ybeY yhiI mlaD eutE tadA yafD ysgA rseA ompT yhhT yajO lpp mlc nfsA phnJ intA yebW chrR yceB fucU rrlA atoD yqfA wcaC panE fumC dmsB preA edd rlmE garR nrfC yjcE potF narL pheL ispA hslR mqsR yahN phoQ metE dnaJ eutP ydcY rtcB yciO ompA ymgI insB1 elbB yhbT mokC ygiB ryfA hemB wcaD flu dgcZ norW aidB hipB ymdB araG iscU ybhN paaZ yjbR yeeX agp ogrK eutJ yfbS soxR ybjC katG rstA mscL proP yfhM rrsD bdcA msrA yeeY phnE yeiG yfcH ydcK hslO ushA yqhC rhsC rarA moaC metQ narI bioB yaiO eutQ mtlD hydN yiaY ydeP hycB gltA ivbL pqiC trpA ydhB iscX zapB nrdD sgrR yfdT rob yhhJ yffP fsr yhaL glxR patD matP mgsA dld yebV yccU rnpB nrfB fryC phnI galK lrhA yciS gldA trpB mtlA ybdG aroM csiD ompC yjbQ pgk araH mlaA yhfG frwB gstB iscA rcsC ydcS eda amiD tusE entB frmR tolB ygiN yccX cyoC rnr dhaM ydhQ yfeO exuR bsmA yddH hiuH eutA dinQ ybcM smg clcA yibA livK thiG xylA ilvN gatY pstB ibsD yhiM clpX yqhD ybjD yebF add mngA yqhG hofN nupG lpd yjjY yffQ yiiS pntB pka phnD fabI bioA yeaC ftsH ydiH sufE rcsD fic kptA epd trpC mlaE cheB sgcE aes malI idi pfkA ycfP ybiO arfB hofM rsmJ mlaF nfo yoeB yjcO yfbV insL1 opgB potD ycfQ lpxL anmK hha tdh moeB yeaI rraB csrA ygfB yeaY ychN sgcB fsaB gcvA yghU yqeB sgcA gntU ybiJ lpoB yggL queE ycgY luxS maeB yiiM frsA crp yoaF mdtM astD nrfA dxs ybiU hipA dmsA rem yjhY moaD yihX tisB ybdF xdhC leuD yehD yfcG sufS clpS ykgE yciB ysaB trxC yjaB ybcW yecH wbbL yodB borD tfaS yraR pck sgbH polB nanM dtpB yahG frmB ompR ybaP glgC nirC sdsR ahpF yraP csgB gor agaV yefM mtn dsdA zraR yaiE sgbU marA eutD yfcO aslA ygeV ydfZ intF dmsC ygiV ydjA ssuC btuR maa ilvB ygeW fadM gnd pntA yqiK cheY ybfB xylH feoA cueR fucO crr lspA rpiA allB tas gntX paaY hyi ytfB yicH mntS bfr yieP nagE yihU uxaC ydiR yjgH zur mutH rtcR gltP iscS ymdA mrp fucK ybcV bioD yqhH yceM rbsB nrfD eutT moaE tolC ugpB copA hcp recA dcp leuL yhhW yaeR pphA hmp hscA pal ygfZ csiR rcnB alaA ykfB secA ygeX ycjG ybaA sufC yifB dgoA ybaO chbR nrdR mdaB yeaR entA fdx yhhX galR ubiA ydbC yjbB smf rdlB livH pptA yecA amyA hpf flc greB yjjI mrcB uspC yjiX ypjA yfdQ yhaV ddpX ybdM rpoC fetA alx glnA gltD rsmE yjbD paaX ung sufA iraD rimK chpS lsrG thiS ygiD yfbT rimJ yeeZ phoR pnuC ygeY ubiH ftsE aaeA fdhF hisJ aroH fbaA sufD hofP trxA yfdV tpiA ydaT tauD frdD tusB feoB macA pykF mhpT hflX pepT envY rutG ybdD feoC nagB ndh potI sgbE nuoF cheZ yhaH glcF mhpE yfbU yqeC nrdH kbI ycgI aceE hinT yidH ihfB kdsA ycfL yoaI fieF fucA rhmA acrB macB yihQ srlM rapA yahF*** *ynbA yghG acrS purE valX purK lysV yraI dctR carA ubiX gltV yhdU purM leuZ insF1 dicB asnU pyrD cspA alaE sfmC xanP argC valY malK insA alaX efeU cusB cusF alaT gltT cysT fepE metV yicT flgB yeaP renD purN argY yedV proV yagI lysY cspH yfjU fecR selC fecI bluF fhuF leuT rpsT rttR hisR ymcE metF leuW cspF cysJ ypjC cmtB argQ citD artP argU ycjW yhiD yeiW argX alaV argZ rluC yidD yedA yjdQ hsdS yciW coaA proM cysD fes lysW yecT valT lysZ tyrV lysT cspG gtrA argV purF rhlE cusA suhB glyW lamB folK gtrB ompF queD rnpA yedL yjhZ tyrT cysC evgS ylIE lysQ aroG pawZ waaL metT yahM mcrA sdiA mdtL yiiQ adeP gfcD mreD mreC flhC yibG metU yihG yojI ynjE dusB cvpA glyU yciX napF fepC yfjW ymiA pcnB gltW flhD rtn icdC queA asnV yneE mcrC yacC oppB gcvT intS rpsU citE gfcB yqeJ yfhR valZ aroA ybeT ydiY rlmA argF cysM opgC metW ynaE ygiQ artJ yibQ leuX yfcC pbl mnmA rimO holD ppdD ybaN yeeE cusC tsaB argA intG tmN gfcC cusS gpt ligT ybfP leuE mltD dppB mdtJ serV lpxT yeeD insK mltF dtpD yoaA artM artQ wecA ybjE iclR fecC yfhL ykfK yghF argD glnU dnaB yoeI ydeE waaA rnb ymfD yhbE yncD ydiE pheP gatR nikB essD cysU apt lysA nikA insJ yhdJ argI fliM sthA malE setB potA dbpA rluE ybcK epmB yqcE lpxH udk recG recQ ghxP yfcA thiI rrD metY yciZ yhjV purT rimI argB cysW fliO hisP yneG gnsA azuC pabB yfjV yeiL yjfM hda malM gspE yecJ ydjN cysI gsk casA yciT yahB glnW wecC hflD fis ymfA mglA yegD fliA fecA opgE metN yjhD wecB ycaL rph dtpA ygaQ napD ais rspR rsmG flgC metA rlmG efeB ghoT