Alkaliphilic Life

Adaptation strategies by Caldalkalibacillus thermarum

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Now what?

Bloat in Pixar's Finding Nemo

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SUMMARY

Alkaliphiles thrive in environments with a pH of 8.5 or above, while maintaining an internal pH closer to neutral. Thus, alkaliphilic microorganisms have a proton gradient inverted with respect to the normal orientation. Intuitively, this would nullify the potential to generate energy *via* respiration with regularly oriented respiratory chains that rely on proton-coupled ATP synthases. Yet, alkaliphilic respiratory chains are oriented traditionally and are actively used. The question therefore is how they are able to create conditions conducive to such behaviour. In addition, attempts to answer that question will hopefully also clarify how alkaliphiles acidify their cytoplasm with respect to the exterior milieu in the first place. This thesis details methods required to study these questions and provides some answers regarding alkaliphilic life. This thesis focuses on a single category of alkaliphiles: the low-salt gram positive alkaliphiles. These microbes have just a single membrane, the proteins therein, and a cell wall to generate conditions suitable for energy generation and other transport mechanisms. In short, it can be regarded as the most basic system to study an alkaline, or basic, problem.

For the study of alkaliphiles, three components are of particular interest: the cell wall, the membranes and the proteins therein. For the first, the cell wall, acidic residues are purported to help alkaliphilic bacteria facilitate local, favourable conditions in the periplasmic space. Though studying the exact mechanism of how it generates local favourable conditions is still required, study of the cell wall falls outside of the scope of this thesis. To drive respiration in alkaliphiles, minimizing proton leakage is crucial. In assessments of the second component, the lipid membrane, only a single observation regarding its observation has stood the test of time. Two studies identified high quantities of neutral lipids in alkaliphiles, positioned in the centre of the lipid bilayer. Due to that orientation, the elevated abundance of neutral lipids served as an insulation layer, thereby further minimizing proton leakage. The third domain of interest is the study of the proteins required to drive respiration and the proteins concerning pH homeostasis. In respiration, a series of proton exporting reactions generates a gradient of the lipids membrane. The energy potential of that gradient is then harvested by the ATP synthase, whose proton capturing domain is specifically adapted to alkaline environments. Because of the regular functioning of the respiratory chain, we state that a localised conducive proton gradient must exist in alkaliphiles. Aside from respiration, pH homeostasis, and in line with that homeostasis of other ions like sodium, are of huge interest in alkaliphilic microbiology. This thesis will cover adaptations of the lipid membrane in increasingly alkaline conditions and additionally extensively elaborate on

alkaliphilic proteomics.

This study revolves around the thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1, whose genome was fully sequenced in this thesis, see **Chapter 2**. *C. thermarum* TA2.A1 is a facultative alkaliphile, meaning that whilst it is capable of growing below pH 8.5, it grows optimally above pH 8.5. In fact the full pH range suitable for cultivation is 7.5 – 11, as discovered in chemostat cultivations in **Chapter 3** of this thesis. In that chapter, we analysed the lipid composition over this range. We did this, because we hypothesized that *C. thermarum* TA2.A1 must have a different method than other alkaliphiles to limit proton leakage, as it is incapable of producing neutral lipids. That statement foregoes the chemical properties of the electron mediator in respiration: menaquinone. Interestingly, **Chapter 3** showed that *C. thermarum* TA2.A1 opts to increase the production of this lipid to replace the insulation role of more traditional neutral lipids. Additionally, the organism produced a three legged lipid, as opposed to the often observed two- or four-legged lipids. This lipid, an acylphosphatidylglycerol, lowered the surface area of the membrane and thus decreases the area through which proton can leak. This thesis is the first report of such a dual mechanism.

The final two chapters of this thesis deal with proteomics. **Chapter 4** focussed on attempting to detect as many membrane proteins as possible. Membrane proteins are especially challenging to detect, as they are more hydrophobic that soluble proteins and due to the fact that they are stuck in the membrane. in **Chapter 4**, we succeeded in detecting most known transporters in the membrane in addition to a complete respiratory chain. This result was achieved by combining whole cell proteomics with a pipeline in which the membrane fraction was separately extracted. These membrane extracts were then solubilized with either SDS or FOS-choline-12 at 65°C or 80°C. The combination of all methods led to the detection of a quarter of entire hypothetical membrane proteome. **Chapter 5** again used chemostat cultivations, this time with varying levels of oxygen available to the cell. Whole cell proteomics then identified intriguing changes within the respiratory chain. At the highest oxygen level, the most efficient terminal oxidase was most abundant (Cyt. aa_3). In the intermediate levels, another terminal oxidase took over (Cyt. ba_3), but that tailed of in the lowest level. Another terminal oxidase was not detected and it is still up for debate whether that would be expected. Finally, this study also observed a decrease in the Mrp H^+:Na^+ antiporter, an enzyme considered crucial for alkaliphiles. We hypothesized that its primary contribution to alkaliphiles is in fact to facilitate sodium homeostasis and not necessarily proton homeostasis. Its lowered abundance was then due to the increased acetate production when oxygen was limiting, and that was also exported in tandem with sodium. While falling short of painting a complete picture, the work in this thesis shows new behaviour in alkaliphiles and teaches us that we are far from understanding their complete physiology.

SAMENVATTING

Alkalifiele microben gedijen in milieus met een pH van 8.5 of hoger. Aangezien deze alkalifielen een zo goed als neutrale interne pH hebben, is de protonengradiënt omgekeerd ten aanzien van de reguliere oriëntatie. De verwachting is dan dat alkalifielen geen energie kunnen genereren met een normaal georiënteerde, proton-gekoppelde ademhalingsketen, maar niets is minder waar. De normaal georiënteerde ademhalingsketens van alkalifiele organismen zijn wel degelijk een actief onderdeel van de energiehuishouding. De fundamentele vraag is dus hoe alkalifielen omstandigheden kunnen creëren waaronder zij deze energie genereren. Of meer algemeen: hoe (over-)leven alkalifiele organismen? Bij het beantwoorden van deze fundamentele vraag, wordt ook verduidelijkt hoe alkalifiele bacteriën überhaupt hun cytoplasma verzuren ten opzichte van de externe omgeving. In dit proefschrift worden de benodigde methoden besproken die zijn gebruikt bij het onderzoeken en beantwoorden van de genoemde vragen. Dit proefschrift richt zich op een enkele categorie van alkalifiele bacteriën: de grampositieve alkalifiele bacteriën komend uit een milieu met een lage zoutconcentratie. Deze micro-organismen hebben slechts één membraan, de daarin aanwezige eiwitten, en de celwand om omstandigheden te genereren die geschikt zijn voor energieopwekking en om andere transportsystemen draaiende te houden. Kortom, dit kan worden beschouwd als het meest eenvoudige systeem om fundamentele vraagstukken over het (over-)leven van alkalifiele microben te bestuderen.

Bij het bestuderen van alkalifielen zijn drie componenten van de cel interessant: de celwand, het celmembraan en de eiwitten in het celmembraan. Zo helpen zure groepen bij de celwand de alkalifiele bacteriën om lokaal gunstige condities te creëren in de quasi-periplasmatische ruimte. Hoewel het exacte mechanisme waarmee deze condities tot stand komen nog onduidelijk is, is in dit proefschrift de keuze gemaakt om verder onderzoek van de celwand buiten beschouwing te laten. Om oxidatieve fosforylering in alkalifiele organismen mogelijk te maken, is het cruciaal om lekkage van protonen door het celmembraan zoveel mogelijk te voorkomen. De samenstelling van het celmembraam bij alkalifiele bacteriën, het tweede interessante component, is meermaals onderzocht, maar slechts één observatie heeft de tand des tijds doorstaan. Twee onafhankelijke studies vonden grote hoeveelheden neutrale lipiden in alkalifiele bacteriën, die zich midden in het celmembraan bevinden. Door op deze wijze georiënteerd te zijn, vormen de neutrale lipiden als een isolatielaag. De twee studies concludeerden dat hierdoor de lekkage van protonen wordt geminimaliseerd. Het derde component betreft de eiwitten in het celmembraam. Interessant zijn de eiwitten die nodig zijn voor

de ademhalingsketen en de eiwitten die betrekking hebben op pH-homeostase. Tijdens oxidatieve fosforylering zorgt een reeks protonen-exporterende reacties voor een gradiënt van protonen over het celmembraan. Het uit dat gradiënt resulterende energiepotentieel wordt vervolgens door de ATP-synthase omgezet in ATP, de universele energiedrager. In alkalifiele micro-organismen is het domein verantwoordelijk voor het binden van protonen aangepast aan alkalische omgevingen. Omdat ademhalingsketens in alkalifielen normaal functioneren, stellen we dat er een gelokaliseerde gunstige protongradiënt *moet* bestaan in alkalifiele bacteriën. Naast oxidatieve fosforylering zijn pH-homeostase en homeostase van andere ionen, zoals natrium, van groot belang in de alkalifiele microbiologie. Dit proefschrift zal aanpassingen van het celmembraan in steeds alkalischere omstandigheden bestuderen en daarnaast uitgebreid ingaan op de proteomica van alkalifiele organismen.

Dit onderzoek draait om de thermoalkalifiele bacterie *Caldalkalibacillus thermarum* TA2.A1, waarvan de volledige sequentie van genoom in deze proefschrift is bepaald (**Hoofdstuk 2**). *C. thermarum* TA2.A1 is een facultatief alkalifiele bacterie. Dit betekent dat deze bacteriën in staat zijn om te groeien bij een pH lager dan 8,5, maar optimaal groeien boven een pH van 8,5. Voor *C. thermarum* TA2.A1 valt het volledige pH-bereik geschikt voor kweek tussen 7,5 en 11, welke bepaald is in chemostaat-kweken (**Hoofdstuk 3**). In **Hoofdstuk 3** analyseerden we de samenstelling van lipiden in elke conditie van dit pH-bereik. We deden dit omdat we aannamen dat *C. thermarum* TA2.A1 een andere methode moet hebben dan andere alkalifiele bacteriën om protonenlekkage te beperken, aangezien het niet in staat is om neutrale lipiden te produceren. Deze stelling gaat echter voorbij aan de chemische eigenschappen van de elektronenbemiddelaar in de ademhaling: menachinon. Interessant genoeg toonde **Hoofdstuk 3** aan dat *C. thermarum* TA2.A1 ervoor kiest om de productie van menachinon te verhogen om de isolerende rol van meer traditionele neutrale lipiden te vervangen. Bovendien produceerde het organisme een driepotige-lipide, als aanvulling op de vaker waargenomen twee- of vierpotige lipiden. Dit lipide, een acylfosfatidylglycerol, verminderde het oppervlak van het membraan en verkleinde daarmee het gebied waarlangs protonen konden lekken. Dit proefschrift is het eerste verslag van een dergelijk dubbel mechanisme.

De laatste twee experimentele hoofdstukken van dit proefschrift gaan over proteomica. **Hoofdstuk 4** richtte zich op het maximaliseren van de detectie van membraaneiwitten. Membraaneiwitten zijn bijzonder moeilijk te detecteren, omdat ze hydrofober zijn dan intracellulaire, meer hydrofiele eiwitten, en omdat ze vastzitten aan of in het membraan. In **Hoofdstuk 4** slaagden we erin om de meeste van de voor ons bekende transporteiwitten in het membraan te detecteren, naast een complete ademhalingsketen. Dit resultaat werd bereikt door proteomica van de hele cel te combineren met een protocol waarbij de membraanfractie afzonderlijk werd geëxtraheerd. De membraanextracten werden vervolgens opgelost met behulp van de detergens SDS of FOS-choline-12 bij 65°C of 80°C. De combina-

tie van al deze methoden leidde tot de detectie van een kwart van het volledige hypothetische membraan-proteoom. **Hoofdstuk 5** maakte opnieuw gebruik van kweken in chemostaat. In dit hoofdstuk varieerden we zuurstofbeschikbaarheid voor de cel. Proteomica van de hele cel resulteerde in de identificatie van interessante veranderingen binnen de ademhalingsketen. Bij het hoogste zuurstofniveau was de meest efficiënte terminale oxidase het meest overvloedig aanwezig (Cyt. aa_3). Bij de tussenliggende niveaus nam een andere terminale oxidase het over (Cyt. ba_3). Echter, ook daarvan nam de aanwezigheid af bij het laagste zuurstofniveau. Een andere terminale oxidase werd niet gedetecteerd en het is nog steeds onderwerp van discussie of dat te verwachten zou zijn. Ten slotte observeerde deze studie ook een afname van de Mrp H^+:Na^+ antiporter, een enzym dat als cruciaal wordt beschouwd voor alkalifiele bacteriën. We vermoedden dat de belangrijkste bijdrage van dit enzym aan alkalifiele bacteriën is om natriumhomeostase te reguleren, in plaats van protonenhomeostase. De lagere aanwezigheid van Mrp was dan te wijten aan de toegenomen productie van acetaat bij toenemende zuurstoflimitatie, hetgeen in combinatie met natrium wordt geëxporteerd. Hoewel de hierboven beschreven observaties en conclusies nog niet een volledig beeld schetsten, beschrijft het werk in dit proefschrift nieuw gedrag van alkalifiele bacteriën en leert ons dat we verre van volledig begrip hebben van hun fysiologie.

1

INTRODUCTION

Samuel I. de Jong

1

1.1. THE BASICS OF BEING AN ALKALIPHILE

Alkaliphile (noun) – al·ka·li·phile| /ˈæl·kə·li·faɪl/ - from the Arabic al-qalī (القلوي) meaning “calcinated ashes”, and from the Ancient Greek philiā (φιλία), meaning “love” or “loving” – an organism that prefers alkaline environments. Theoretically, the presence of a high amount of alkali metals (e.g. sodium and potassium) or of alkali earth metals (e.g. calcium and magnesium), or of a combination of both might cause an environment to become alkaline. This statement foregoes the physicochemical properties of the individual ions. An alkaline habitat can be for instance be calcium-based due to a formation of $Ca(OH)_2$ during leaching of serpentine rocks. As soon as this water comes into contact with air however, it is neutralized by atmospheric CO_2, which precipitates with calcium to form $CaCO_3$. The only stable highly alkaline surface habitat are so called soda lakes which sodium carbonate/bicarbonate brines exists in a dynamic equilibrium with the atmospheric CO_2 and have a pH values from 9 and up to 11 [1]. An example of a niche alkaline environment is the KOH based milieu in the gut of termites [2], another is where urea degradation to NH_3OH is responsible for the high pH [3, 4]. In spite of the etymology, a non-alkali (earth) metal environment with a high pH is also called an alkaline environment, see the NH_3OH environment described. Theis discrepancy receives little attention in the literature of microorganisms living at high pH, called alkaliphiles. This is partly due to the fact that soda lakes are the predominant stable naturally occurring high pH environment caused by inherent high alkalinity levels or by high alkalinity levels induced by water evaporation. Additionally, any alkaliphile not originating from a soda lake is likely still an organism adapted from a soda lake ancestor [5, 6].

To our knowledge, first published report of an alkaliphilic bacterium is that of *Alkalihalobacillus alcalophilus* (formerly *Bacillus alcalophilus*) back before World War II [7]. Undoubtably hindered by worldwide developments, the field of alkaliphiles was only truly founded over twenty years later by Koki Horikoshi. His initial work in the field of alkaliphiles revolved around the endo-1-3-β-glucanase of *Niallia circulans* (formerly *Bacillus circulans*), which lyses *Aspergillus oryzae* in sake fermentation [8–10]. From those initial discoveries, the field of alkaliphiles diverged into two categories: firstly the use of alkaliphilic enzymes in biotechnological applications, most notably in laundry detergent [11]; secondly the H_2S removal from bio- and industrial off-gases (Thiopaq) [12]; thirdly the fundamental study of alkaliphilic microbes, including functional diversity, ecology and bioenergetic adaptation mechanisms. This thesis falls within the latter category.