pdxA proY yijE potB yfaT mrcA tmpR wcaL fepG purR plsY yafT yjiC glyX yeiR nudJ fliF nikC alsR lpxP mepS tusD tdk trmL metZ yehH yhdT cysP ygeQ fecE prs ycfZ entS fabA ecnA rsxD essQ yjjQ ybfE pncB ydaY yfiP nikE tusC ydjX ydfA yqjH cdh yniD ygcB rsxC dacB yahL oppC glpE nrfG yhdE yegK rlmH vsr alr yafK yjcZ yddG ygaH ygeN proW malF trmH yieH ydgC ydbD mrdA idnR ybjS citF bcp yfjH dcm fecD yccE purH fhuD ycdT holE rluB sdhC clcB uhpC dppC cysN glpG gnsB barA yfcJ ydjO yiiG uraA gltU yqiG uhpB hisM rzpD yjjG yadS ydeM trkG ansA ynfG recB cspB galS leuV agrB rrrQ plaP lysP yoaK yfeA ydiL waaC yjfL gspF gsiD gpp yicL ybdO glsB pinQ sbcD ybhA pdxY leuO scpA pepE yjgN ydfU rsfS ymjA yccF mdtI fdhD ampG lacI fepD yheT typA yfjI plsX bglG gfcA hsdM yqeI prfC yggI pyrC brnQ pphB ryhB ebgR citX rzpQ cusR gtrS ynfF adk gspD yhbY zapE yqcC fliH rplU yfdF argS yadV yddA yoaJ ftsP eptC motA hybF serB nudC kch mepA ybhC yoaE yehL yjaA nikD ansP yehA lpxK yggU rseC zapC ynjD rpmH yejE infA hyfA tsaD ybgC arpA panF dosP yncI flgN yheO mreB dinF mltA sbp nikR ynaK yigB argG glyV yidC fepB rlmL rsmF glyY tsaA yjfK oppD recO yfdR ycaQ ynjC dusA yhcC yaiV rimM ydjE ycaD pinR asnS obgE ghoS ydcD ydfK rdgC elaD exbD mltC yheS rlmN yeiS serT rpmB allA purU ybhK dsrA ycgX entD pheV appY lepB thiP intZ ydjL ybgP cobC truC hypT yjjZ priA xseB dppD pyrE thiQ insD1 yjeN yciA tilS yagM cmoB rlmC djlB rbn purL ygjI yaeI yjiH tolR ompG yjiL ychF bdcR ygjQ xseA aat fhuB mioC tonB ybeF wzzE gluQ rnc fhuC flgJ caiC yqeF ymfE amiC hcaT ynjI napA bcsQ ydjY rpsP rffG argH ydiM lomR dnaE ndk ybdL yejM elfD yqeG insZ ydgI slmA secE idnT ydjJ yciH ppx lepA cmoA efeO ribB ygcW hisA mnmG hybG mepH purB yghS feaR prc fumB ydbJ menD yhbU yejF emtA ydfR rhtA yqiH pppA ybfG tolQ yghO envC avtA yqgB dmsD ygaY folB panD menE rsmC rarD ppdA ytfA pitA tmcA yejB sbcC yohO yijF rpsJ rnt insI1 mdtO tsgA uvrC pitB fliL symR prfB mppA proL ilvA can ykfJ hcaE yijO msrC ygcE yggW ygdG yeiB insC1 lolC ygdB ynaI motB radA yghW carB ynfH ybcI yedE potC gpsA exbB fimC nrfF yidZ rplY lpxD aspV thrT yraJ gspC gspA holA yggS ydcP valV napH ydjK plsC ychQ yigG hisF ydjF dnaX murJ gsiA ydiJ ydiV tyrP nanS tnaB rep serC rdlD rplC ispD peaD pssA yibN rne ebgA ycaN rluF tgt cysH yafP rcnA rsxG ycaM uup yraQ ybiR ydcR leuQ pyrF pth ylcI yqgF rssA cadC bcsA fimI yedW hisL sdaB agaC yqiI mltB ilvD ybiT tfaX yjfI ycaO rhsH torY eamA glyT secG yqfE yhbV yliF lysC xerC valW iaaA prmC ogt bamD ampC gspH nudE ychJ lolE nmpC ypjD rho yahE purC yjiK yagB yfhG yccT rplD rpmA yicC yfbO tig argK ppdB msbA aer nudG serU ycgH ygcN yjhR apbE endA fabB ynbE pmrD ycdZ yagN rnlA trmD era ydbL fimD hybE dnaA glrR dinI metB yghR aspT alaW lplT nth gspM rhtC ygaZ rpmG hisG yjeO recR yehI emrK ybeU hybD yecR glpX ydjG modC proQ hisH yeeN gfcE yjcF rsmA yceA yehT hemA ygfF parE ydjH rimP ygcU alsA yjhE pdhR yhcD folC yhiN tyrU yjiG speC ycgG ydhP recC allD djlA yjiM yoeG rnhA ycdU panM mnmC yeaV secD elfC yqaB acrE yjiR yqaA yobF yehU yjiT fliI yjeJ birA murR yegQ yaiL cydD rnd ubiB yfgF ygcP rfbB caiB hisI tatC yccS metG gspL fliG rplW codB nusG oppF ydhK yggT rsxB ybbC ispB purD frvR dnaN aas cysS ybgQ dusC gltX yiiX cspC pgaB ydcZ glyA yiiF parC abgR yaaU pgaC nadD nlpI yeaJ ppk murB hisS yobD pyrH fliN prmB fadL yjbM accB yjbI arpB ytfL accC waaF yjeM nadC yjeT rnhB gyrA caiT ydjZ lpxM mutT cysB csgD yjcC proK mntR ycbZ rng cyaR yjgL acpT lolD degS ydjI btuF fadD yajD yegE leuP alaS ybjJ yfjL pnp cytR mmuP mutY ptrA yddW yecC hisQ menF ascG napG yebB nadB ycjT yggM hyfB caiA pncA cyaY ypdA sfmZ mrdB cbrA ftsB emrE mepM apaH ryjB yhhQ yneJ glmU ytfP ppdC yjfZ yejL rsxA flgD prfH rffH dut glnX agaI chiP folA rlpA coaD truB cmtA yfjD yghQ yhjH serW mglB cysE ampH yhcA ygiS yagU flgM leuU gspB rlmI spoT rplB wecD ygcQ djlC yaiP ilvG metL yzgL kdgT ybjT malG npr hisB yidX gcvR bglH srmB umpH yceK metI ygcO folD ribF pqqL arnA ydfJ phr hypE yhfA yhaJ glnV ettA thyA cmk lnt agaR rpsA***

Table 11.7 Continued…

	fecB nrdA prmA dsbB ddlA ydbA yfgH ybiV fimB yfiN ybhI pbpC hemG yeiP ynfN pta yfiH ycgN
RNM-23	***yhjX asr yibT ybgS pspA pspD pspC ybhG pspB ydgU ibpB pspE ibpA raiA ychH ycaC ynfM ypdJ ygdI pspG ybiH ariR ybhF ldhA ytjA ybfA glgS yahO osmB osmY ynfD osmC yhbO ynfO dps ymdF bssR tqsA fruB yjiY yhhA uspG acs rmf insH1 yneM ypeC ygaU ycfJ yghA hchA ypfM yegP sra loiP ydeI micF glpA galP yqaE yacH ymgC yohC sucD ybhS aroF osmE rutA katE pliG spy ymgA hdhA uspB mliC ytfK uspF yciG ldtE marA yaiY bhsA ygaM puuE puuB wrbA sucB ycgB ilvC fruK yohK gabP soxR sdhB sucC tktB glpB qmcA ybaY ygiW ivy yfeO dacC rpoS yiaG ydcF ybcO betI yccJ betB gabD pstS ybhL sucA yeaQ yodD bdm chaA aldA marB ymgE yohJ talA ggt phnI aldB gstA yjfN glpD adhP ldtC mgtL higA phoP ybjP dcrB gabT patA paoA puuC yebE pgpC fbaB sodB yfiL yagE yajI yohP clpB yeaG yhbW uhpT ybbJ narW ybeL ybeD tyrA yhdV fadE sseA elaB psiF cdd rclA galU cspD glk higB ynfB dhaK uspA glnH yagG paoB mcbA yeaO zraP mlaC narG hspQ yhaK fxsA rutE qorA putA yedP yjdN narU bssS betA rbbA dhaL dgoR yafC ybdR eutK marR yniA uspE curA aceF yjcH narV slyB narY glpF treF ybiC rraA yjbJ ptwF adiY ydcH ygdR norV ldtD hslJ phoH omrA yahK ycgZ gadC csrB yfdY ahr sodC ybdK slt yciF yceI clpA hokD udp ycbJ eutL fucP ecnB actP ybhR ykgE yeaH ftnB lhgO dkgA glpC paoC sdhA melR ryeA degQ gpr patD mgtA yaeH relE csrC mgrB dnaK yhjY narZ araC dinJ ydiZ yagH ybbA gltA yohF eutT mdh lldD hipB csgA yegS grxB trpR yrbL csiE ydeJ otsB yjdJ yggE bolA grxA dgoK omrB gatY ycjX eutC yqjD lolA yddK yacL yccA yphA cfa blc eutR norR dmlA hemL ompX fadH htpX ompC yffR ylil glmZ hcr panE aceA mlaB cyoD otsA nlpE iceT ybhQ yajL cyoE mlaA ydcL yebO fadA ldtA yhbS ydgA yjfY manA trpL bsmA ompR eutB yqjE ygaV ecpA ygaP hslV hycI yibH yagF ompT rpoH yfdC ydhR tpx fruA yfcZ csgB yqjK yahN yhjD yhhJ cbpM sodA dicF shoB ybiJ rutF ybbN rhsO trpE hdeA rof relB ubiF preT icd bglA htpG rob lpxL lgoR yqjG pstC yhjG cpxP yhbT eutH aroL lpp potI ybfC intF ssrA deoC pka norW livJ clsB yhfY yaeP yeaC csiD ybhB ybjQ yhfZ ryfA ucpA agp rstA flu ycjF yidF ypfH nuoK yqhC sufE glpK glnK gltI speG yffQ selD oweE yddH ydcO ygeV pfkB gpmA uspD ybiB ydiQ yjbR gadB deoA pagP zntR ybbP nuoL pfkA ydcY nuoJ yfhM mlc intA yhjE cyoC potF ysgA fucU phoU mqsA yqiK wzb yajQ yceH poxB cbpA yhiI kptA ydgD rutB deoB pepP miaA yjcO yaaX ybaE yhcN ydhB ydeR cbdX moeA ivbL grcA elbB ykgT degP ybhP yffB yfbV yfdV insB1 yegR fryC uspC yjbQ ycjO ypdF paoD rrlD lpd yihQ rspB rclR trpD mgsA dmsA eutD ynfE mscL yibI glsA nlpD ydhS mqsR yfbS rbsD hipA glnP matP sufS fumC ihfA phoB ybfB qorB rnpB uvrB mlaD eutQ gshB ushA frwA mscS eaeH rbsB nuoM deoD ftsZ yciN ydcV dksA hcp nuoI smg srlA yfdQ phoQ yhhN gstB osmF iscU treR feoA eutG yqcA yjaB aaeX ycjM yeiE ynjH rutD sufD yeeX ycjN yqiJ rcsB hdeB dinQ chaB mokC nuoH sgrR yciO leuD galE manX eutN pyrI ibsB ydjA cheR ytfB zitB fsr nfsA ybjD xylB nfuA metQ hiuH ybaT nuoN ycfP yobB garR lpxC yebF ydiT mutH sfsA mdaB pepB slp yjbE glcG cpsG tomB pck nupG rtcR pstA amyA yeeY sbmC msyB psiE mlaE yaiA dhaM hupA ychN manY ygiB glcB cysQ hfq sgcE opgB pgI iscA thrC yciS feoB sufC yihX rstB yfcG rcnB hemB baeS maeB fadB yccX ilvN phnK ybiO dgoA yghU mlaF yccU zraR ymdB glmY prlC asIA bfr ymgI ydbC rutC amiD moeB metE mtlA yjiX hisJ hslU ycfQ fetA ahpF wbbL ybjC rrlC glxR lsrA ygiN prlF ilvB ndh aceK pheL sfmA wbbK yeiG yqhD yajO astC yjdI clpP asnC yqfA epd msrA rhsC yeeR pqiC yciE malT ycik mtlD manZ mokB yhaV ynaJ iscS sgbE fsaB yoeB ryjA paaZ potD lsrD azoR aidB potH php yihO yiiS ydcS dmsB zur wbbJ ymgG yrfF nirB yjjB msrB ygfB sgcA ydeP eutA ftsA ttdR yqjC grpE ybiU ubiA mprA pstB adhE dcp yjhY fiu fucO ydcK mntS rrsH yedY malX rrlH afuC moaB dkgB pgk araH wzc add yjdP lsrK nuoG glcA preA smf pyrB yhaH ygiV yhdN tisB ybeZ paaX nrdR entB yfcH ldtB dld wbbI rraB abgA gadA ridA atpC aceE nfsB yfgO idnD atoD yqjA abgB fnrS chbG yhcO rtcB clpS ymgD sufB csiR yaiO galT afuB dedA kduI marC yceM lon folX gudD yecD yraR pntB erpA yecH yjjI pntA queE betT thrA torR ypjA nanM emrD cueR rseA hsdR frsA mtn cstA ogrK lpoB slyA crp malY yebW clcA ydhQ xylH groL zapB ppsR yicH eutE ybdF fabI yieP iraM sufA rpiA lsrC ghrA cynS cheY glgC glpT hokB argT yceF yncG yhiM eutM yffP ydiO yefM trxC mtfA tolC ybiX amtB yibA mdtM glcF ldcA hofM wcaJ yjjY ompA yjcE potE fic gloA tyrR ynfK leuL acnA mrr yfbR rimJ mall isrC ybhN paaY rssB ygeW exuR tam rbsC frwB kdgR ung dsbA ykfB kefC araF yagA rayT groS yhhT acnB ybdG ribD mak entA ibsD glcE yeeZ yqgA chrR mrp xapA yfbT gltS yiiM recA yjbD tadA nupX emrA ybdD mug appA truA ibsA yihT ybjH ypdE gatZ alaA ldcC uxuR fumA pgi yffL hscA yeeS yffO hmp ycaP yfcO tdcC murQ gnd dcuS ascB ydeO yqhG recT ulaB iscX galK dgcZ ygiD*** *yjdQ purE yraI napH alaX napG insE1 rttR cspA cspH purK napF argU insF1 insA alaE asnU ymcE nrfG carA napA yecT asnV ygeH cspF cspG napD nrfF