In the microbiological definition, an organism growing optimally at pH of 8.5 or above is usually considered as an alkaliphile. Alkaliphiles can be subcategorized into facultative or obligate alkaliphiles. Obligate alkaliphiles grow *exclusively* above pH 7.5-8, whilst facultative alkaliphiles grow *optimally* at pH 8.5 or above but still are capable of moderate growth at pH-neutral conditions [13]. No lower pH limit for facultative alkaliphiles has been defined. Empirical observations dictate what is considered the upper limit conducive for

growth. Currently, the record pH from which growing microbes were isolated pH 13.2 [14]. This value is close to the limit suitable for survival, considering that a study of a hyperalkaline lake in Poland (pH_{max} = 13.3) detected no life, in spite of reducing conditions [15]. Managing to isolate bacteria from a sample with such a pH says nothing about stable growth at that pH, considering bacteria can sporulate. Proof of stable growth at pH 13.2 is lacking. The highest pH at which microbes stable grew in a chemostat is 11.4 observed in *Alkalihalophilus pseudofirmus* (formerly *Bacillus* (*pseudo-*)*firmus*) [16]. What we can conclude is that at some point, the alkaliphilic challenge becomes too great.

Alkaliphiles are present in all domains of life, though eukaryotic alkaliphiles are rare [4, 17]. Due to the fact that alkaline conditions are often due to high concentrations of sodium, alkaliphiles can also be halophiles [18]. Haloalkaliphiles often differ intracellularly from neutrophilic organisms as they resort to methods such as the salt-in strategy or the use of compatible solutes [19]. Since these bacteria are well studied, this thesis will focus on the adaptations of low-salt alkaliphiles, specifically low-salt alkaliphilic bacteria. Within the bacterial domain, alkaliphiles can be either gram-positive or gram-negative. Gram negatives have the advantage of modulating their periplasmic pH, where gram positives do not, as they have only a single membrane. How gram-positive alkaliphiles manage to survive, and even respire, is therefore still not fully understood. Furthering the understanding of this question is the central focus of this thesis. The rest of this introduction will focus on introducing the various challenges that alkaliphilic gram positives deal with. For simplicity, the term 'alkaliphile' refers to gram-positive alkaliphiles for the remainder of the thesis, unless otherwise specified.

1.2. ENERGY GENERATION IN ALKALIPHILES

Life generates ATP, the universal energy currency, *via* two distinct methods: substrate-level phosphorylation and oxidative phosphorylation. During substrate-level phosphorylation, the energy to produce ATP is typically directly harvested from a certain reaction, e.g. phosphoenolpyruvate to pyruvate. For oxidative phosphorylation, as dictated by the chemiosmotic theory [20, 21], ATP is generated by the ATP synthase, an enzyme capable of utilizing the transmembrane potential [22, 23]. The transmembrane potential is a sum of two components: the electrochemical potential over the membrane and gradient of a cationic species over the membrane, most often protons. Transmembrane gradients of cations are predominantly generated by proteins in the electron transport chain (ETC). This cationic species can be either protons of sodium ions, of which the latter is generally more abundant in alkaline environments. Curiously though, all aerobic alkaliphiles mostly utilize protons for their ETC, with notable exceptions. In *Thioalkalivibrio versutus* for example, a sodium translocating cytochrome *c*:oxygen cbb_3 oxidoreductase complex was isolated [24]. In mitochondria and unicellular life, proton gradients

are easily established, since the internal pH is similar to, or lower than, the intramembrane space or external environment. The common denominator in alkaliphiles is that they naturally have an inverted proton gradient; the outer pH is high, whilst the cytoplasmic pH is kept close to neutral. In spite of this, aerobic alkaliphiles still rely on regularly oriented and functioning proton-coupled ATP synthase mediated respiration [25].

Alkaliphiles have evidently adapted to circumvent the problem of the inverted proton gradient. Considering that the cytoplasm of an alkaliphile is similar to that of a neutrophile [26], the adaptations in alkaliphiles to their surroundings must be within the following three domains: the cell wall, the cell membrane and the proteins within the membrane. Alkaliphile theory states that the cell wall is responsible for creating a local conductive proton motive force [27]. The membrane lipids are responsible for preventing proton leakage [28, 29]. The proteins within the membrane ensure that the cytoplasm is acidified with respect to the exterior. We will elaborate on each of these aspects separately.

1.3. THE CELL WALL AND THE CELL MEMBRANE

Alkaliphiles generally have a negatively charged cell wall made up of constituents such as teichoic acid in *Halalkalibacterium halodurans* (formerly *Bacillus halodurans*) and the S-layer protein SlpA in *A. pseudofirmus* [30–35]. These negatively charged molecules should establish more acidic conditions close to the cell. That in turns ensures a localized, more conducive proton motive force [27]. Measurements of such a local proton motive force are challenging and so far, so this theory lacks experimental proof. Though a mechanism remains to be proven, a local proton motive force *must* exist, considering the regular functioning of the ETC. The cell wall is not subject to investigation in this study.

Aside from alkaliphiles' ability to build a local proton motive force, preventing leakage of protons over the membrane is essential for survival. When a proton leaks through a bacterial membrane, it has to be reimported either directly using active transport, or via another mechanism that requires energy. In short, losing a proton through leakage is an energy loss. For regular bacteria, preventing membrane leakage is important, but not immediately problematic considering the abundance of protons in the exterior environment. For alkaliphiles however, the quantity of protons in the external milieu is decreased 100-fold at pH 9 (compared to pH 7). Research on *A. pseudofirmus* and *Bacillus* sp. 007/AIA 02/001 (formerly *Bacillus* sp. A-007) therefore focused on possible adaptations alkaliphiles might harbour to minimize proton leakage. The incorporation of squalene, dihydrosqualene (neutral lipids) and diacylglycerol in the middle of the lipid bilayer, perpendicular to the rest of the lipids, was highlighted as an option alkaliphiles might have to form an additional insulating layer [28, 29]. Additionally, high concentrations of cardiolipins might benefit alkaliphiles as well, considering their negative charge, though the data supporting the theory of Clejan and colleagues is contradictory [29]. This

theory was later also disproven by the same research group [36]. Whether these are universal adaptations and whether other adaptations to mitigate the issue of proton leakage exist, is currently unknown. A focal point within this thesis is to investigate the concept of leakage minimizing membrane features further.

1.4. SODIUM POWERED MEMBRANE MACHINERY

In terms of proton homeostasis, membrane proteins can be categorized into three sections: those of the ETC, proton-cation antiporters, and cation-anion symporters related to metabolism. The proteins of the ETC, whose main goal is to avoid alkalinisation of the cytoplasm, will be discussed further below. In the category of proton-cation antiporters, sodium-proton antiporters are considered particularly important [37, 38], with the availability of Na^+ considered a prerequisite for pH homeostasis [39]. The main sodium-proton antiporters in alkaliphiles are Mrp family (also called Mnh) and a variety of Nha and CPA1 and CPA2 systems. Mrp is multisubunit antiporter (multiple resistance and pH) [40, 41] initially found in the neutrophile *Escherichia coli* [42]. In *A. halodurans* a mutation in this protein caused a complete lack of growth under alkaline conditions [43, 44]. This multisubunit H^+:Na^+ antiporter has since been considered an obligatory tool for alkaliphily [45–47]. NhaC is most prominently associated with alkaliphiles, being first identified in *A. pseudofirmus* [48, 49]. NhaD, which moonlights as a Li^+:H^+ antiporter, was first found in the haloalkaliphile *Alkalimonas amylolytica* [50]. NhaE, originally found in the non-alkaliphile, thermohalophilic bacterium *Rhodothermus marinus* [51], might also function as an Na+/H+ anti-port mechanism in alkaliphiles, and has been found in *A. pseudofirmus*. Lastly, a NhaA homolog rechristened as NapA found in *Priestia megaterium* (formerly *Bacillus megaterium*) [52], might also function in alkaliphiles as a sodium-proton antiporter. None of these mono-subunit proteins have been implied to fulfil the same vital function as the Mrp system, though alkaliphilic growth without Mrp is also possible as demonstrated in *Arthospira platensis* [53].

While the ETC of alkaliphiles is usually proton-coupled, organic metabolite transport is typically mediated by Na^+-dependent symporter's. The initial reports on sodium mediated symport centred on the import of α aminoisobutyric acid, found in *A. alcalophilus* and *Cytobacillus firmus* (formerly *Bacillus firmus*) [54–57]. In the thermoalkaliphile *Caldalkalibacillus thermarum* (formerly *Bacillus* sp. TA2.A1), sodium driven sucrose and glutamate transporters were found [58, 59]. Nowadays, it is generally accepted that almost all solute transport in aerobic alkaliphiles is mediated by sodium [25, 60]. Additionally, in alkaliphiles the flagellum motor functioning is also usually sodium mediated, as first discovered in *C. firmus*, *Evansella polygoni* (formerly *Bacillus* sp. YN-1), *Bacillus* sp. 8-1 and *Bacillus* sp. 202-1 [61–63]. Aside from the lipids, this thesis will also focus on the ETC of alkaliphiles and more broadly on the membrane proteins of alkaliphiles. The alkaliphile that will be researched

specifically is the thermoalkaliphile *C. thermarum* TA2.A1, which comes from a low-salt environment.

1.5. INTRODUCING Caldalkalibacillus thermarum TA2.A1

The thermoalkaliphilic bacterium *C. thermarum* TA2.A1 is a facultatively alkaliphilic and moderately thermophilic Gram-positive bacterium, native to Mt. Te Aroha in New Zealand [58]. It is capable of growing between pH 7.5 and 11 (**Chapter 3**). Over that range, the amount of free protons will decrease over 1000-fold. The organism is a chemoorganoheterotroph, capable of consuming a wide range of substrates [64]. Like most aerobic alkaliphiles, it depends on a proton translocating F_1F0_3 ATP synthase, which has been extensively studied [65–73]. The rest of its ETC and the bioenergetics in general have also been studied to great extent [26, 74, 75]. Aside from the abovementioned ATP synthase, it contains a type I and a type II NADH dehydrogenase; a succinate dehydrogenase and putatively a fumarate reductase; a hybrid menaquinone:cytochrome *c* oxidoreductase b_6c_1 complex; a cytochrome *c*:oxygen oxidoreductase aa_3 complex, a cytochrome *c*:oxygen oxidoreductase ba_3 complex, a cytochrome *c*:oxygen oxidoreductase bb_3 complex and a menaquinone:oxygen oxidoreductase *bd* complex (**Chapter 2**). Genomic analysis revealed that the organic solute transporters of *C. thermarum* TA2.A1 are likely all sodium driven, like the sucrose and glutamate transporters mentioned above (**Chapter 2**) [76]. *C. thermarum* TA2.A1 is additionally introduced in the separate chapters of this thesis. Considering all its traits, it fits well within the aims of this thesis, which is to study the ETC and the membrane proteins in general of an alkaliphile. Furthermore, its ability to grow at such a broad pH range makes it an ideal candidate to study the concept of leakage through bacterial membranes. The study of its lipid membranes will give exciting insights, considering *C. thermarum* is incapable of producing any of the neutral lipids mentioned above.

1.6. OUTLINE OF THE THESIS

Chapter 2 will detail the completion of the genome of *C. thermarum* TA2.A1 and put the findings into the context of alkaliphiles as the evolutionary precursor to all life. **Chapter 3** is a study on the membrane lipids of *C. thermarum* TA2.A1 grown in chemostat at various pH levels (7.5 – 11). This chapter shows that acylphosphatidylglycerols are produced at high pH to lower the cell membrane surface area and thereby reduce proton leakage. We hypothesize

that this is especially useful in curved areas of the membrane. Furthermore, at pH 11, menaquinone concentrations are increased dramatically without oxygen consumption concomitantly increasing. Our theory is that the elevated menaquinone concentrations act in a manner similar to squalene and other neutral lipids and that they act as additional insulating layer. **Chapter 4** details the membrane proteome of *C. thermarum* TA2.A1. This study makes use of solubilization techniques to optimize the number of detected proteins. In conclusion, the combined techniques manage to give a near complete overview of all membrane proteins required for transport of organic and inorganic solutes. The membrane proteome also found more ETC proteins expressed than specifically required. **Chapter 5** zooms in on the supposedly inefficient expression of ETC proteins. In this study, *C. thermarum* TA2.A1 was grown in chemostat in the microaerobic range, where the maximum oxygen level approximated full aerobiosis [77]. We discovered that the cytochrome *c*:oxygen oxidoreductase aa_3 complex is used primarily at full aerobiosis. Under intermediate oxygen conditions, the cytochrome *c*:oxygen oxidoreductase ba_3 complex dominates abundance profiles. The profile of ba_3 tails off at the end, putatively because of its function being taken over either by the cytochrome *c*:oxygen oxidoreductase bb_3 complex or by the menaquinone:oxygen oxidoreductase *bd* complex. Detection of both bb_3 and *bd* remained elusive. Rather surprisingly, both type I and type II NADH dehydrogenases were expressed at similar levels throughout the microaerobic range. *E. coli* for instance primarily depends on the type I NADH dehydrogenase at high aerobiosis levels, whilst the type II NADH dehydrogenase become more active at lower oxygen levels [78, 79]. Lastly, the Mrp complex, which is considered to be essential for the functioning of alkaliphiles, was downregulated at low O_2. We hypothesized that this is due to the increased activity of the acetate exporter, which is sodium powered and thus competes for available sodium. This competition is enable due to the lower proton export levels induced by shifting from aa_3 to ba_3. In all, this thesis revealed new insights into the functioning of alkaliphiles. As with all research, many new questions arise from this study, which will be discussed in **Chapter 6**.

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2

GENOMIC ANALYSIS OF *Caldalkalibacillus thermarum* TA2.A1 REVEALS ALKALIPHILIC METABOLISM AND EVOLUTIONARY HALLMARKS LINKING ALKALIPHILIC BACTERIA AND PLANT LIFE

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ABSTRACT

The aerobic thermoalkaliphile *Caldalkalibacillus thermarum* strain TA2.A1 is a member of a separate order of alkaliphilic bacteria closely related to the Bacillales order. Eforts to relate the genomic information of this evolutionary ancient organism to environmental adaptation have been thwarted by the inability to construct a complete genome. The existing draft genome is highly fragmented due to repetitive regions, and gaps between and over repetitive regions were unbridgeable. To address this, Oxford Nanopore Technology's MinION allowed us to span these repeats through long reads, with over 6000-fold coverage. This resulted in a single 3.34 Mb circular chromosome. The profle of transporters and central metabolism gives insight into why the organism prefers glutamate over sucrose as carbon source. We propose that the deamination of glutamate allows alkalization of the immediate environment, an excellent example of how an extremophile modulates environmental conditions to suit its own requirements. Curiously, plant-like hallmark electron transfer enzymes and transporters are found throughout the genome, such as a cytochrome b_6c_1 complex and a CO_2-concentrating transporter. In addition, multiple self-splicing group II intron-encoded proteins closely aligning to those of a telomerase reverse transcriptase in *Arabidopsis thaliana* were revealed. Collectively, these features suggest an evolutionary relationship to plant life.

2.1. INTRODUCTION

The alkaline world is a fascinating and geologically ancient environment [1]. In line with this, Russell and Hall first theorized that life on earth might well have started in alkaline hot springs [2, 3], but whether this is true for all life is still a matter of intense debate [4–6]. Support for this theory appears to be 'branched', and is built on the plant-specific processes of carbon fixation [7], which we will not discuss at length here, and cellular energy generation. Interestingly, the latter provides some the most substantial genetic evidence that plant life finds its origin in alkaline environments [2]. The original proposal centers on electron transport via iron-sulfur clusters in a primitive 'electron transport chain' (ETC). In commonly studied bacteria such as *Escherichia coli*, the electron transport chain consists of an electron-donating reaction, generally either an NADH dehydrogenase (complex I) or a succinate dehydrogenase (complex II). The other reaction logically is an electron-accepting reaction, catalyzed by a terminal cytochrome oxidase complex IV, which transfer the electrons to an oxidized compound – oxygen in case of aerobic microorganisms. In some bacteria and in all animal life, the electron donating and accepting reactions have an electron splitting reaction, a cytochrome bc_1 complex (Cyt. bc_1; complex III) in between them, as a form of regulation and also energy generation. In bacteria and archaea, classical complexes I, III and IV can translocate H^+ or Na^+ over the membrane, thereby generating an ion-motive force [8, 9]. The resulting gradient is harvested by the F_1F_o ATP synthase in most bacteria (complex V). The soluble F1 domain is responsible for the catalytic activity, while the Fo domain is responsible for importing the translocated H^+ or Na^+. Translocation of protons is via the *a* subunit and a proteolipid c-subunit ring of organism specific size [10, 11].

A clue about evolution stems from the stoichiometry of the rotating, membrane embedded, *c*-subunit ring of ATP synthase. This ring consists of a multimer of eight in *Bos taurus*, to fourteen-mer in chloroplasts and onwards to fifteen-mer in cyanobacteria. Interestingly enough, having a large *c*-ring is actually not advantageous, if purely assessing the amount of ATP produced per proton pumped. Regardless, a large *c*-ring is a common trait for alkaliphiles, cyanobacteria and plants [12–15]. One of the most deeply rooted member of the Bacilli class, the thermoalkaliphile *Caldalkalibacillus thermarum* strain TA2.A1 [16]. *C. thermarum* TA2.A1 has a large thirteen-mer *c*-subunit ring [17], and its ATP synthase has been extensively researched [18–20]. Relatively little has been described on its preceding ETC however and current genetic data is incomplete. Thus, we cannot to determine whether it contains all possible components of the ETC, which is crucial for drawing evolutionary conclusions, and for further research in general.

The NCBI database actually does state that sequencing data from 2011 constitute a representative genome. However, considering the 18x fold 454 GS FLX and 261x Illumina coverage, it was actually denoted as only a high quality draft, with some small regions possibly remaining undiscovered [21]. The draft could not be assembled due to complexity of the repetitions

present, leaving the genome scattered over 251 contigs. Repetitive regions are not unusual in a genome and can indicate multiple features. In bacteria they feature as control region for expression [22] or as regions regulating recombination [23]. Another, quite noteworthy example for bacteria, is that of the CRISPR-associated regions [24, 25]. All of the three aforementioned features contribute to bacterial fitness, something that is expected to be of prime importance for a polyextremophile.

Oxford Nanopore Technology's MinION platform enables us to sequence long reads (up to 200 kb), which should resolve the problem of repetitive regions in the genome of *C. thermarum* TA2.A1. A drawback of the technique is the fact that it does have an error rate of up to 12% [26].Therefore, only when supplementing this technique with the precise Illumina technology will we obtain a representative genome. In this research paper, we sequenced *C. thermarum* TA2.A1 using the abovementioned techniques. We use the improved genomic data to outline new features and its implications for observed physiology. We also discuss additional evolutionary perspectives this genome provides for the alkaline hot pool theory.

2.2. MATERIALS AND METHODS

2.2.1. BACTERIAL STRAIN, GROWTH CONDITIONS

Caldalkalibacillus thermarum strain TA2.A1 was cultured as described previously [27]. *C. thermarum* TA2.A1 was grown aseptically in a shaking incubator (180 rpm) at 65°C in an alkaline basal medium containing 10 g L^{-1} trypticase peptone at pH_{65C} 9.5 or as indicated. Growth was initiated with a 0.1% inoculum from an overnight culture. For DNA extraction and pH-shift studies 10 g L^{-1} L-glutamate was used as the major carbon source and cells were grown aerobically in a round bottom shake flask overnight. When L-glutamate was excluded from this medium, it was replaced with sucrose to a final concentration of 10 g L^{-1}. Where necessary, growth was monitored by aseptically extraction of samples and measuring the optical density at 600 nm (OD_{600}; 1-cm light path length) or measuring dry weights. Dry weights were obtained by filtration through a filter (0.2 µm Millipore) and drying thereafter in a 105 °C oven for at least 24 h.

2.2.2. DNA EXTRACTION, SEQUENCING, AND ASSEMBLY

DNA was isolated according to the QIAGEN® Genomic DNA Handbook, from 5 mL overnight culture. Sequencing was performed with both Illumina® MiSeq and Oxford Nanopore Technologies MinION platform. Genome was *de novo* assembled with Canu [28] and thereafter annotated with Prokka, RAST [29–31] and BlastKOALA [32, 33]. A single annotation file was made by combining the three annotations and manually curating discrepancies between algorithms.

2.2.3. PHYLOGENETIC ANALYSIS

Protein-based phylogeny. A list of 120 bacterial core genes was taken from Genome Taxonomy DataBase (GTDB) [34]. These marker genes were identified in selected genomes, aligned and concatenated using GTDBtk v0.3.2 [35]. Alignment was automatically trimmed using trimAl 1.2rev59 by using automated1 and gt 0.95 options[36]. The resulting alignment consisted of 21,432 amino acid residues. Phylogenetic tree was built using IQ-TREE 1.6.12 program [37] with SH-aLRT test [38] as well as ultrafast bootstrap with 1,000 replicates [39] and ModelFinder to determine the best-fit model [40].

For *Bacillus* sp. genome comparison, whole genome sequences were downloaded from NCBI and processed by kSNP3 [41–43]. A parsimony tree was inferred by kSNP3 with default settings and a kmer of 21, which was determined by Kchooser. The resulting newick file was visualized with MEGA 8 resulting in a phylogenetic tree.

2.3. RESULTS AND DISCUSSION

2.3.1. CONTEMPARY SEQUENCING METHODOLOGIES ENABLE THE ASSEMBLY OF A COMPLETE CIRCULAR CHROMOSOME

MinION and Illumina sequencing resulted in 6173x and 621x coverage respectively, with an average read length of 10.50 kb and 301 bp respectively. Especially the high coverage with the MinION, when compared with the old sequencing data (**Figure** 2.1a), aided us in spanning repeat regions present in the genome. The successful assembly resulted in a single circular chromosome (**Figure** 2.1b) of a total sequence length of 3.34 Mb, a 15.5% increase in length over the 2011 study. 3652 coding sequences were found, up from 3160 genes reported by deposited data in the NCBI database [21]. These were annotated as described above. The new, annotated *C. thermarum* TA2.A1 sequence was uploaded to the NCBI database, with accession number: PRJNA638815.

2.3.2. PHYLOGENY

A full genome gives us the possibility of analyzing the phylogenetic relationship of *C. thermarum* TA2.A1 within the Firmicutes phylum (**Figure** S1) and the Bacilli class (**Figure** 2.2). For this analysis, we chose to use a core set of 120 reference genes [34] taken from the Genome Taxonomy DataBase (GTDB), with the taxonomic trees constructed as described above. The phylogenetic analysis shows that based on amino acid sequences of single copy marker genes *C. thermarum* TA2.A1 is part of the “Caldalkalibacillales” order, almost all by itself. The GTDB classification is in contrast with what NCBI reports, which states common ancestry with Bacillus species only diverges at genus level. We propose that the GTDB classification, confirmed by the findings of this study, to be the correct classification. *C. thermarum* TA2.A1 is joined in its order by Bacillus mannanilyticus JCM 10596T, which is an mesophilic,

A	*Caldalkalibacillus thermarum* TA2.A1 (this dissertation)	*Caldalkalibacillus thermarum* TA2.A1 (Kalamorz *et al.*, 2011)
Genome size (Mbp)	3.34	2.89
Contigs	1	251
Contigs N50 (bp)	3,344,914	28,715
Contigs L50	1	31
Max contig size (bp)	3,334,914	104,216
GC content (%)	47.62	47.58
Coding sequences (CDS)	3,652	3,160
rRNA copies	113	98

B

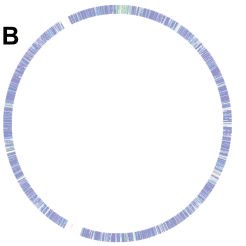

Figure 2.1: Comparison and Alignment of old and new genetic data of *Caldalkalibacillus thermarum* strain TA2.A1. **A**, Statistical analysis of the assembled and annotated genome sequence of *C. thermarum* TA2.A1 from this manuscript compared to the 2011 publication 24. **B**, The new circular genome is used a reference to which the old data is aligned. Green/blue indicates a 90 – 100 % alignment, red/pink/absence of color indicates no sequence overlap. This alignment shows how the old, fragmented high-quality draft compares to the complete, new singular chromosome. As is visualized, some regions were represented well in the old draft, while some were not represented at all, which leads to the observed discrepancy in genome length between the two studies. The alignment was visualized using the online global alignment function of RAST [29, 30, 44].

moderately halotolerant, alkaliphilic chemoorganotroph [45].

Based on our phylogenetic reconstruction, two representatives of the ”Caldalkalibacillales” order with available genomes form a separate deep phylogenetic branch of the order level within the Bacilli class (**Figure** 2.2). Most other known halophiles and alkaliphiles are part of “Bacillales” order, which curiously is the most closely related order. We also note a far more distant relationship with other (an-)aerobic thermophiles within the Firmicutes phylum (**Figure** S1).

However, to further corroborate genomic information with functional data, well-characterized bacterial systems are required for comparison. In light of this limitation we decided to compare *C. thermarum* TA2.A1 with environmentally similar well-describe alkaliphilic *Bacillus* sp., the mesophilic *Bacillus* sp, and the thermophilic *Geobacillus stearothermophilus*. For this analysis, we chose to use a reference free whole genome single nuclear polymorphim (SNP) based phylogeny on *Bacillus* species. SNPs are the most common type of genetic variation and allow a rapid, but more wide reaching than classical 16S rRNA, and are frequently used as biological markers [46]. In agreement with the analysis in Figure 2.2 and Figure S1, *C. thermarum* TA2.A1 is the most phylogenetically ancient member of the genus *Bacillus* examined (**Figure** S2).

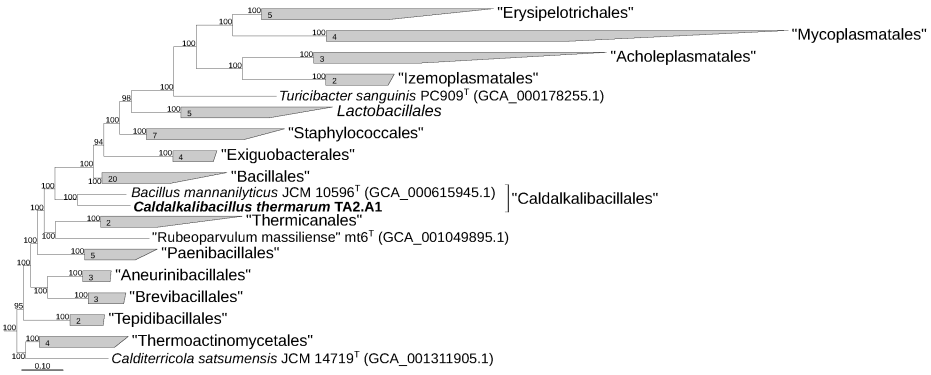

Figure 2.2: *Caldalkalibacillus thermarum* strain TA2.A1 is in a novel order "Caldalkalibacillales".Placement of *C. thermarum* TA2.A1 within the class Bacilli based on phylogenetic analysis of concatenated partial amino acid sequences of 120 bacterial conservative proteins 40 by maximum likelihood inference; taxonomic designations correspond with Genome Taxonomy DataBase 40. Bootstrap values are shown at the nodes. Bar, 0.10 changes per position.

2.3.3. ORIGIN OF REPLICATION

Initially, we attempted to find the origin of replication of *C. thermarum* TA2.A1. For related alkaliphiles such as *Bacillus halodurans* C-125 and *Bacillus pseudofirmus* OF4, 'origin regions' have been specified, not a bona fide oriC regulatory region such as that for *Escherichia coli* K-12 [47]. The oriC region is of significance, as this is the binding site of dnaA, the chromosomal replication initiation enzyme [48], which starts DNA unwinding and subsequent loading of the replisome. The model organism in the Bacilli class, *Bacillus subtilis*, has a fragment of DNA denoted as an oriC sequence (accession: X02369), yet an annotated oriC region is not discretely identified. The putative oriC region roughly ends with the coding region of gyrB.

Another putative origin of replication for a species in the Bacilli class, *B. halodurans* C-125 (accession: AB013492), similarly shows gyrB close to its end, and additionally shows dnaA close to its start (**Figure** 2.3). The dnaH and dnaN genes are directly involved in the replisome [49], whilst DNA gyrase, a topoisomerase encoded by gyrA and gyrB, is crucial for unwinding DNA during cell replication [50]. The genome of *C. thermarum* TA2.A1 also has a region spanning from dnaA to gyrB, which could contain the origin of replication (i.e. the oriC region). We do note that in the case of *E. coli* K-12, the oriC region is over 45 kb away from the replication initiating gene dnaA (**Figure** 2.3). The 4.7 kb space between gyrB and gyrA is seemingly unique to *C. thermarum* TA2.A1 and it contains import machinery for magnesium and molybdate, and, most crucially, the regulator cysL, which has been described to regulate the sulfite reductase operon in *B. subtilis* [51]. This alignment reveals a commonality exists within the Bacilli class regarding the origin of replication, and that a

defined oriC sequence is not a commonality might indicate that this sequence is order-specific within the taxonomic class.

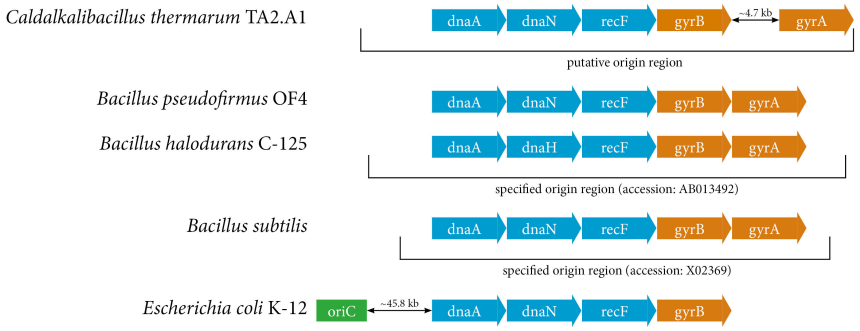

Figure 2.3: Alignment of putative origin regions of various *Bacillus* species and *E. coli*. Organisms included are *C. thermarum* TA2.A1, *B. pseudofirmus* OF4 (accession: CP001878), *B. halodurans* C-125 (accession: AB013492) and *B. subtilis* (accession X02369). The genes dnaA, dnaN/H and recF are part of the bacterial replisome, whilst gyrA and gyrB code for DNA gyrase, a topoisomerase, which is not related to chromosomal replication. *E. coli* K-12 (accession AP009048) is included as comparison, as this has an oriC region, which removed ∼45.8 kb from its dnaA gene.

2.3.4. REDUNDANCY IN CRISPRS, PLANT-LIKE SELF-SPLICING INTRON-ENCODED PROTEINS, AND OTHER GENES

Surprisingly, a set of genes is found scattered over the genome called ltrA (numbered 1 to 13), which is a self-splicing group II intron-encoded protein. This caught our attention, as this is presumed to be a precursor to the eukaryotic intron mechanism. The enzyme is multifunctional, as it has reverse transcriptase activity next to maturase and endonuclease activity [52]. This reverse transcriptase activity aligns for 53% with the TERT gene of *Arabidopsis thaliana* (query cover based on amino acid sequence). The TERT gene encodes for telomerase reverse transcriptase, a gene crucial for maintaining linear architecture in eukaryotic chromosomes. We therefore question whether this gene, or related genes, could also be an evolutionary precursor of eukaryotic telomerase machinery. The presence of ltrA in Bacilli is seemingly ubiquitous, as for most domains of life in general [53], though we observe notably higher copy numbers in fellow alkaliphiles *B. halodurans* C-125 (5 copies) and *B. pseudofirmus* OF4 (4 copies) than in neutrophilic *B. subtilis* (2 copies) or thermophilic *Geobacillus stearothermophilus* 10 (0 copies; accession number: PRJNA252389). Apart from the copy number, the similarity between alkaliphilic variants of ltrA and the TERT gene of A. thaliana is much higher

(see above) than that of the neutrophilic *B. subtilis*, which has only 4% query cover based on amino acid sequence, meaning that in terms of evolutionary relevance, the study of an alkaliphilic ltrA is much more interesting.