narI serU purM leuX xanP argY fliP proM cusB napB valX pyrD leuV glyU ppdD rnpA efeU tyrT argZ yidD arpA argQ ynfN ubiX argC metU leuT yfhL yicT bluF tyrV metV yedV leuZ yahM rrlB argV gltT yfhR rhlE ybfP gfcB queD dtpA ynaE yqiI sfmZ lysQ leuW cysT ynbB nrfE ynjE artP yneE narJ yeiW rpsT citX fepE yagI hisR rrrQ lysY gspI yihG lpxP argX mcrA valT metT valV alaW dusB cspB purN azuC yeaP cusF selC sbp opgE cdh yiaW yliE rluC queA ymiA gnsB nikE yadN evgS cysJ purF lysZ essQ lysT ycaL gtrA lysW insJ oppB glyW ybfE yfcC cusC serV ydfU yfjV ycgX intS mreC yciW yibG ygeQ fecI fecR mdtL yqgC yacC hsdS tsaB rtn nikD cysD yahL gltV pinE dctR yciT gtrB mreD ileX adeP gcvT brnQ tusC cusS rrsB ydjN hisI mcrC gltW insK glyY peaD ydiL hyfA rlmG lysV hisF valW cvpA alaV ccmH rlmA mnmA valZ ybeT yedL metY yqeJ epmB hisA metW dbpA mdtJ holD coaA fliM ymgF tusD ybjE dnaB opgC rpsU ydiY apt pinQ ghxP rimI gfcA yfcA cbl valY pcnB rimO leuE yhbE flhC nikC thiI pinR ccmF rph sdiA ygiQ yhjV yneG yqeI yhdJ ygbE lpxH ampG oppC gpt fis gfcC yedA proY aroG nrfD recQ yciX adk ymgJ potA yrhC essD iclR yjfK mdtI ais ydeE citE yhiD uhpC flgB waaL ycfZ ynbA gnsA aroA argF rsmG yghE yfiP yojI yibQ ybdO argA ygeK ydbD yecJ dppB uhpB rrrD yaeI nadB dsbE yjaA serT ydfK yciZ ybaN fecA nikR asnW rzpD ribB metF trmH ispD trmN hisP yncI pheP cysC nrdG rnb yceO waaA yqiG flhD agaI pepE ligT tsgA yafK argW yijE yddA glnU pawZ wecB purT yoaA yliF entD artM tauA fabA wecC ybeF purH folK yhdX rseC yhhH rluB ppbB gatR ykfF rluE dtpD fliA ytfA pabB yeiL glpE fes ymfA oppD yafP gpp fepC pyrE pheV yeiR insZ ftsB hflD ynjD ppdA cusA lpxT glnW yhdU rlmH fecC yheO wecA ffs hypE udk zinT scpA hisH emrY ecnA yfjW proV yjjG ygjI malK yoaJ ryhB yoeI yhdE fecE pncB cysG kdgT artQ yggI ygaY yfbO ebgA entS rsmF fhuB uraA icdC caiT dusC yegD yqjH mltC yoaK mrdA rsfS xseB ybgC pdxA rnc yghS yqiH citF yhjH nikB gluQ tsaD rlmC ybcK rsxC yigB dsrA tdk mltD lysA prmC tilS yoaE yjeO ydaF ycjW yqcC yeeE rplU argD ycaQ fhuF ansP ynjI alsR recG yigG codB yciH amiC yhdW menE ynjC plsX proL fhuD fau infA atpI yiiQ yqgB ychQ thrW flgD potB gsk tauB recO yfcJ purR serB argI ghoT yeeL acpT zapE suhB tmcA cysM alr prs yghG rrlG artJ serW nrdA yraJ hsdM nudJ hybG hybD cobC glsB hybF fimC metZ yadS ygaH yfeA yiiG yjiL gtrS ycaD yccF cspI gpsA tolQ ydgI yggW mnmG tfaX flgC yncD setB yjeT hypT rep gspE flgJ yagM ygeI mepS ymjA vsr yeiS nudC mutY mltA pheU yjiR ampC tsaA lacI bcp xerC yjiK endA yhbY ydiE elfD rclB yhdT insC1 fliO yahB rpmH yciA fecD ymfD gltU argG dacB oppF pyrF leuU gspC hybE yjiH truC ydjO ydcD mrcA rspR rsxA yobH hcaT dppC yegK yjeN yheT fepG tdcA ghoS thiP ebgR rlmN avtA ylbI flgF yjiM yjhD prfB dinF rimP rpmB rsmC yegQ rnlB lysP ccmE ppdB secE wcaM mreB ycgH prc flgE tolR mioC renD tyrU yeeD yidX fdhD mepA lomR ygaQ hypD asnS ompF ompN galS ydfJ rffG cysI rdlC rlmL dusA priA ygaZ mltF kch nikA yahE hda flhB argB yohO lpxK mntP fimI yheS yjgZ ykfJ eamA dnaG recB obgE rho yibN gntU hisM trmL panF ygcB yjeM yidC fliN ybcI pppA glpX typA yghR aspV sokC insI1 ftsP torY waaC ydcZ fliI efeB rnlA pyrC dcm appY hofC lnt murJ clcB tmpR cadC ybhC cysN cmoA idnK secG cmoB yjeV hcaE gspD rnt agrB arpB proK era hybC ccmB yjhR prfC npr lepA folC nrdD fliL metA fadL nrdF yghQ yhcC yhcA ybhK yhfL ydhK yidZ ndk yjfI dnaA eptB yecF narH parE ansA glyA rcnA trkG acrE msrC yieH ydhP yqfE ydjJ glyX yafT yfbM intZ plaP hisB yaaU yhbV pdhR yiaV ytfI purC purL dnaE yejF rpmA ybjS fpr casA efeO mltB nrdE ycdT gspM fliH yfjU rsmA dnaX fliF rsxD yjcZ cusR yehA bdcR ygjQ rpsJ yeiB smmB rdgC ycdZ yjgL ycdU cmk rsxB yebB emtA uvrC cysU rhsH pdxY rimM cysE yjjJ pbpG ynfG mmuP thrT dnaN sfsB exoD yhiN ygfF fdnG plsY ydcR glpG leuO accB leuQ fldA cbrB proQ fhuC lipB rhsB rluF rhtC degS nepI wzzE lysC yfiM ilvD yqgF rne yeiI lepB gspA nadA rplC pth purU sbcD argS elaD ygdB aat intR yehB mtr fliG ydfR pssA apaH ttdA recF yggU yfdF yhcD eptC yhaI lpxD ydiJ pitA tyrP rdlD nirC wcaL rhtA ygiL yiaY yafO recR yihF bamD ypdK yfjH yjaH atpB envC glyT aas ybgP dppD proW tonB yjjQ rpsP yraQ radA fadD cysW nusG ydgC nanS metN rlmI ybiT ynbE rnhB yeaJ purB spoT yibB yzgL yfhG yehH yfiE fimB yfbN rpmG yicG ycgV rplD gfcD can ydjE yedE yhbU nudE fldB accC nsrR yicC yccS ydaY ycgN fabB yjfJ motA tig yggT yrdF recC idnT hisQ lolC rnd yijF yejE yghF ccmD potC yaeF gsiD tgt yniD ygeN leuP ogt birA yejM ycbU ynfH uof lplT nusA pgaC metG yhcG yjaZ ubiB pitB flk ydiV cysP purD entC ispF prfA rpoA ycaO yoeG sthA rlmF ppx ybfG ccmC yfjI gcvP plsC hypC yfaE wecD dmsD feaR epmC ytfP yfgH yfcL flgN hemA yggS ygdQ ypjD menD codA xseA yehI panD yfaA yagB rplQ holE pta yebQ nlpI yehL fliZ rdgB ydbJ btuF pyrH thrU chiP rpsK glyV ygjV yqcE ribF yfiH napC holA ugpA truB modC yjeJ rplW lysS rseB glrR yedW ygdG yqiA thiQ ydjZ rssA ydaG tatC fecB serC yijO yjcB ydfO nadD sibD miaB ispB yfaT ydjY yadD tauC lolE ycbL pnp gspF gadE*</td>