Within the genome of *C. thermarum* TA2.A1, we find nine genes encoding for CRISPR-associated proteins; three copies of the Cas1 and Cas2 adaptation genes, two copies of Cas3 and a single copy of Cas9. The presence of two Cas3 genes suggests that *C. thermarum* TA2.A1 has two different type I systems and the single Cas9 gene indicates a single type II system. Collectively these give *C. thermarum* TA2.A1 a strong capacity for defense against phages [54]. Interestingly, while CRISPR is best known for phage resistance, Weinberger et al have shown it is only present in ∼45% of mesophilic bacterial genomes available on databases, whereas it is found in ∼90% of thermophilic bacterial genomes available on databases, and are prevalent in thermophilic Bacilli [55]. Mesophilic Bacilli have no such degeneracy as far as we are aware. The authors used models to show that as higher mutation rates in viruses increase, the rate of CRISPR spacer addition decreases. They suggest that because both mesophilic viruses and bacteria mutate more frequently that this effectively outruns CRISPR/Cas based immunity [55]. With this in mind, the presence of three CRISPR/Cas systems in *C. thermarum* TA2.A1 suggests that phages existing in thermoalkaliphilic conditions must have relatively low mutation rates making them potentially useful molecular tools.

This observed genetic redundancy is not confined to CRISPR/Cas systems. The genome of *C. thermarum* TA2.A1 contains a total of six copies of smc, the gene responsible for chromosome partitioning and at least three annotated operons dealing with spore germination, encoded by gerABC and yndE. *C. thermarum* TA2.A1 has three complete copies of pdhABCD, the operon for pyruvate dehydrogenase. The microorganism also has multiple sets of the gsiBCD genes, encoding for glutathione binding and import. Interestingly enough, only one copy of the gsiBCD genes constitute an operon, which begs the question what the other copies are for. This phenomenon is prevalent with other transporters as well; we give the C4-dicarboxylate, lactose, arabinose and trehalose importers as examples. The same incomplete duplication is something we observe for genes encoding for DNA polymerase III – some genes are present only once, whilst others have three copies scattered across the genome. While degeneracy of transporters are relatively common in Bacilli, such as multidrug efflux in *Bacillus subtilis* or methods of transport such as a symporter and PTS systems capable of transporting the same substrates (see Fig 2.4), and metal transport [56], spore germination and chromosomal partitioning is usually restricted to a single operon in described Bacilli to date [57, 58].

2.3.5. KEY FEATURES OF THE C. thermarum TA2.A1 GENOME PHYSIOLOGY AND METABOLISM

Previous publications on *C. thermarum* TA2.A1 have heavily focused on particular aspects of cellular physiology in isolation, such as transporter activity [16, 59], cell physiology[27], siderophore production [60], and the F-type ATP synthase [19, 61]. Here, we take the opportunity of having a holistic picture to assemble these into a thorough description by reflecting on the genome. Alkaliphiles, like most groups of highly extremophilic organisms are highly fastidious in growth, so the utmost care must be taken when cultivating them. One peculiarity of alkaliphiles is the reliance on specific types of peptone/tryptone extracts and the continued lack of ability to find a general chemically defined growth media for cultivation. It has been proposed that this is due to specific need for oligopeptides or dipeptides, yet extensive studies on alkaliphiles have not yet revealed what these are and they may indeed be organism-specific [62–65]. *C. thermarum* TA2.A1 is no exception, while being reported to grow on a wide variety of carbon sources while remaining strictly aerobic (**Figure** 2.4), and has a particular proclivity for growth on glutamate [27].

2.3.6. TRANSPORTERS

C. thermarum TA2.A1 has a broad variety of substrate/sugar transporters, for (in-) organic substrates and siderophores. Most of the organic substrate uptake machinery is chemical energy dependent, ATP-utilizing ABC type (e.g. maltodextrin and trehalose) and Phophoenol pyruvate (PEP) phosphotransferase type transporter dominate (e.g. fructose and mannose) (see **Figure** 2.4). Interestingly, glutamate and sucrose, the substrates on which the microorganism grows best, can be imported through secondary symporter transport systems (**Figure** 2.4), utilizing a sodium-motive force (SMF), in agreement with what has been described experimentally for both glutamate [16] and sucrose [59]. C4-dicarboxylates, such as succinate and malate, have also been demonstrated be imported into the cell utilizing an SMF [27], a flux which is likely controlled by a voltage-gated Na^+ channel [66]. Interestingly we have only found a single candidate transport system capable of this function in a Tripartite (TRAP)-type transporter (**Figure** 2.4). While symporters are mass-transport type, TRAP transporters are regarded as scavenging, indicating that while *C. thermarum* TA2.A1 is clearly capable of growth on C4-dicarboxylates [27], it is unlikely that these are very bioavailable in an environmental setting. We also note a TRAP-type sialic acid transporter (**Figure** 2.4). While sialic acids in bacteria were previously associated exclusively with pathogens in immune response avoidance [67], recent research shows far more diversity within this class of sugars [68]. Intriguingly, *C. thermarum* TA2.A1 also has ccmL and ccmM (**Figure** 2.4), genes used for inorganic carbon concentration and capture [69]. Capturing inorganic carbon should be wholly useless for a heterotrophic, aerobic organism such as *C. thermarum* TA2.A1, these

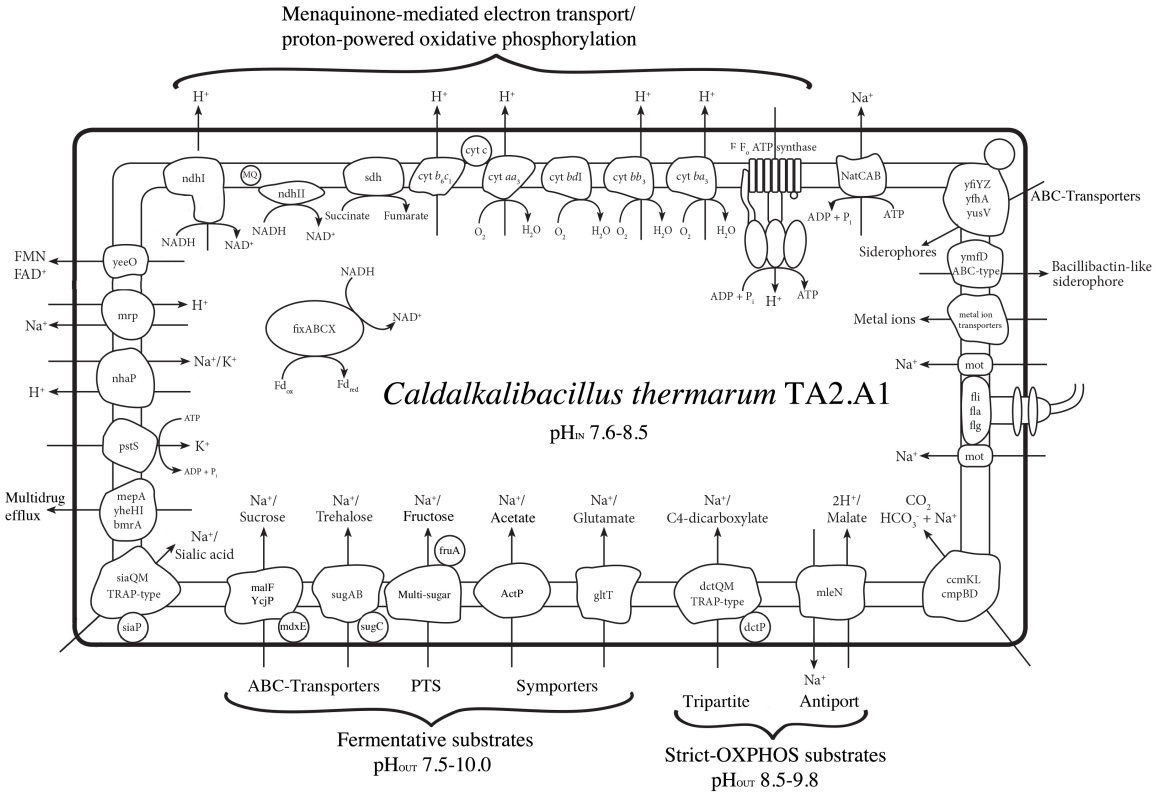

Figure 2.4: A selection of transporters and membrane proteins present in *C. thermarum* TA2.A1 based on the annotated genome. Note that two different substrate categories are present: fermentable and non-fermentable. Fermentable substrates enable *C. thermarum* TA2.A1 to adapt to a far greater variety of conditions than substrates that have to be consumed through the TCA cycle.

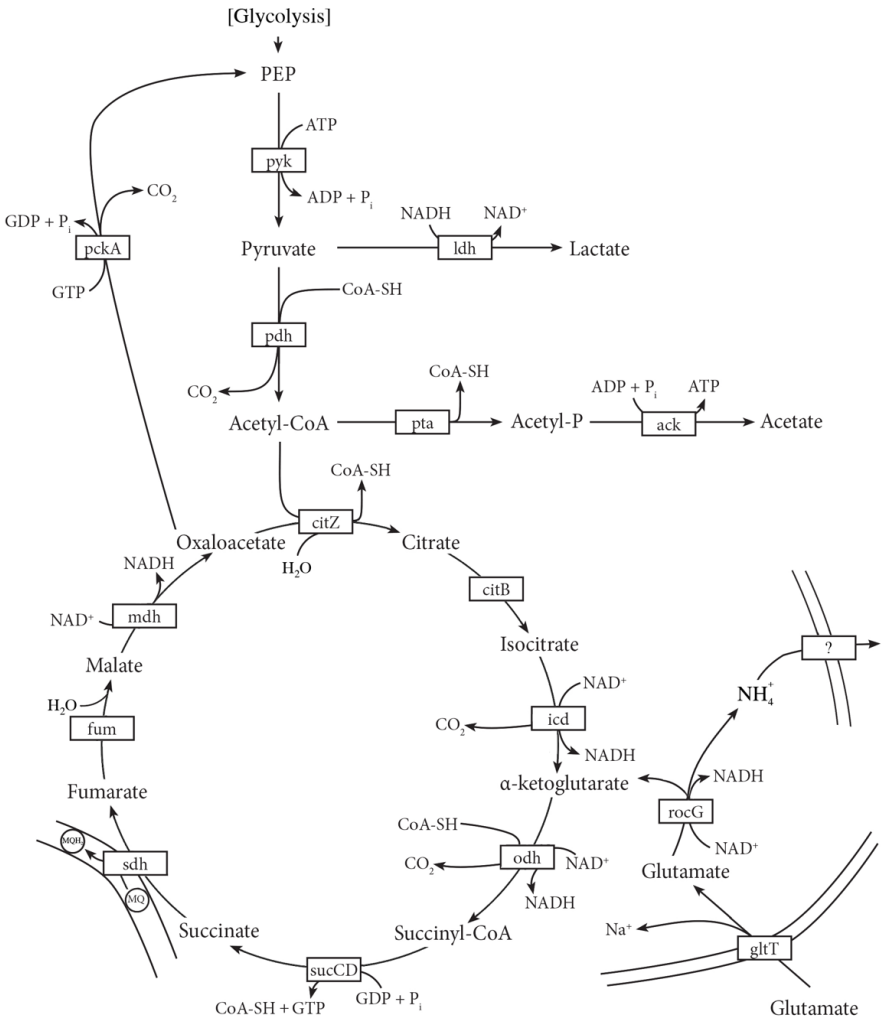

Figure 2.5: Proposed catabolic model for *C. thermarum* TA2.A1 The model consists of an Embden-Meyenhoff-Parnas type glycolysis (see Figure S1 for details), and a tricarboxylic acid (TCA) cycle, including a one-step shunt to connect glutamate to the TCA cycle. It also shows fermentative routes towards acetate and lactate. Furthermore, the glutamate uptake system is shown, and a pathway to alkalize local environment using $NH4^{+}$. The parallel lines indicate membrane-bound proteins; MQ/MQH2 is the membrane-bound electron carrier menaquinone used in the electron transfer chain (see **Figure** 4).

systems are usually found in photosynthetic organisms. Considering this, its presence could originate from a more ancient environment, one in which the ability to scavenge any carbon, organic or inorganic, was a prime competitive advantage.

Iron sequestering is an extreme challenge in an alkaline environment with iron being almost totally insoluble. *C. thermarum* TA2.A1 has been reported to produce a siderophore composed of catecholate and hydroxamate [60] chemical groups, but unfortunately this molecule could not be isolated. Here, we find the presence of a bacillibactin-like exporter, which is a catacholate siderophore, giving insight into new approaches for siderophore purification. Unsurprisingly, *C. thermarum* TA2.A1 has multiple siderophore import mechanisms to gather siderophores produced by other microbes (see **Figure** 2.4). Lastly, *C. thermarum* TA2.A1 also has FAD/FMN export machinery (yeeO), the purpose of this is totally unknown.

2.3.7. CENTRAL CARBON METABOLISM

C. thermarum TA2.A1 is an aerobic, chemoheteroorganotrophic organism, preferably growing on glutamate or sucrose, which are mainly converted to CO2 and acetate [27]. After substrate import, *C. thermarum* TA2.A1 has a relatively straightforward catabolism consisting of the glycolysis and the tricarboxylic acid (TCA) cycle (see **Figure** S3 and **Figure** 2.5). For classical glycolytic sucrose consumption, the compound is imported *via* a phosphotransferase system, but in *C. thermarum* TA2.A1 symport is the dominant mechanism [59] (see **Figure** 2.4), and performs the split into fructose and glucose thereafter (**Figure** S3). Glutamate catabolism in *C. thermarum* TA2.A1 is intriguing, as it seems to differ from known pathways [70, 71]. These pathways include the strictly anaerobic mesaconate pathway first described in *Clostridium tetanomorphum* [72], a pathway *via* 2-hydroxyglutarate discovered in *Peptostreptococcus asaccharolyticus* [73] and a variation on the pathway via 2-hydroxyglutarate found in *Fusobacterium nucleatum* [74]. *C. thermarum* TA2.A1 lacks the genes for those pathways and instead we hypothesize it feeds glutamate directly into the TCA cycle *via* a one-step catalyzed by glutamate dehydrogenase (**Figure** 2.5), an enzyme that confusingly enough has glutamate deaminase activity [75].