Notes: Differentially upregulated genes correspond to genes with logFC ≥ 1.0 and pvalue <0.05, while diffferntially downregulated genes correspond to genes with logFC ≥ 1.0 and p-value <0.05. Text in bold italic were differentially upregulated genes, while the text in regular italics were differentially down regulated genes.

11.6 BMA production calibration standards

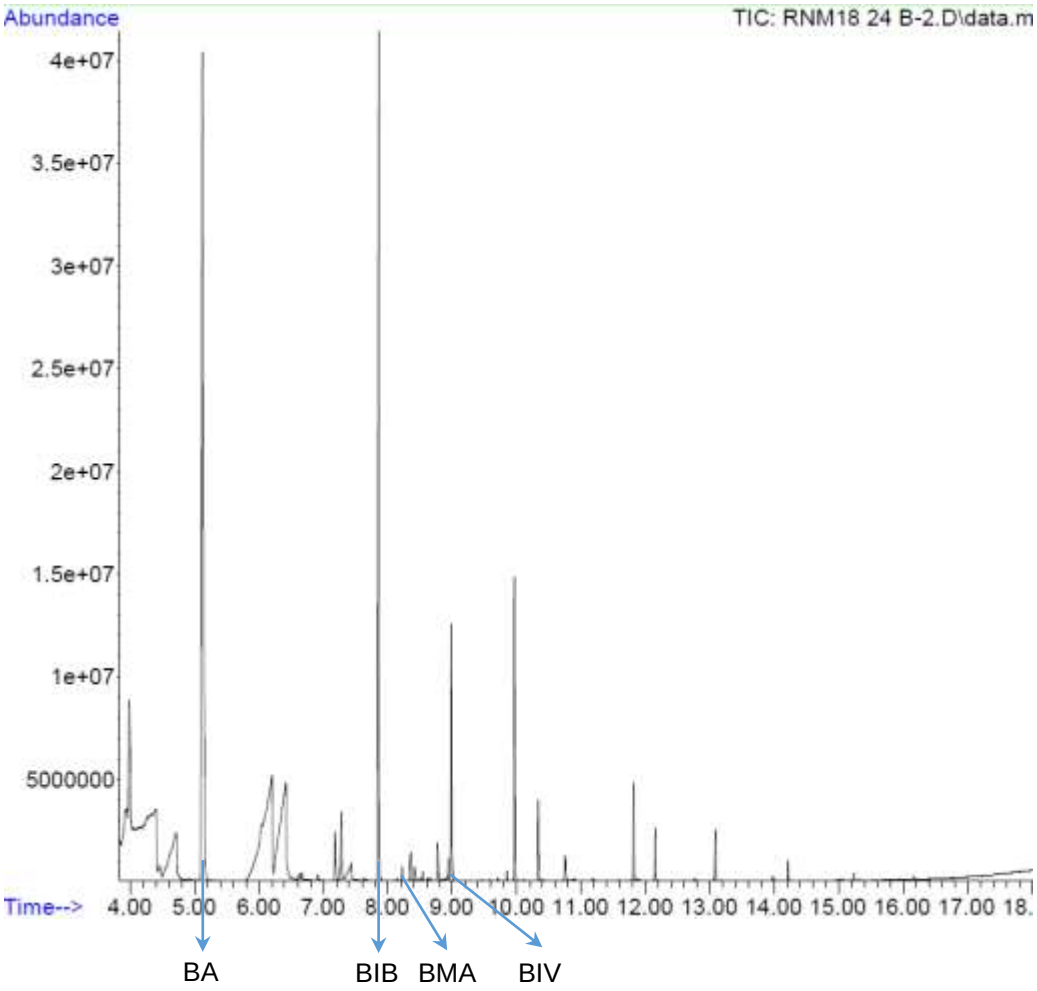

Figure 11.7 A sample chromatograph for BMA production.

Legend: BA = butyl acetate, BIB = butyl isobutyrate, BMA = butyl methacrylate, BIV = butyl isovalerate

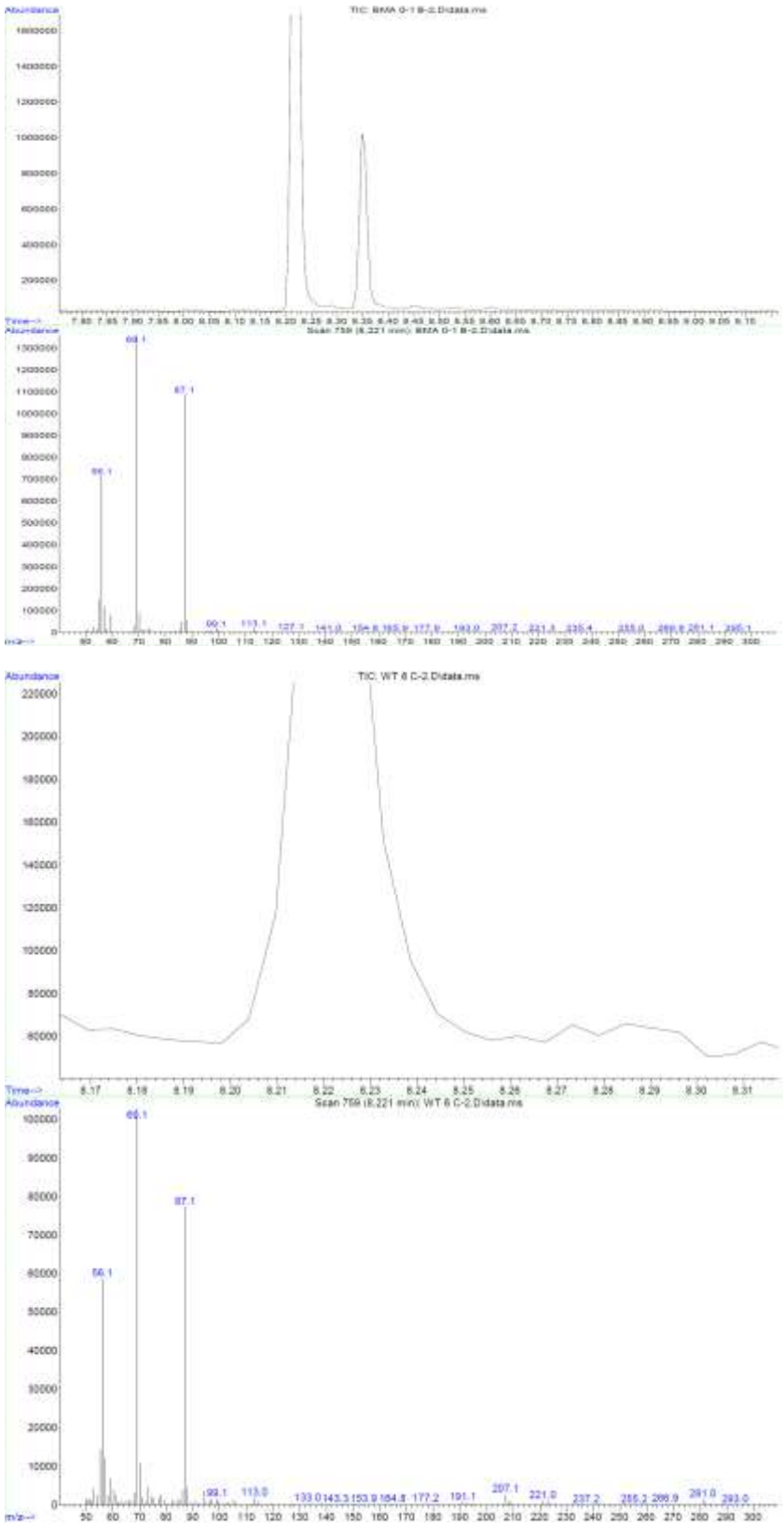

Figure 11.8 Chromatograph and spectra of butyl methacrylate. (A) sample (B) standard

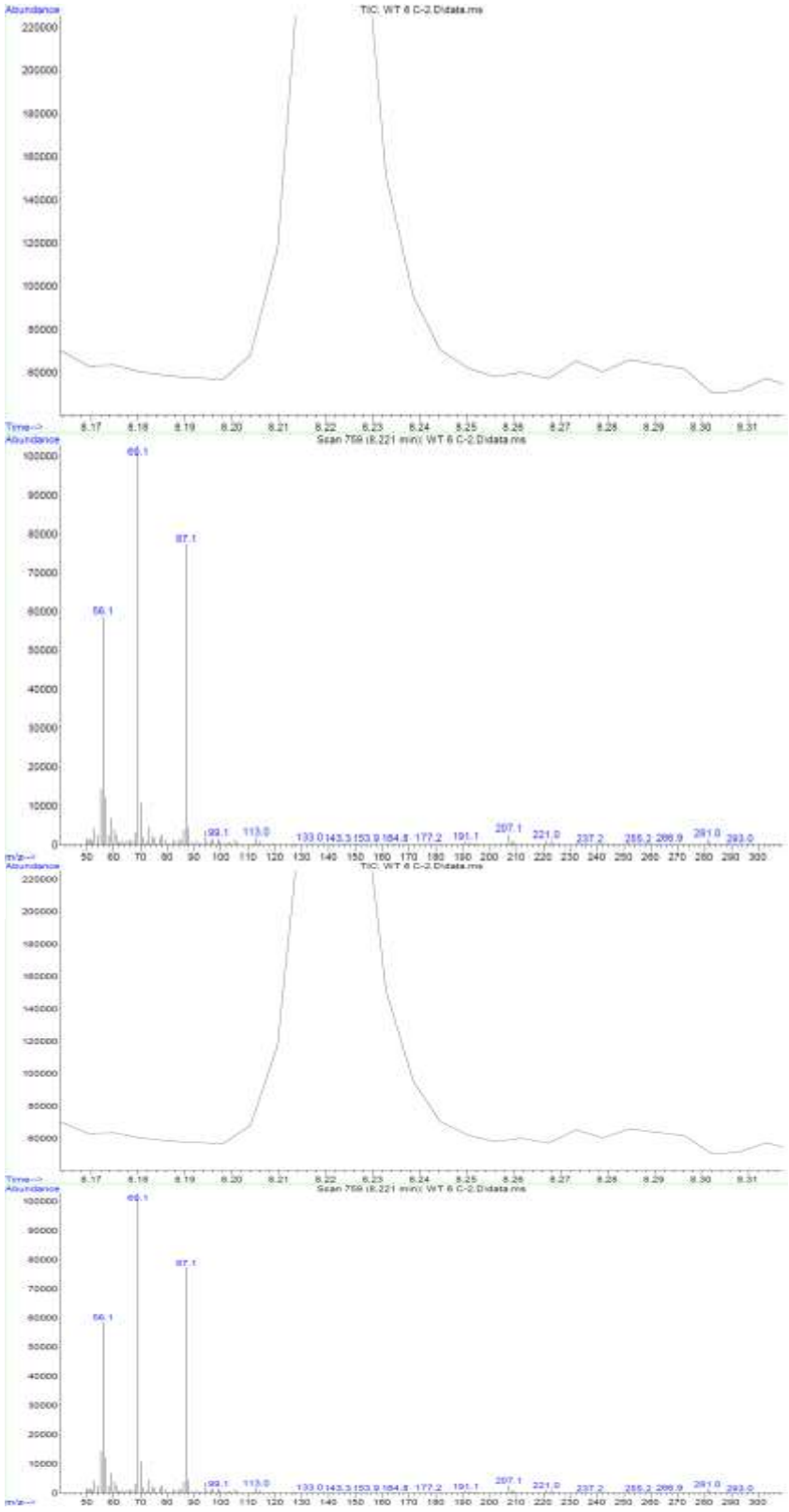

Figure 11.9 Chromatograph and spectra of butyl acetate. (A) sample (B) standard

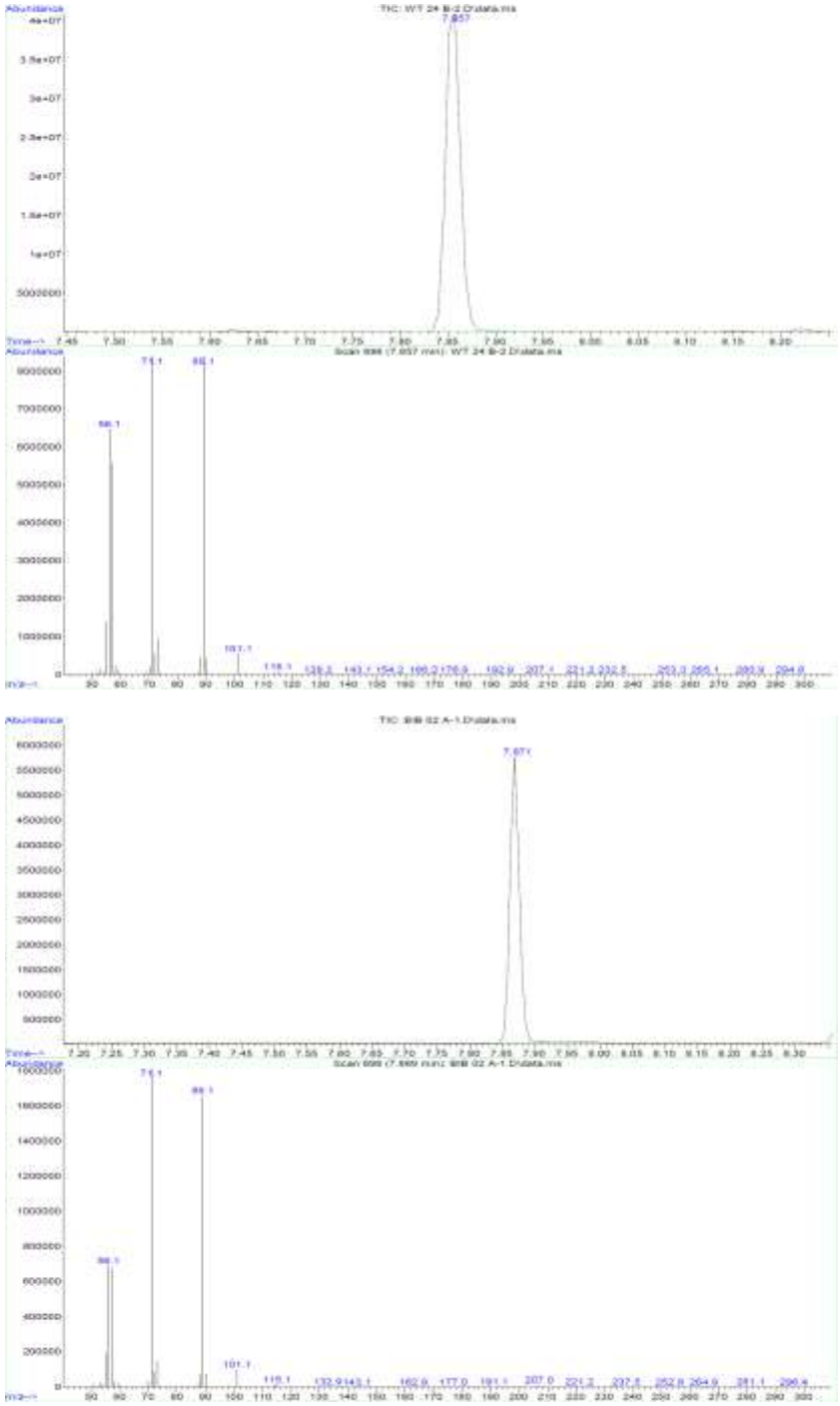

Figure 11.10 Chromatograph and spectra of butyl isobutyrate. (A) sample (B) standard