Entering the TCA cycle *via* α-ketoglutarate will require some production of acetyl-CoA *via* gluconeogenesis in order to circumvent the problem of the unconventional TCA cycle access point. Being an aerobic organism, excess reducing equivalents are produced during dissimilation and these are respired in the ETC (see **Figure** 2.4). However, nothing in the central metabolism suggests why *C. thermarum* TA2.A1 growth using glutamate as a carbon source has a consistently shorter lag phase and grows ∼30% faster than when using sucrose (**Figure** 2.6a-b), especially since both carbon sources are imported using ΔNa^+-driven symport (**Figure** 2.4). In addition, sucrose should theoretically yield more energy/mol since 2 rounds of glycolysis and 4 rounds of

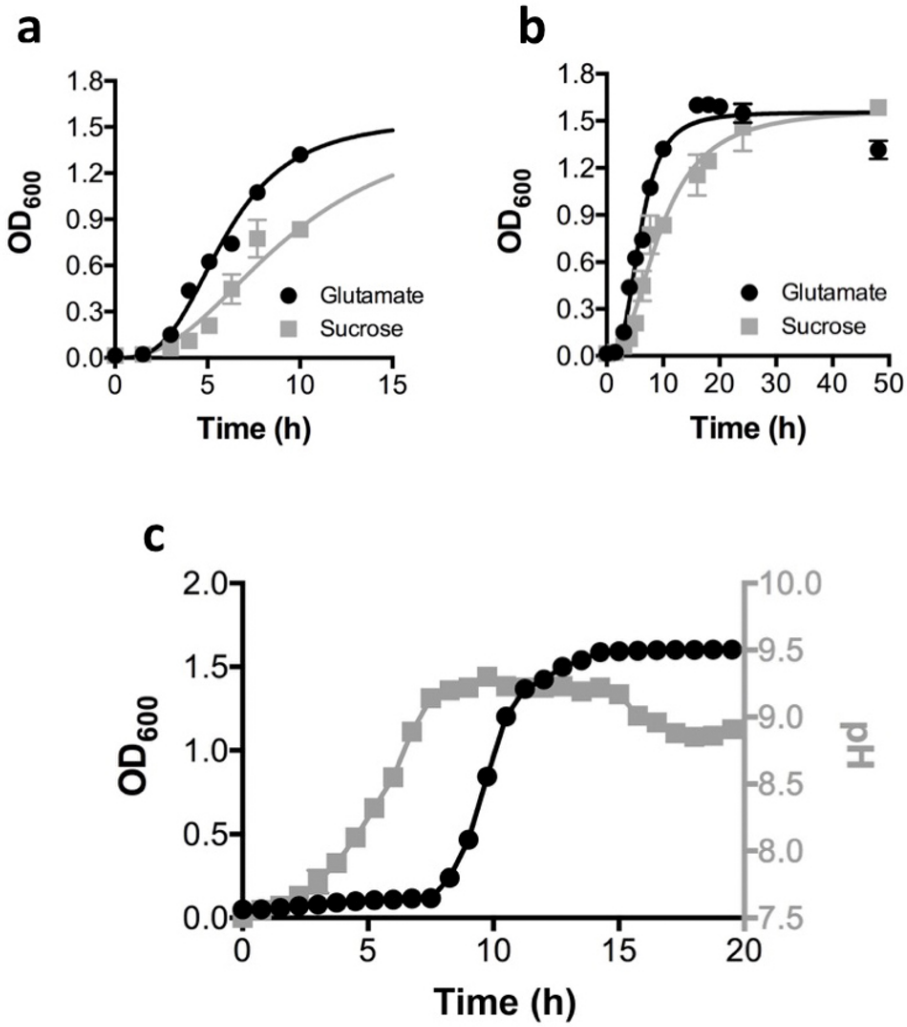

Figure 2.6: *C. thermarum* TA2.A1 growth on glutamate vs sucrose and influence of starting pH. Effect of carbon source on the growth of *C. thermarum* TA2.A1 in flask batch-culture using alkaline basal medium supplemented with either 10 g L^{-1} L-glutamate or sucrose. In **A** and **B** the initial starting pH was 9.5 where as in C it was 7.5. **A**, Growth over the first 10 hours of culturing; **B**, Full growth curves; **C**, Growth on glutamate with initial pH at 7.5 showing a pH shift before onset of growth. The values reported are the means of four replicate experiments with the standard error of the means shown.

the TCA is possible (**Figure** 2.5 and **Figure** S3). It is noted that this is not due to a change in cell size, since the dry weight also has a ∼30% higher mass when *C. thermarum* TA2.A1 is grown on glutamate after 16 hours (**Figure** S4).

We propose that the answer does not lie in the substrate that yields the most energy, but the substrate that allows another physiologically beneficial function. Therein we suggest two phenomena are likely at work. Firstly, the discrepancy between the onsets of growth could be due to the bacterial variety of the 'resource allocation theory' [76] – fewer enzymes have to be expressed for glutamate consumption (**Figure** 2.5), compared to sucrose (**Figure** S3 and **Figure** 2.5), and thus growth with this substrate may be kinetically advantageous. Secondly, the glutamate deamination into α-ketoglutarate by glutamate dehydrogenase releases ammonium, which has a pK_a of 9.25. This tends not to matter under alkaline conditions in a hot-pool, but when grown in a sealed volume, this does matter, as metabolic by-products are not washed away. Interestingly, we observe a longer lag-phase when grown on glutamate at pH 7.5 (**Figure** 2.6c) that does not occur when grown on sucrose [27]. Upon onset of eventual growth, retesting pH shows a significant increase in extracellular pH. In short, deamination of glutamate is an excellent example of an extremophile modulating environmental conditions to suit its own requirements and to outcompete possible competitors. This allows alkaliphiles to be found in a number of non-alkaliphilic environments [64].

2.3.8. OXIDATIVE PHOSPHORYLATION

In the electron transport chain an unexpectedly extensive variation in available respiratory enzymes is found with seeming redundancy (see **Figure** 2.4). Since only menaquinone biosynthetic machinery could be identified (see **Figure** S5), it is highly likely that all the enzymes involved in membrane-bound electron transport are reliant on menaquinone for physiological function (**Figure** 2.4). *C. thermarum* TA2.A1 has both putative type I (Ndh1) and characterized type II (Ndh2) NADH dehydrogenases [77, 78] (**Figure** 2.4). The Ndh1 is likely capable of translocating ions over the membrane, presumably protons, which would be used by the proton-linked F_1F_o ATP synthase [19]. Using a proton-translocating variant of NADH dehydrogenase yields more energy, begging the question why the microorganism has an Ndh2 as well. Reports into the physiological function of the type II NADH dehydrogenase has yielded various explanations from simply balancing NADH/NAD+ concentrations [79], to responses to changing oxygen levels [80]. In agreement with membrane measurements [27], a putative sdh1 was identified (see **Figure** 2.4) which is capable of the succinate to fumarate conversion, however any fumarate reductase activity of this enzyme is unknown. In the previous genome an operon annotated as a cytochrome b_6f was annotated. However, while genes indeed include a cytochrome b_6, there is no homology to a cytochrome *f*, but there is to a cytochrome c_1 (**Figure** S6). This leads us to believe that this organism has a cytochrome b6c1, which is a novel-hybrid type

complex III also found in *G. stearothermophilus* [81]. Curiously, the iron-sulfur cluster of *C. thermarum* TA2.A1's cyt.b6c1 also shows similarities to the iron-sulfur cluster in cyt. b_6f of the cyanobacterium *Synechocystis* sp. PCC 6803 (**Figure** S7), and may be an evolutionary precursor. Supporting this proposition, while a b_6c_1 has not been examined, a synthetically constructed b_6c_1 was constructed and was able to functionally replace a cytochrome b_6f in *Rhodobacter capsulatus* in the process of cellular photosynthesis [82].

At the electron-accepting end of the respiratory chain, *C. thermarum* TA2.A1 has extreme plasticity. Four terminal oxidases were identified; cytochromes aa_3, ba_3, bb_3, and *bd* (see **Figure** 2.4). In cytochromes aa_3, ba_3 and bb_3 *C. thermarum* TA2.A1 has three types of proton-translocating terminal oxidases. Previous reports have identified that these enzymes pump different amounts of protons per oxygen molecule reduced [83, 84]. This, together with a non-proton pumping cytochrome bd_3 leads to the notion that the variation is there for extreme optimization since *C. thermarum* TA2.A1 lives in an extremely proton-poor environment and that membrane potential is likely playing a greater role than pH for oxidative phosphorylation. Lastly, *C. thermarum* TA2.A1 has an extensively studied [17, 18, 85–87] proton-coupled F_1F_o-type ATP synthase (see **Figure** 2.4) which is only capable of ATP synthesis, but not hydrolysis [19, 20]. The operon has previously been shown to have the typical canonical subunits and atpI, but here we also report the presence of atpZ. The functions of atpI and atpZ are unknown, and although both have been proposed to link to magnesium transport [88], no concrete biochemical assays have been conducted to verify this proposal.

Although not membrane bound, it is indeed curious in the context of the link to plant evolution that we also found an electron bifurcating enzyme of the *fix* class (see **Figure** 2.4). This enzyme transfers electrons from NADH to ferredoxin, and is generally found in plant-associated microbes [89]. In the context of *C. thermarum* TA2.A1 the use of ferredoxin may be involved in glutamate synthesis from glutamine and α-ketoglutarate [90], giving another possible reason why the microorganism grows so well on this substrate (i.e. it does not have to synthesize glutamate because it is bioavailable).

2.3.9. ION HOMEOSTASIS

An important feature of *C. thermarum* TA2.A1 is that while it is indeed an obligate alkaliphile when grown on C4-dicarboxylates, it is a facultative alkaliphile when grown on fermentable substrates such as sucrose. This naturally means *C. thermarum* TA2.A1 has to be able to adapt to neutral pH conditions in which more protons are located outside of the cell than in [91], and an inverted ΔpH at alkaline conditions [92]. Interestingly enough, *C. thermarum* TA2.A1 does this without altering its maximum specific growth rate, propping up the dearth of its proton motive force – a decline of -164 mV at pH 7.5 to -78 mV at pH 10 – with a -100 mV sodium motive force [93]. Since the F_1F_o-type ATP synthase is only capable of importing protons, and

cytoplasmic pH is maintained between pH 7.8-8.5, we envision a substantial role for monovalent cation antiporters. This view is strengthened by an earlier hypothesis describing that antiport of proton vs. potassium/sodium contributes to energy generation via the ETC in alkaliphiles [94]. Indeed, to maintain cytoplasmic pH at pH 9.5 we identified nhaC and mrp for Na^+ extrusion and H^+ uptake (see **Figure** 2.4). However, to maintain cytoplasmic pH at pH 7.5 we identified a cation antiporter (nhaP) capable of importing K^+, and possibly Na^+, at the cost of exporting H^+ (**Figure** 2.4). If the balance of cations is wholly disrupted, H^+ export is undesirable, or H^+ import impossible, *C. thermarum* TA2.A1 has 'emergency override systems'. ATP-dependent uniporters for Na^+ (natCAB) and K^+ (pstS) are present for such a situation (see **Figure** 2.4).

2.3.10. REGULATORY SYSTEMS

C. thermarum TA2.A1 has three annotated toxin-antitoxin systems. Toxin/antitoxin systems are generally regarded as 'selfish genes' deriving from plasmids. It has been proposed that if after replication the plasmid is absent in the daughter cell, the toxin-antitoxin system promotes its own survival over the survival of the cell as a whole [95]. When integrated into a bacterial genome, they have also been shown to regulate translation as a response to environmental conditions, having some bearing on cell metabolism and cell death [96]. Taking this into account, the loci of the annotated toxin-antitoxin systems are intriguing. The toxin-antitoxin mazEF system was found just downstream of the operons for Ndh1 and the F_1F_o-ATP synthase. A second toxin-antitoxin ndoAI/ndoA system [97] was revealed by this new genome and found upstream of an operon encoding for tRNA's for asparagine, serine, glutamate, aspartate, glutamine and leucine. The third system, a doc toxin/antitoxin system (death on curing) is located just upstream of a few transporters, including that of the C4-dicarboxylates. We hypothesize that these systems might regulate expression, of for instance oxidative phosphorylation (mazEF), when environmental conditions become adverse, or even program cell-death of a subpopulation [98].

2.4. CONCLUSION

This article outlines the first report of a complete circular chromosome of the thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1. Considering the increase in genetic data of 15.5% over the previously available data, we decided to perform an in-depth analysis of its features and placement within a novel order. Consequently, we give a hypothesis for a putative origin region and we outline similarities to plant genomes. These similarities include a cytochrome b_6c_1 complex that is a possible homolog of the plant cytochrome b_6f, the similarity in F_1F_o-ATP synthase *c*-subunit rotor ring size, and the means to capture inorganic carbon. Furthermore, we outline a catabolic pathway *via* the oxidative TCA cycle, something that has not been reported yet, to the best of our knowledge. We also find interesting regulatory systems, such as CRISPRs and toxin/antitoxin systems, the latter of which could have

a bearing on regulation of cellular processes, like oxidative phosphorylation. Finally, we describe how its many monovalent cation antiporters are capable of enabling the facultative alkaliphilic lifestyle of *C. thermarum* TA2.A1. These features are just a tip of the iceberg of new data made available by this updated genome, indicating the value of continuously re-sequencing genomes present in the NCBI database, as our sequencing methods are ever-improving.

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3

ELEVATED ACYL-PG AND MENAQUINONE LEVELS STRUCTURALLY STABILIZE MEMBRANES TO MINIMIZE PROTON LEAKAGE IN *Caldalkalibacillus thermarum* TA2.A1

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ABSTRACT

Alkaliphiles must minimise proton leakage through the membrane to maintain a near-neutral internal pH in a proton scarce environment. Neutral lipids like triterpenes have been implicated to serve as an insulating layer to minimise proton loss. However, not all alkaliphiles have the genetic machinery to produce triterpenes. In this study, we present findings from chemostat cultivations and lipids analysis of the triterpene-negative thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1, cultivated at a pH range from 7.5 to a new pH_{max} of 11.0. Alkaline pH levels of 9.5 and above triggered the over-production of acylphosphatidylglycerols (acyl-PG), constituting up to a 26-fold increase compared to pH 7.5. Molecular dynamics simulations revealed that acyl-PG reduces the 'solvent accessible surface area' of the membrane by 15%, thereby mitigating proton leakage. Additionally, we observed a 8.5-fold increase in menaquinone concentration at pH 11 from pH 9.5, which we propose acts as insulating layer at highly alkaline pH, akin to the role of triterpenes. In light of these observations, we hypothesize that survival at alkaline pH can be facilitated by a dual mechanism involving membrane smoothening by acyl-PG and menaquinone-based proton-blocking. This work contributes to a deeper understanding of strategies employed by alkaliphiles to thrive in their extreme environments.

3.1. INTRODUCTION

Alkaliphiles reside in a proton scarce environment and face the challenge of maintaining a neutral or mildly alkaline internal pH in spite of this low external proton concentration. This paradox has two key implications: firstly, aerobic alkaliphiles must overcome an inverted proton gradient for respiration; secondly, they must limit proton leakage to a bare minimum. Extensive research into the cell wall of alkaliphiles uncovered a role for teichuronic acid in *Halalkalibacterium halodurans* [1–5], and for SlpA in *Alkalihalophilus pseudofirmus* [6]. Although a comprehensive characterization of the cell wall remains elusive, the observations in *H. halodurans* and *A. pseudofirmus* have identified a pattern of utilizing acidic residues in their cell walls, which suggests the capability of alkaliphiles to generate a localized, conductive proton motive force [7].

Minimizing proton permeability of the cell is a vital process in both energy conservation and pH homeostasis. Research into the membranes of alkaliphiles therefore primarily focussed on mechanisms to limit proton leakage. Employing branched-chain fatty acids as an additional proton barrier is suggested as viable strategy for alkaliphiles [8], such as reported for thermoalkaliphile *Anaerobranca gottschalkii* [9]. For organisms such as *A. pseudofirmus* and *Bacillus* sp. A 007 [10, 11] incorporation of up to 10% (mol/mol) neutral lipids like squalene, dihydrosqualene, and diacylglycerol has emerged as a notable feature. The orientation of these lipids, in the middle, perpendicular to the polar lipids that form the bilayer, creates an additional insulating layer that proton diffusion must overcome [8, 12]. Koga and colleagues observed a neutral lipids to polar lipids ratio of 4:6 [11]. While studies on alkaliphilic membrane composition highlighted specific lipids, overarching adaptations for alkaline conditions, compared to neutral ones, remain inadequately explored, particularly whilst using controlled conditions such as chemostat bioreactors.

Caldalkalibacillus thermarum TA2.A1, a gram-positive thermoalkaliphile, confronts the additional challenge formed by heat in parallel to high pH. It is an obligate thermophile and a facultative alkaliphile; the reported pH growth spans from pH 7.5 to at least pH 10, possibly extending to pH 10.5 [13]. *C. thermarum* TA2.A1's adaptation to this pH range, spanning a 1000-fold reduction in free proton concentration, remains an unsolved mystery. This environmental pressure is profound because aerobic cell respiration is proton-coupled [13, 14]. *C. thermarum* TA2.A1 respiratory proteins are highly adapted and regulated, with a host of mechanisms in various enzymes such as the F_1F_o ATP synthase to prevent proton loss [15–18].