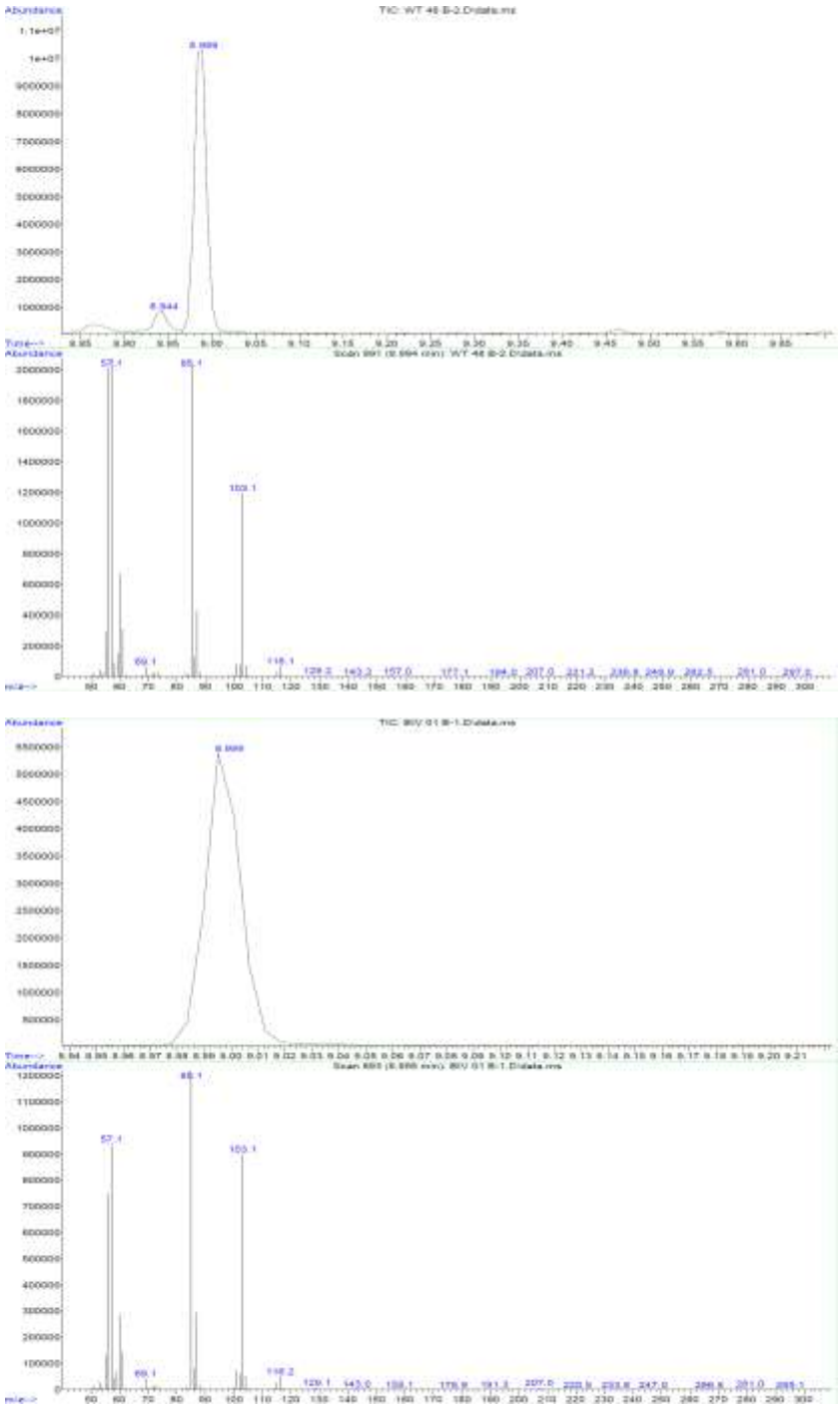

Figure 11.11 Chromatograph and spectra of butyl isovalerate. (A) sample (B) standard

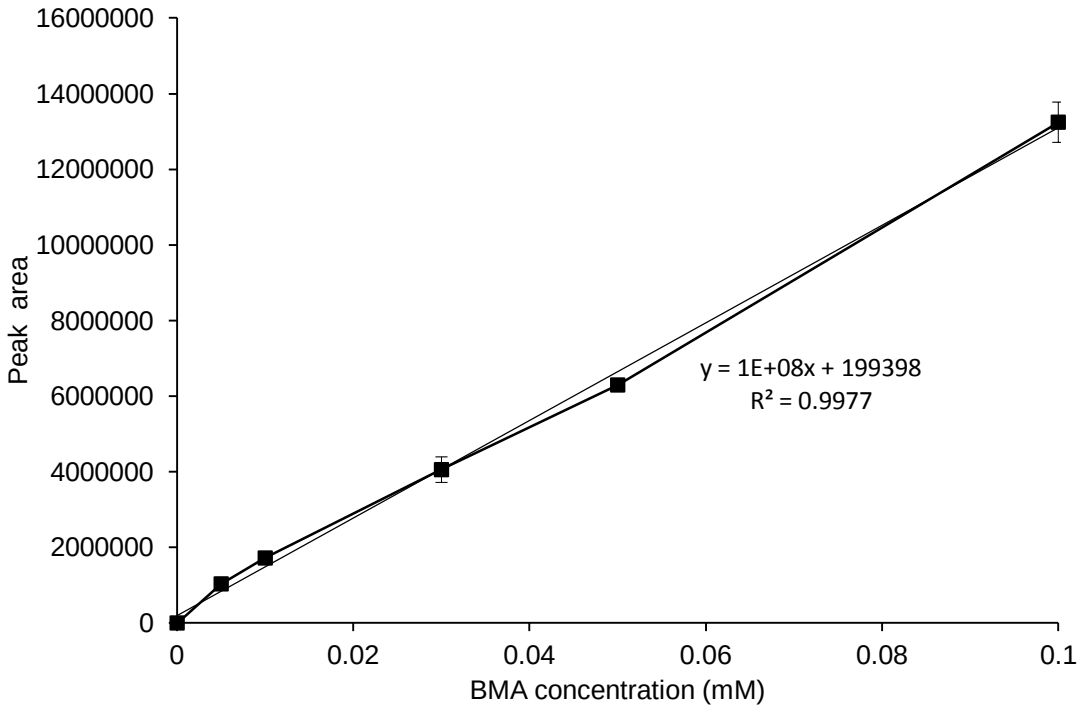

Figure 11.12 Standard curve for butyl methacrylate (BMA).

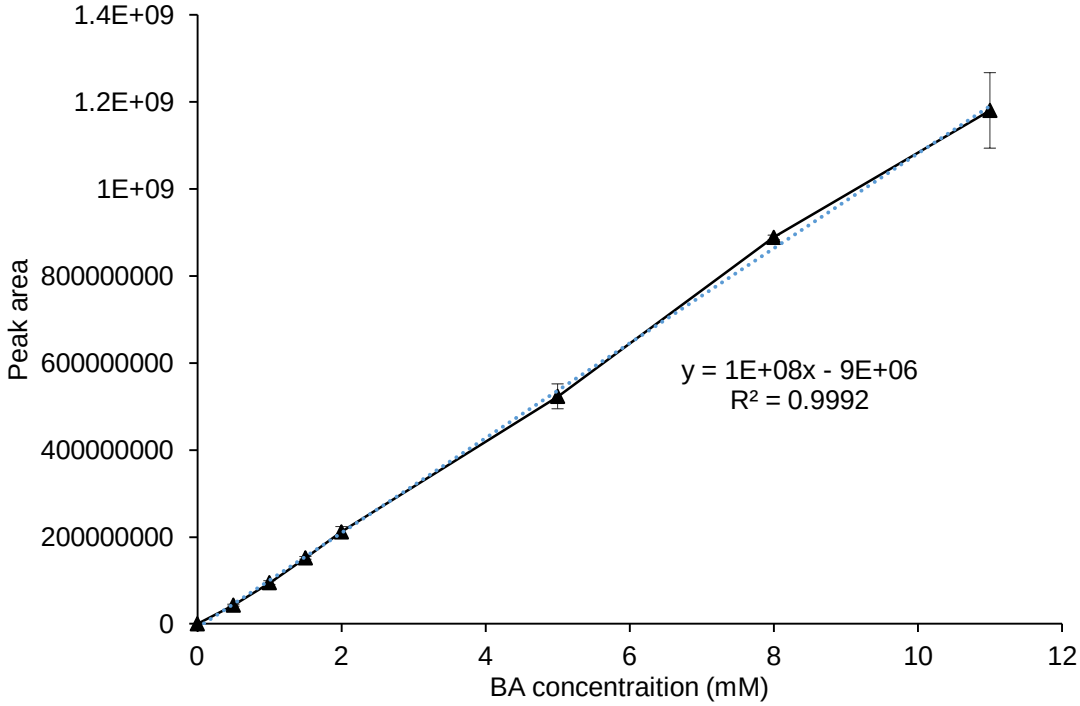

Figure 11.13 Standard curve for butyl acetate (BA).

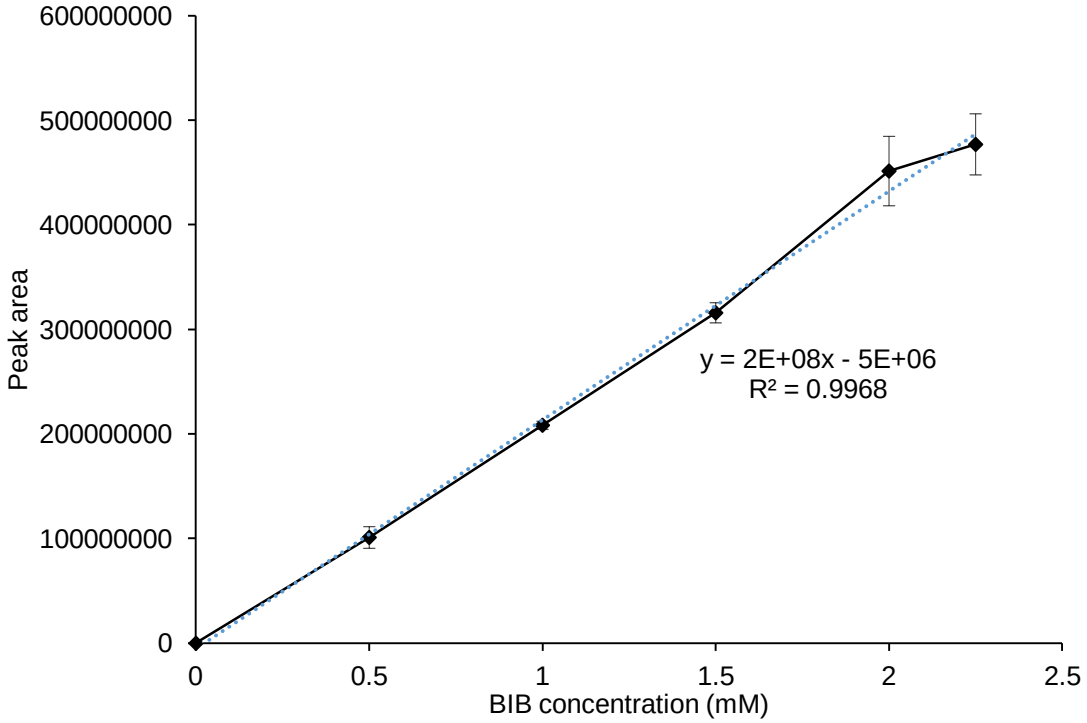

Figure 11.14 Standard curve for butyl isobutyrate (BIB).

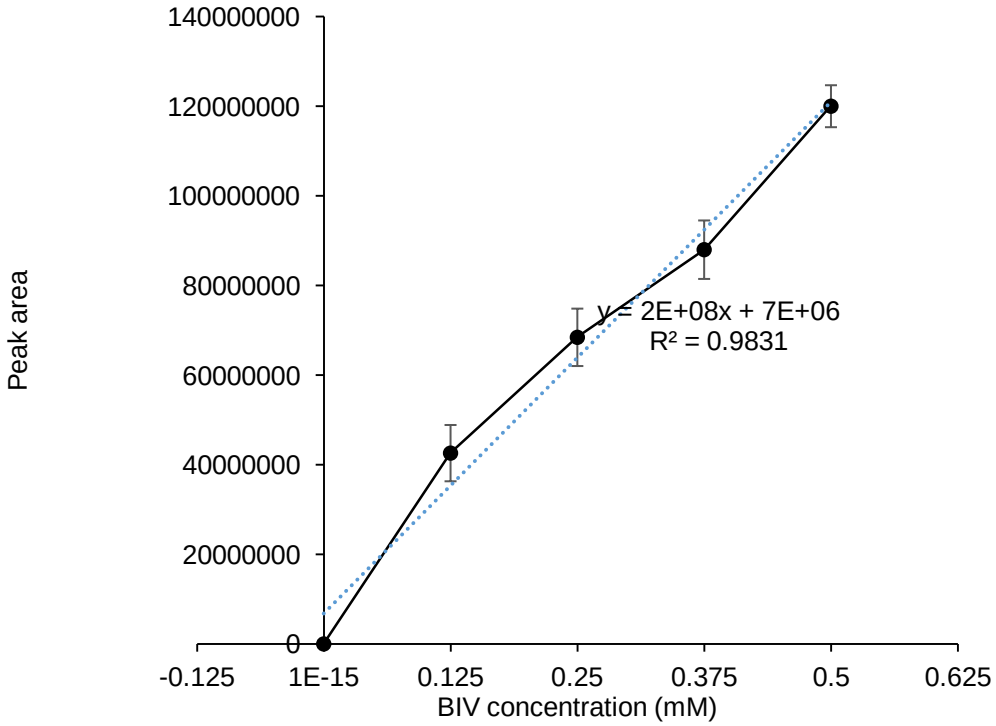

Figure 11.15 Standard curve for butyl isovalerate (BIV).

11.7 Growth of *E. coli* with butyl isobutyrate

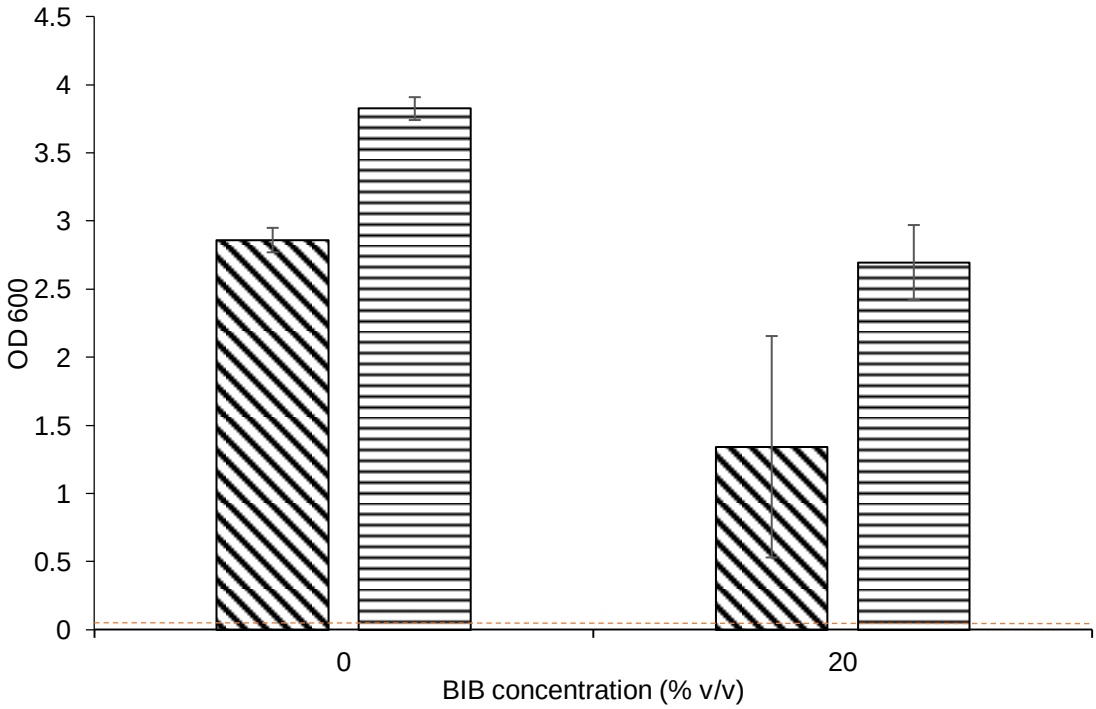

Figure 11.16 Test for growth of *E. coli* strains in butyl isobutyrate (BIB). Cultures were grown for 24 h in M9 minimal medium (5 mL) supplemented with 10 g/L glucose at 37°C and 200 RPM in 30 mL sealed glass vials containing n-butanol at a starting OD600 of 0.05 (dashed line). OD600 after 36 h is indicated in the figure. Legend: *E. coli* BW25113-WT (diagonal bars) and RNM-18 (horizontal bars).