Only two studies described the fatty acid composition for the *Caldalkalibacillus* genus [19, 20]. These investigations reported a prevalence of branched fatty acids, a common trait among thermophilic microorganisms [21]. Given its dependence of branched-chain fatty acids for thermophilic survival, *C. thermarum* TA2.A1 cannot utilize the strategy of tooling with the level of branching, nor can it synthesize the neutral triterpenes. This raises the intriguing question of

which adaptations it employs to thrive across such a broad pH range (from 7.5 to 10.5). To study this question, we conducted chemostat cultivations of *C. thermarum* TA2.A1 within this pH range and attempted to expand it beyond pH 10.5. In this study, we present the results of these cultivations, provide an overview of the membrane lipid composition, and support experimental results with a molecular dynamics simulation that compares the membrane at the extremities of the pH spectrum. Our research gives insights on the distinctive adaptations of *C. thermarum* TA2.A1 to thrive under alkaline conditions.

3.2. MATERIALS AND METHODS

3.2.1. CULTIVATION MEDIUM

C. thermarum TA2.A1 was cultured in a rich medium adapted from McMillan and coworkers [13]. The modified medium consisted of the following components: 10.0 g Tryptone Peptone (Difco), 10 g $C_{12}H_{22}O_{11}$, 9.0 g $NaHCO_3$, 0.2 g K_2HPO_4, 0.5 g Na_2SO_4, 0.1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7 H_2O$, $5.0 \cdot 10^{-5}$ g $MnCl_2 \cdot 4 H_2O$, $1.4 \cdot 10^{-5}$ g $ZnSO_4$ and $1.2 \cdot 10^{-5}$ g $Na_2MoO_4 \cdot 2 H_2O$. During bioreactor operation, 0.25 mL L^{-1} of Antifoam C (Sigma-Aldrich, Missouri, USA) was added to the medium. The medium, without sucrose, was autoclaved at 121°C for 15 minutes and sucrose was separately autoclaved at 110°C in a 50% (w/v) concentrate. Then, the sucrose was aseptically added to the autoclaved basal medium, which was concentrated accordingly to accommodate sucrose addition. The pH of the medium was set to the desired levels: either pH 7.5, 8.5, 9.5, 10.5 or 11. For pH 7.5, the $NaHCO_3$ was replaced with 6.26 g NaCl; for pH 10.5 and 11, the $NaHCO_3$ was replaced with 11.33 g Na_2CO_3.

3.2.2. BIOREACTOR OPERATION

Chemostat cultivation were conducted in a 3.0 L jacketed bioreactor (Applikon Biotechnology, the Netherlands), stirred at 800 rpm using two Rushton impellers attached to the shaft. Stirring was controlled by an ADI 1012 (Applikon Biotechnology, the Netherlands). To maintain a working volume of 1.0 L, fresh medium was continuously added, and effluent continuously removed. The target dilution rate inside the reactor was D = 0.1 h^1. In cases where reactor commencement with this dilution rate led to washout, the cultivation began at D = 0.03 h^1 after batch phase (see below) and was incrementally (0.015 h^{-1} increments every 1-4 days) raised to the target dilution rate of D = 0.1 h^1. Temperature was controlled at 65°C by an Ecoline Staredition E 300 thermostat (Lauda, Germany). The reactor was sparged at a rate of 0.1 L_n min^{-1} air. Off-gas was cooled using an RM6S Refrigerated Circulating Bath (Lauda, Germany) as a cryostat. Off-gas composition was quantified using an NGA 2000 off gas multiplexer (Rosemount Inc., Minnesota, USA). The pH was maintained by automatic addition of either 2 M H_2SO_4 or 2 M NaOH, controlled by an ADI 1030 Bio Controller (Applikon Biotechnology, the

Netherlands). pH was measured on-line by a 235 mm AppliSens pH+ probe (Applikon Biotechnology, the Netherlands).

For the inoculation procedure a fresh ± 1.6 mL glycerol stock of *C. thermarum* TA2.A1 was taken from a -80°C freezer and thawed. The same batch of glycerol stocks was used for all cultivations in this paper. Cells were reconstituted in 100 mL pH 9.5 medium in a 500 mL round bottom shake flask and incubated at 65°C and 140 rpm for 18 hours. Another 500 mL shake flask, this time with the pH of the medium of the chemostat cultivation, was inoculated with 2% of the overnight culture and also incubated for 18 hours. The second cultivation was used as reactor inoculum in its entirety. The initial batch phase in the reactor lasted roughly 18 hours for the pH 8.5, 9.5 and 10.5 chemostats. For the pH 7.5 and pH 11 chemostats, batch phase was extended to 24 hours. Following the batch phase, reactor was set to chemostat, as described above. Note that the pH 7.5 and pH 11 cultivations commenced at D = 0.03 h^{1}. To confirm steady state, samples were aseptically collected for optical density and dry weight measurements. A supernatant sample was stored at -20°C for potential future analysis. Provided off-gas profiles, optical density and dry weight measurements were stable for 3 consecutive retention times, steady state was assumed. Upon reaching steady state, approximately 400 mg of wet weight was harvested and freeze dried. The whole experimental procedure was conducted aseptically, with biological duplicates for all conditions.

3.2.3. ANALYTICAL METHODS & FREEZE DRYING

Optical density was determined at 600 nm wavelength (OD_{600}) in a Biochrom Ultrospec 2100 Pro UV Vis Spectrometer (Amersham, United Kingdom) with a 1 cm light path. 0.20 µm Supor® PES Membrane Disc Filters (Pall, New York, USA) were dried as preparation for dry weight measurements. A 10 mL sample was filtered under vacuum, and the filter was dried overnight at 105°C. Dry weight was determined by subtracting the weight of the pre dried filter from the weight of the dried filter with sample. The 400 mg sample intended for freeze drying was washed thrice with 50 mL of a 0.85% NaCl solution. After the third washing step, the pellet was once again resuspended in a 50 mL 0.85% NaCl solution and divided over two self-standing Falcon® tubes. The tubes were flash-frozen, while rotating on their sides, in liquid nitrogen and stored at -80°C. The rotating was conducted to maximize the surface area for freeze drying. Freeze drying took place in an Alpha 1-5 LDplus apparatus (Martin Christ, Germany) at 0.050 mbar and -50°C. The freeze-dried samples were collected afterwards and stored for subsequent analysis.

3.2.4. LIPID ANALYSIS

For fatty acid analysis, freeze-dried biomass was hydrolysed by refluxing with 1 N KOH in methanol (MeOH) solution for 1 hour. After neutralization with a 2 N HCl/MeOH (1/1, v/v) solution, the fatty acids were methylated with diazomethane in diethyl ether which was removed under a stream of

N_2. Before analysis the extracts were treated with pyridine (10 µL) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 10 µL) to derivatize alcohol groups and brought to a final volume with ethyl acetate to a concentration of 1 mg mL^{-1}. Fatty acid methyl ester (FAME) quantification was carried out on an Agilent 7890B gas chromatograph (GC; Agilent, Santa Clara, CA, United States) with an Agilent CP Sil-5 silica column (25 m × 0.32 mm) and helium as the carrier gas at a constant flow rate of 2 mL min^1. Initial oven temperature was 70°C, and increased first at a rate of 20°C min^1 to 130°C, and next at a rate of 4°C min^1 to the final temperature of 320°C, which was held for 10 mins. FAME identification was carried out on an Agilent 7890A GC coupled to an Agilent 5975C VL MSD mass spectrometer (MS) operated at 70 eV, with a mass range *m*/*z* 50–850 and 1.9 scans per second. The column and oven settings were the same as for the quantification GC analysis. FAMEs were identified based on literature data and library mass spectra.

Intact polar lipids (IPLs) were extracted from freeze-dried biomass using a modified Bligh-Dyer procedure [22]. Briefly, the biomass was extracted in a solvent mixture of MeOH, dichloromethane and phosphate buffer (2:1:0.8, v:v:v) in an ultrasonic bath for 10 min and then the supernatant was removed by centrifugation (3000 rpm, 2 mins). This was repeated two more times. The combined supernatants were then phase-separated by adding additional dichloromethane and phosphate buffer to a final solvent ratio of 1:1:0.9 (v:v:v). The organic phase containing the IPLs was collected and the aqueous phase re-extracted two times with dichloromethane. The biomass residue was then re-extracted following the same procedure but starting with a solvent mix of MeOH, dichloromethane and trichloroacetic acid pH 2-3 (2:1:0.8, v:v:v). Finally, the combined extract was dried under a stream of N_2 gas. Before analysis, the extract was redissolved in a mixture of MeOH:dichloromethane (9:1, v:v). Subsequently, aliquots were filtered through 0.45 µm regenerated cellulose syringe filters (4 mm diameter; Grace Alltech, Deerfield, IL, United States). Analysis was carried out using Ultra High Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) according to the reversed phase method of [23] with modifications as per [22]. We used an Agilent 1290 Infinity I UHPLC equipped with a temperature-controlled auto-injector and column oven, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (ESI) probe (Thermo Fisher Scientific, Waltham, MA, United States). Separation was achieved on an Acquity BEH C18 column (2.1 x 150 mm, 1.7 µm; Waters Corporation, Milford, MA, United States) maintained at 30°C. The eluent composition was (A) MeOH/H_2O/formic acid/14.8 M NH_{3aq} (85:15:0.12:0.04 [v:v]) and (B) isopropanol/MeOH/formic acid/14.8 M NH_{3aq} (50:50:0.12:0.04 [v:v]). The elution program was: 5% B for 3 min, followed by a linear gradient to 60% B at 12 min and then to 100% B at 50 min, this was maintained until 80 min. The flow rate was 0.2 mL min^{-1}. Positive ion ESI settings were: capillary temperature, 300°C; sheath gas (N_2) pressure, 40 arbitrary units (AU); auxiliary gas (N_2) pressure, 10 AU; spray voltage, 4.5 kV; probe heater temperature, 50°C;

S-lens 70 V. Target lipids were analysed with a mass range of *m*/*z* 350–2000 (resolving power 70,000 ppm at m/z 200), followed by data-dependent MS^2 (resolving power 17,500 ppm), in which the ten most abundant masses in the mass spectrum were fragmented successively (stepped normalized collision energy 15, 22.5, 30; isolation width, 1.0 *m*/*z*). The MS was calibrated within a mass accuracy range of 1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution. During analysis dynamic exclusion was used to temporarily exclude masses (for 6 s) in order to allow selection of less abundant ions for MS^2. IPLs were quantified in terms of their MS peak area response. The peak areas were determined from extracted ion chromatograms of the combined $[M+H]^+$, $[M+NH4]^+$ and $[M+Na]^+$ ion (where present) for each individual IPL species. The raw peaks areas (see supporting data) underwent an approximate correction based on the relative response of three standards: (S,R)sn-(3-myristoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3′-(1′,2′-dimyristoyl)-glycerol (for the acylphosphatidylglycerols), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (for the phospholipids) and 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-glycerol (for the cardiolipins). The amount of quinone was corrected based arbitrarily on the response behaviour of the cardiolipin standard.

3.2.5. MOLECULAR DYNAMICS

Atomistic models of the lipid bilayers for the Molecular Dynamics (MD) simulations were prepared using the CHARMM-GUI Membrane Bilayer tool [24–27]. The online graphical user interface facilitated easy utilization of the original CHARMM software [24, 28, 29]. A rectangular lipid bilayer was generated with 200 lipids per leaflet, with an equal composition for both leaflets. For simplification, each type of lipid detected in the experiments was modelled with a set chain length, thus yielding one representative type per category; see **Supplementary Table I**, which is calculated based on the experimentally observed changes in the lipid distribution (**Table 3.1**). A water layer of 20 Å thickness was added on both sides of the membrane. An ion concentration of 0.1 M Na_2CO_3 was used as for the pH 10.5 and pH 11 simulations; whilst 0.1 M NaCl was used for the pH 7.5 simulation, all in accordance with culturing conditions. The resulting lipid bilayer models were used as input for molecular dynamics, where the lipid and ions were represented by the CHARMM36m force field and water by de TIP3P force field. The simulations were performed in GROMACS 2021.3-spack [30–34] for 105 ns with a time steps of 1 fs. A Particle-mesh Ewald algorithm with a 1.2 nm cut-off was used for electrostatic interactions [35]. A reciprocal grid of 120 x 120 x 64 cells was used with 4^{th} order B-spline interpolation. A cut-off of 1.2 nm was used for Van der Waals interactions. Temperature was kept constant at 338.15 K with a Nose-Hoover thermostat [36, 37]. Atmospheric pressure was maintained with the Parrinello-Rahman barostat [38, 39].

To produce density plots, the '*gmx make_ndx*' tool generated separate groups for water, total lipids, polar lipid headgroups, polar lipid chains and

menaquinone. '*gmx density*' was used to calculate the density profile for each group in each condition. Density profiles were centred around the lipid bilayer. To approximate surface area, '*gmx sasa*' (sasa = solvent accessible surface area) was used [40]. Images of lipid bilayers were produced with Visual Molecular Dynamics (VMD) software.

3.3. RESULTS

3.3.1. CULTIVATION OF C. thermarum TA2.A1

For investigating the role of specific lipids in facilitating growth at high pH in *C. thermarum* TA2.A1, we first aimed to determine the limits of growth in a chemostat operating at D = 0.1 h^{-1}. Previous research reported that the boundaries of growth were pH 7.7 and pH 10.5 [41]. The results of Peddie and coworkers [41] were based on shake flask batch cultures, which lacks pH control. Results of McMillan and colleagues were in batch bioreactors without active aeration [13]. Chemostat bioreactor operation controls pH and aeration. Additionally, considering continuous broth removal, cells must divide at the set conditions. Our study discovered that the minimum pH at which growth was possible was pH 7.5 and the maximum was pH 11. Within this range, no large deviations in biomass yields nor in oxygen consumption were observed. Next to cultivations at pH 7.5 and 11, cultivations at pH 8.5, 9.5 and 10.5 were also performed. Once steady state was confirmed, cells were sampled and freeze dried for lipids analysis.

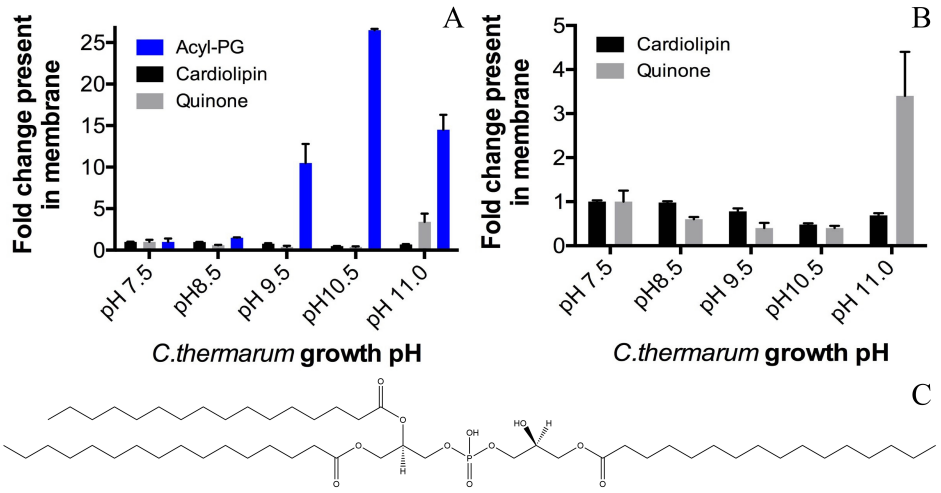

Figure 3.1: Fold changes of Acyl-PG (**A**) and cardiolipin and quinone (**A** and **B**) abundances relative to pH 7.5. Natta projection (**C**) of an all $C_{16:0}$ acyl-PG: 1,2-dipentadecanoyl-*sn*-glycero-3-phospho-(3'-pentadecanoyl)-1'-*sn*-glycerol (also used in molecular dynamics simulations in this study.

3.3.2. POLAR LIPID COMPOSITION

The strain contained menaquinone MK7:7 (MQ_7), identified from its accurate mass and characteristic fragment ion in MS^2 [42]. The major intact polar lipids observed were a range of commonly-observed glycerophospholipids, predominately phosphatidylcholine (PC), and phosphatidylethanolamine (PE) with trace amounts of phosphatidylglycerol (PG), methylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE) (**Table 3.1**; trace components that represented < 0.1% of the total lipids are not given.). These ranged in their total acyl carbon number between 28 and 34. Additionally, a range of cardiolipins were observed, ranging in their total acyl carbon number between 60 and 68. The composition of the phospholipids and cardiolipins saw little change over the pH range. Eluting between the phospholipids and cardiolipins was a series of acylphosphatidylglycerols (acyl-PGs; **Figure 3.1C**; **Supplementary Table II**). From pH 7.5 to pH 10.5, the relative proportion of the acyl-PGs increased with increasing pH, while the proportion of cardiolipins decreased. At pH 11, the relative proportion of MQ_7 increased substantially, whilst the acyl-PG proportion decreased (**Table 3.1**, **Figure 3.1A & 1B**).

3.3.3. FATTY ACID COMPOSITION

The core lipid profile (base hydrolysed) is given in **Table 3.2**. The major fatty acids detected were *iso*-$C_{17:0}$ and *iso*-$C_{15:0}$ with lower amounts of *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$. The detection of *anteiso* and $C_{16:0}$ lipids is an addition to what was reported earlier for *C. thermarum* [19]. A range of other fatty acids present in low amounts (generally < 1% of the total) are also given in **Table 3.2**. Trace components that represented < 0.1% of the total lipids are not given. Overall, the distribution was quite stable for the pH 7.5-10.5 experiments. At pH 11, the fatty acids chain length profile leant towards slight shorter chains.

3.3.4. MOLECULAR DYNAMICS

To gain insights into the effect of elevated acyl-PG concentrations and of elevated MQ_7 concentrations in the membrane, we performed a molecular dynamics simulation. Considering the objectives of testing elevated acyl-PG and MQ_7, only the pH 7.5, pH 10.5 and pH 11 membrane compositions were simulated. The pH 7.5 lipid composition served as the reference simulation. Simulations of the pH 10.5 lipid composition allowed for studying the effect of elevated concentrations of acyl-PG. The pH 11 membrane simulations uncovered the contribution of increased MQ_7 levels. All models were simulated for 105 ns to provide ample time for stabilization of quinones (Kaurola et al., 2016). Mean Squared Displacement (MSD) and Root Mean Squared Displacement (RMSD) analysis validated the stability of conducted simulations

Table 3.1: IPL composition of C. thermarum TA2.A1 in chemostat bioreactors under various pH levels. Displayed values are relative abundances of biological replicates. AEC = Assigned elemental composition; mmu = milli mass unit; Δ mmu = (calculated mass - observed mass –) x 1000. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, MQ7 = menaquinone 7:7. Trace components that represented < 0.1% of the total lipids are not given. In the lipid name, x:y denotes the total of acyl carbon atoms (x) and the number of unsaturations (y). All masses and AECs represent an [M·+·H]+ ion except PCs which are [M]+

Lipid	AEC	Calc. mass	Obs. mass	Δ mmu	pH 7.5	pH 8.5	pH 9.5	pH 10.5	pH 11
PE 33:0	$C_{38}H_{77}NO_8P$	706.5381	706.5390	-0.9	0.7 ± 0.3	0.6 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
PE 34:0	$C_{39}H_{79}NO_8P$	720.5538	720.5548	-1.0	1.2 ± 0.1	0.6 ± 0.7	0.7 ± 0.1	0.4 ± 0.0	0.1 ± 0.0
				Total	**1.9 ± 0.3**	**1.2 ± 0.8**	**1.0 ± 0.1**	**0.5 ± 0.0**	**0.2 ± 0.0**
PC 30:0	$C_{38}H_{77}NO_8P$	706.5381	706.5386	-0.5	2.0 ± 0.1	2.5 ± 0.2	2.6 ± 0.0	1.7 ± 0.4	2.7 ± 1.1
PC 31:0	$C_{39}H_{79}NO_8P$	720.5538	720.5549	-1.1	0.9 ± 1.0	1.7 ± 0.2	1.3 ± 0.1	0.9 ± 0.2	2.4 ± 1.2
PC 32:0	$C_{40}H_{81}NO_8P$	734.5694	734.5704	-1.0	4.5 ± 0.0	4.9 ± 0.5	4.9 ± 1.1	3.4 ± 0.6	3.6 ± 1.0
PC 33:0	$C_{41}H_{83}NO_8P$	748.5851	748.5862	-1.1	3.3 ± 0.6	2.8 ± 0.3	1.9 ± 0.3	1.7 ± 0.2	2.3 ± 0.8
PC 34:0	$C_{42}H_{85}NO_8P$	762.6007	762.6018	-1.1	4.5 ± 0.6	5.1 ± 0.7	4.5 ± 1.1	3.4 ± 0.5	2.1 ± 0.0
				Total	**15.3 ± 1.0**	**17.0 ± 1.9**	**15.2 ± 2.3**	**11.1 ± 2.0**	**13.1 ± 4.0**
PG-acyl*	$C_{53}H_{107}NO_{11}P$	964.7576	964.7590	-1.4	0.4 ± 0.3	0.8 ± 0.1	8.8 ± 5.3	13.9 ± 0.5	10.9 ± 1.1
PG-acyl*	$C_{54}CH_{109}NO_{11}P$	978.7733	978.7745	-1.2	0.1 ± 0.1	0.2 ± 0.0	1.1 ± 0.5	3.5 ± 0.9	4.8 ± 0.7
PG-acyl*	$C_{54}H_{109}NO_{11}P$	978.7733	978.7750	-1.7	0.1 ± 0.1	0.2 ± 0.0	1.0 ± 0.0	3.6 ± 0.7	1.2 ± 0.6
PG-acyl*	$C_{55}H_{111}NO_{11}P$	992.7889	992.7907	-1.8	1.2 ± 0.7	1.5 ± 0.2	9.7 ± 1.1	19.9 ± 0.9	7.4 ± 3.3
PG-acyl*	$C_{56}H_{113}NO_{11}P$	1006.8050	1006.8070	-2.0	0.2 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	2.0 ± 0.7	1.0 ± 0.4
PG-acyl*	$C_{56}H_{113}NO_{11}P$	1006.8050	1006.8060	-1.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	1.2 ± 0.5	0.3 ± 0.0
PG-acyl*	$C_{57}H_{115}NO_{11}P$	1020.820	1020.8210	-0.7	0.8 ± 0.0	0.6 ± 0.1	1.3 ± 0.3	4.5 ± 1.2	1.7 ± 0.8
				Total	**3.0 ± 1.3**	**3.6 ± 0.1**	**22.6 ± 7.1**	**48.6± 4.4**	**27.3 ± 5.6**
Cardiolipin 62:0	$C_{71}H_{142}NO_{17}P_2$	1342.9750	1342.9770	-2.2	10.8 ± 1.6	12.7 ± 0.7	12.8 ± 0.9	6.8 ± 1.4	12.9 ± 3.3
Cardiolipin 63:0	$C_{72}H_{144}NO_{17}P_2$	1356.9900	1356.9930	-2.5	5.8 ± 0.5	6.7 ± 0.3	5.4 ± 1.2	3.8 ± 0.2	10.0 ± 3.9
Cardiolipin 64:0	$C_{73}H_{146}NO_{17}P_2$	1371.0060	1371.0090	-2.9	16.9 ± 0.9	18.3 ± 0.2	16.8 ± 1.4	10.5 ± 1.1	12.4 ± 2.2
Cardiolipin 65:0	$C_{74}H_{148}NO_{17}P_2$	1385.0220	1385.0240	-2.7	11.1 ± 0.1	9.0 ± 1.5	6.6 ± 0.5	4.7 ± 0.4	7.5 ± 0.3
Cardiolipin 66:0	$C_{75}H_{150}NO_{17}P_2$	1399.0370	1399.0400	-2.3	16.8 ± 0.4	17.1 ± 0.5	12.8 ± 3.0	9.1 ± 0.2	8.5 ± 3.7
Cardiolipin 67:0	$C_{76}H_{152}NO_{17}P_2$	1413.0530	1413.0550	-1.6	7.8 ± 0.1	6.1 ± 0.3	2.7 ± 0.2	1.2 ± 1.2	2.0 ± 1.0
Cardiolipin 68:0	$C_{77}H_{154}NO_{17}P_2$	1427.0690	1427.0700	-0.9	8.7 ± 0.0	6.7 ± 0.1	3.4 ± 2.0	3.1 ± 1.0	0.8 ± 1.0
				Total	**77.9 ± 2.2**	**76.6 ± 2.5**	**60.5 ± 5.1**	**39.2 ± 2.4**	**54.0 ± 3.9**
MQ-7	$C_{46}H_{65}O_2$	649.4979	649.4993	-1.4	**1.9 ± 0.5**	**1.6 ± 0.1**	**0.7 ± 0.4**	**0.6 ± 0.1**	**5.4 ± 2.3**

Table 3.2: Fatty acids profile of *C. thermarum* TA2.A1 in chemostat bioreactors under various pH levels. Displayed values are relative mass fractions of biological replicates.

	pH 7.5	**pH 8.5**	**pH 9.5**	**pH 10.5**	**pH 11**
iso $C_{14:0}$	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
$C_{14:0}$	0.0 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
iso $C_{15:0}$	21.8 ± 0.2	24.2 ± 0.5	30.3 ± 7.0	22.8 ± 1.9	36.0 ± 4.4
anteiso $C_{15:0}$	3.6 ± 0.2	3.5 ± 0.2	4.8 ± 0.6	5.5 ± 0.4	6.8 ± 6.4
$C_{15:0}$	0.5 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.1 ± 0.7
diMe $C_{14:0}$	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.6
4,12-diMe $C_{14:0}$	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
iso $C_{16:0}$	5.8 ± 1.0	6.0 ± 0.6	4.4 ± 0.3	5.3 ± 0.4	14.3 ± 6.7
$C_{16:0}$	3.1 ± 0.4	4.2 ± 0.6	2.5 ± 1.3	5.0 ± 0.5	1.7 ± 0.9
iso $C_{17:0}$	52.5 ± 1.4	51.9 ± 0.1	47.9 ± 4.7	47.8 ± 1.0	33.1 ± 14.9
anteiso $C_{17:0}$	7.4 ± 0.1	6.9 ± 0.6	7.3 ± 1.3	10.2 ± 0.5	6.1 ± 5.8
$C_{17:0}$	0.7 ± 0.1	0.8 ± 0.0	0.4 ± 0.2	0.5 ± 0.1	0.0 ± 0.0
diMe $C_{16:0}$	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.0 ± 0.0
4,14-diMe $C_{16:0}$	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.0 ± 0.0
iso $C_{18:0}$	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
$C_{18:0}$	3.6 ± 0.7	0.1 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	0.0 ± 0.0

(**Supplementary Figure I**).

For assessment of membrane adaptations, we employed two key analysis methods. One method compared the densities of water, the polar lipid headgroups, the polar lipid chains and MQ_7 and provided insights into how the membrane accommodates lipidome changes. Due to their hydrophobicity, quinones prefer to be located within the lipid bilayer [43] and the density profiles show a peak MQ_7 in the centre (**Figure 3.2A – 3.2C**). In the centre of the simulated pH 11 lipid bilayer, MQ_7 density increases, whilst the density of the polar lipid chains decreases. The combined density of polar lipid chains and MQ_7 in the centre of the two leaflets is roughly 660 kg/m^3 for all three simulations.

However, at pH 11, MQ_7 contributes 7.9% to the total density, compared to just 2.2% for pH 10.5 and 0.1% for pH 7.5. Visual inspection of the lipid membranes of pH 7.5, featuring the acyl-PGs and MQ_7 (**Figure 3.3**), and pH 11, displaying solely MQ_7 (**Figure 3.4**), further emphasizes the increased levels of MQ_7 at pH 11. At pH 11, the MQ_7 are present at a higher concentration over the entire intralayer surface. **Supplementary Figure II** displays the distribution of acyl-PGs in the pH 10.5 membrane, the condition at which acyl-PG levels are highest.

Regarding lipid headgroups, the decrease in cardiolipin content corresponds to an increase in acyl-PGs. This rearrangement led to a reduction in the

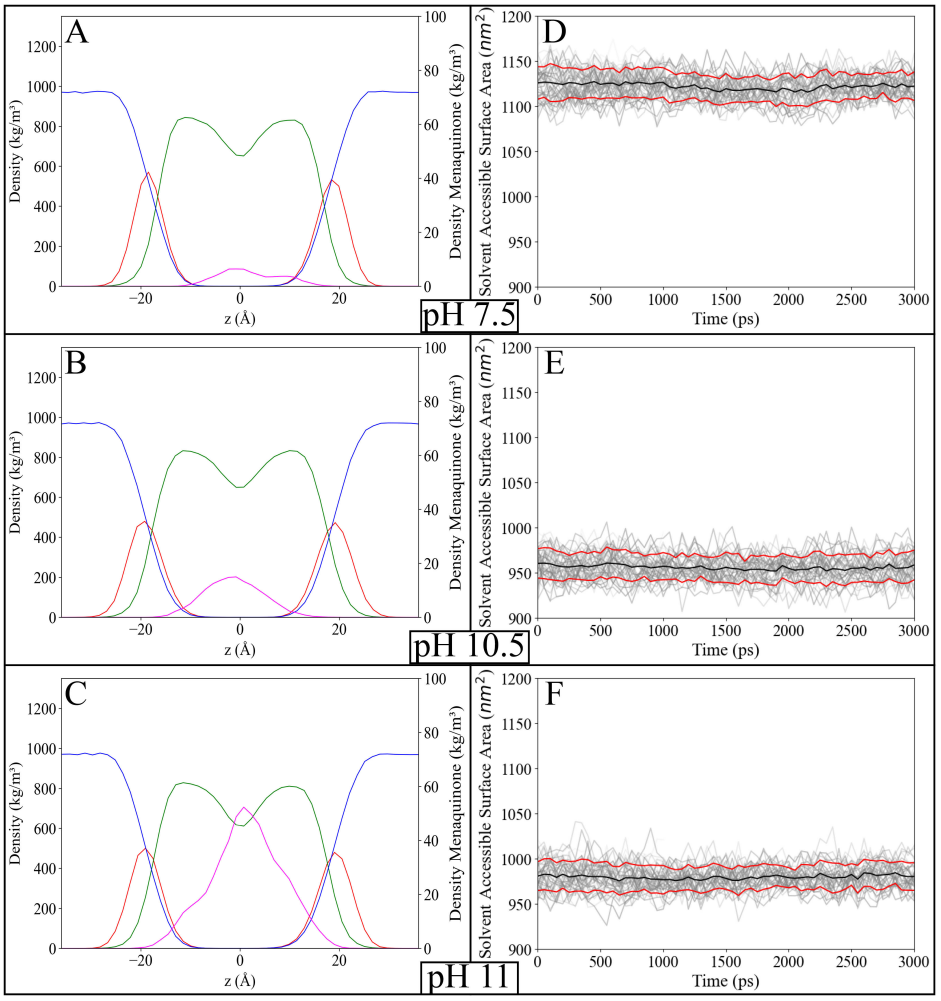

Figure 3.2: Results of molecular dynamics simulations in GROMACS. Density profiles of water (blue), polar lipid headgroup s(red), polar lipid aliphatic chains (green) and menaquinone (pink) of pH 7.5 (**A**), pH 10.5 (**B**) and pH 11 (**C**) simulated membranes. Ions are not shown. Water and both polar lipid groups share the left y-axis, whilst menaquinone is plotted on the right y-axis. Profiles were calculated only over the final 3 of the 105 ns of simulations. Solvent Accessible Surface Area of pH 7.5 (**D**), pH 10.5 (**E**) and pH 11 (**F**) simulated membranes. Plotted are all 45 segments of the trajectory in grayscale, from which the average Solvent Accessible Surface Area is calculated in black (with standard deviation in red). Note that the first 15 segments of the trajectory are 1 ns each, whilst the following 30 are 3 ns each.

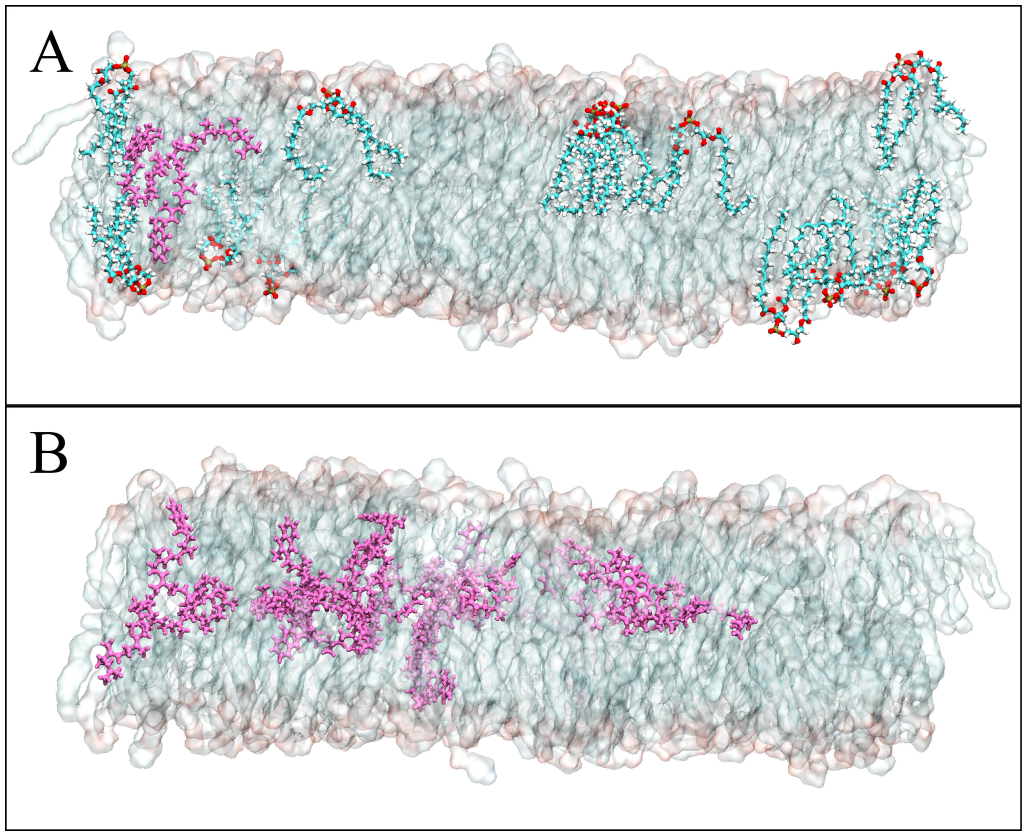

Figure 3.3: Illustration of the membrane of *C. thermarum* TA2.A1 at pH 7.5 (**A**) and pH 11 (**B**). Shown in pink is menaquinone-7 (absolute abundance = 2 (pH 7.5) or 16 (pH 11) out of 400 lipids) and only shown in the pH 7.5 picture, in default lipid colours, are the acyl-PGs (absolute abundance = 14 (pH 7.5) out of 400). Breakdown of default lipid colours: aqua is carbon, white is hydrogen, red is oxygen and yellow is phosphorus. The transparent background matter is the rest of the simulated membrane. The frame used for pH 7.5 is that after 87 ns of simulation, while a frame after 103 ns of simulation was taken for the pH 11 illustration.

maximum surface density at the lipid bilayer. The second method, the 'solvent accessible surface area' (SASA) gave a measure for the roughness of the membrane. The reference pH 7.5 membrane SASA averaged 1122 ± 16 nm^2 for the entire bilayer surface (**Figure 3.2D**). Upon introduction of elevated acyl-PG levels, the SASA decreased to roughly 956 ± 15 nm^2 and 980 ± 15 nm^2 for the pH 10.5 and pH 11 simulations, respectively (**Figure 3.2E** & **3.2F**). The experimental and computational results regarding the lipid membrane adaptations of *C. thermarum* TA2.A1 warrant further discussion concerning the adaptations made by this alkaliphile.

3.4. DISCUSSION

Alkaliphiles must prevent proton leakage in order to survive. This challenge becomes more pronounced at higher pH levels. In this light, we discuss the adaptations found within this study. The discussion focusses on: the tendency towards shorter fatty acids at pH 11, the increased MQ_7 concentration at pH 11, and the role of acyl-PGs at pH 9.5 and above.

3.4.1. FATTY ACID REARRANGEMENT AT PH 11

The fatty acid profile of *C. thermarum* TA2.A1, featuring branched-chains with lengths ranging from C_{15} to C_{17}, displays characteristics typical of thermophiles [21]. The composition of the fatty acids profile as detected in this study, is largely stable between pH 7.5 and pH 10.5 and in line with previous findings [19]. However, at pH 11, the concentrations of *iso*-C17:0 and *anteiso*-C17:0 decrease, supplanted by increased levels of *iso*-$C_{16:0}$ and *iso*-$C_{15:0}$. Shorter acyl chain lengths are linked to higher membrane permeability [44]. Since the penalty for proton leakage is steepest at pH 11, the shortening of acyl chain length appears counterintuitive. Plausibly, the shortened acyl chain length is a result of the increased MQ_7 content. **Figures 3.3** and **3.4** showed that at pH 11, MQ_7 takes up considerable space within the centre of the lipid bilayer. The dimensions of membrane proteins will be independent of the growth pH, thus we therefore argue that the total membrane is unlikely to change. Thus, this adaptation appears to be driven by a trade-off between higher amounts of MQ_7 and the shortening of acyl chain lengths. Menaquinone as insulating layer Alkaliphiles frequently opt to incorporate triterpenes in their membrane, most notably squalene, as means to bolster membrane integrity [10, 11, 45]. The ability to produce significant quantities of neutral lipids has been suggested to serve as a basis for (obligate) alkaliphily [46]. The prevalent theory is that the isoprenoids are located in the middle of the lipid bilayer, oriented perpendicular to the phospholipid bilayer [12]. Due to the orientation, the neutral lipids act as an insulating layer, providing additional protection against proton leakage. *C. thermarum* TA2.A1 harbours the terpenoid backbone biosynthesis pathway [14], but like most species within the Bacillota phylum, lacks any genes required for subsequent triterpene biosynthesis, [47, 48]. Instead, the bacterium uses

the terpenoid backbone for MQ_7 synthesis.

Quinones primary function is to serve as an electron carrier within the electron transport chain [49, 50]. They are neutral lipids, though capable of accepting electrons, evidenced by their contribution to respiration [51, 52]. Protons are incorporated in tandem with the electrons to maintain the neutral charge. *C. thermarum* TA2.A1 contains exclusively MQ_7 as its electron-mediating component [19]. The core responsibility of MQ_7 within this bacterium is to shuttle electrons from the primary type I and type II NADH dehydrogenases and from the succinate dehydrogenase towards the menaquinol:cytochrome *c* b_6c_1 oxidoreductase complex [14, 53]. Our previous works on this organism showed that the respiratory chain is expressed and most active at pH 9.5 [13, 17, 54], meaning that the MQ_7 levels detected at pH 9.5 in this study are sufficient to drive cell respiration. pH 9.5 is the level at which where the F_1F_o ATP synthase optimally functions [17]. Thus, the lack of additional oxygen consumption at pH 11 indicates an alternative role for increased MQ_7 concentrations. One notable aspect our molecular dynamics study indicates is that MQ_7, like the triterpenes, is located at the centre of the lipid bilayer [55]. Our hypothesis is that the increased MQ_7 concentration acts as insulating layer much like triterpenes function in other alkaliphiles. Whether the ability of quinones to accept protons has any influence on this proposed barrier function is unknown. A review on proton leakage suggested that prokaryotes might modulate ubiquinone levels with the aim of controlling proton leakage through the (inner) membrane [56] and this research further alludes to that function. Although *in vitro* experiments should still validate this hypothesis, the current research adds depth to the potential multifunctional roles of quinones.

3.4.2. PROPOSED ROLE OF ACYLPHOSPHATIDYLGLYCEROLS (ACYL-PGS)

A notable discovery in our dataset is the detection of a group of acylphosphatidylglycerols (acyl-PGs). Containing three acyl chains, the presence of acyl-PGs has been confirmed in a number of organisms, of which none are alkaliphiles. The organisms harbouring acyl-PGs are (in chronological order of discovery) *Salmonella tryphimurium*, *Pseudoalteromonas espejiana*, *Escherichia coli*, *Caulobacter crescentus* and *Corynebacterium amycolatum* [57–65]. For most bacteria, the acyl-PG is a minor component of the membrane. In *C. amycolatum* and *Corynebacterium* sp. in general, acyl-PGs are a major component of the lipid membrane [66]. None of the studies mentioned above elaborated on the need for incorporating acyl-PG. In *Acinetobacter radioresistens* and in Roseobacteria species, phosphorus limitation was implied to induce acylphosphatidylglyceride biosynthesis [67, 68]. Luo and coworkers hypothesized that the acyl-PG was solely incorporated in the outer leaflet of the membrane, opposite to cardiolipin [67]. Since this lipid takes up less core lipid space than cardiolipin *per* headgroup, the authors suggested that the acyl-PG would aid in stabilizing the curvature of the

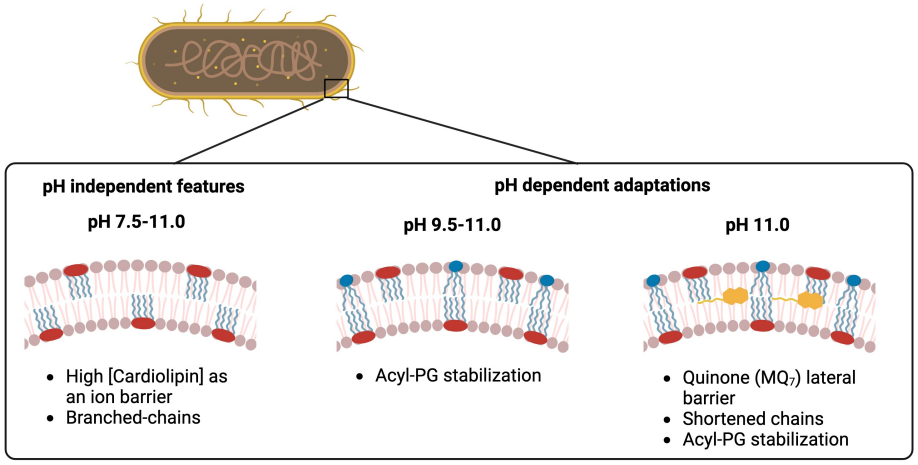

Figure 3.4: Proposed mechanisms to limit proton leakage in *C. thermarum* TA2.A1. At pH 7.5, *C. thermarum* TA2.A1 has an ordinary membrane consisting of cardiolipin and phospholipids, with branched-chain fatty acids, conforming to thermophile requirements. At pH 9.5 and above, significant quantities (> 2 %) of acyl-PGs are included to stabilize the membrane in the curved regions. At pH 11, menaquinone levels are increased to act as an insulating layer, thereby further minimizing proton leakage.

membrane. This hypothesis was later also suggested for *E. coli* [69]. Though both these organisms are gram-negatives, this explanation might extend to the curved margins of a gram positive. This hypothesis is supported by our molecular dynamics simulations, and in this acyl-PG seems to address this challenge by plugging these gaps in curved areas. This adaptation aligns with the observations of decreased solvent accessible surface areas at elevated pH conditions. The incorporation of acyl-PG could thus serve to counteract proton leakage, contributing to membrane stability.

3.4.3. BIOSYNTHESIS OF ACYLPHOSPHATIDYLGLYCEROLS

Little is known about bacterial acyl-PG biosynthesis. In *E. coli*, acyl-PG is produced *via* two different methods. One candidate is PldB, which catalyses a one-step reaction in the inner membrane, transferring one acyl chain from lyso-phoshatidylglycerol or lyso-phosphatidylethanolamine to

phosphatidylglycerol. The other candidate is PagP, which also catalyses a one-step reaction, but in the outer membrane. In the case of PagP, the donating lipid is phosphatidylglycerol [70]. N-acyl-phosphatidylethanolamine (NAPE) synthase from plants forms an alternative method of catalysis [71]. This membrane bound enzyme scavenges free fatty acids and transfers them onto phosphatidylethanolamine. BLAST detected no alignment for a NAPE synthase from *Arabidopsis thaliana* [72]. Regardless, examination of the *C. thermarum* TA2.A1 genome fails to reveal homologs of PldB, PagP, or NAPE and the gene(s) responsible for the biosynthesis of the acyl PG in *C. thermarum* TA2.A1 remain elusive.

3.4.4. Molecular adaptations of C. thermarum TA2.A1 to mitigate proton leakage

Collectively, the three adaptations identified in our study – fatty acid chain length alterations, increased MQ_7 concentration, and the presence of acyl-PGs – shed light on the mechanisms that *C. thermarum* TA2.A1 employs to ensure membrane stability and impermeability. The content of cardiolipin in the membrane of *C. thermarum* TA2.A1 decreases as pH increases. Considering that, cardiolipin content is unlikely to be an adaptation to alkaline conditions, as was stated before [73]. *A. pseudofirmus* OF4 has 25% cardiolipin at pH 10.5 and 20% cardiolipin at pH 7.5 making it tempting to speculate cardiolipin is a pH-adaptive lipid. However, equally, *A. pseudofirmus* OF1 has 8% cardiolipin at pH 10.5, but 13% cardiolipin at pH 7.5 [10]. We note that neutrophilic E. coli growing at pH 7.5 contains 9.7% cardiolipin [74] and that *Bacillus subtilis* contains 4% cardiolipin at pH 7.0 [10]. We also note follow-up work on a mutant of *A. pseudofirmus* that further debunked the cardiolipin theory [75]. Therefore, we propose that cardiolipin is acting more as an agent to stiffen the bilayer and by merit of this core function also acts to limit ion limitation. At pH 7.5, the membrane is relatively uncomplicated, relying on cardiolipin and various phospholipids for survival (**Figure 3.4**). At pH 9.5 and above, the production of acyl-PGs increases to maintain the integrity of the membrane surface in the curved regions (**Figure 3.4**). At the extreme pH 11, *C. thermarum* TA2.A1 requires the additional measure of increased MQ_7 concentrations to act as an insulating layer (**Figure 3.4**).

Our inability to grow *C. thermarum* TA2.A1 stably above pH 11 underscores the limit of these measures to minimize proton leakage. It will be interesting to evaluate if the use of quinones to reduce proton leakage is a common phenomenon in other organisms to both resist alkaline, but also acidic conditions. Additionally, the role of (other) neutral lipids to combat extreme conditions should also be explored. Overall, these insights enrich our understanding of the intricate adaptations alkaliphilic membranes undergo to secure the survival of organisms like *C. thermarum* TA2.A1.

3.5. ACKNOWLEDGMENTS

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4

MEMBRANE PROTEOME OF THE THERMOALKALIPHILE *Caldalkalibacillus thermarum* TA2.A1

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ABSTRACT

Proteomics has greatly advanced the understanding of the cellular biochemistry of microorganisms. The thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1 is an organism of interest for studies into how alkaliphiles adapt to their extreme lifestyles, as it can grow from pH 7.5 to pH 11. Within most classes of microbes, the membrane-bound electron transport chain (ETC) enables a great degree of adaptability and is a key part of metabolic adaptation. Knowing what membrane proteins are generally expressed is crucial as a benchmark for further studies. Unfortunately, membrane proteins are the category of proteins hardest to detect using conventional cellular proteomics protocols. In part, this is due to the hydrophobicity of membrane proteins as well as their general lower absolute abundance, which hinders detection. Here, we performed a combination of whole cell lysate proteomics and proteomics of membrane extracts solubilised with either SDS or FOS-choline-12 at various temperatures. The combined methods led to the detection of 158 membrane proteins containing at least a single transmembrane helix (TMH). Within this data set we revealed a full oxidative phosphorylation pathway as well as an alternative NADH dehydrogenase type II (Ndh2) and a microaerophilic cytochrome oxidase ba_3. We also observed *C. thermarum* TA2.A1 expressing transporters for ectoine and glycine betaine, compounds that are known osmolytes that may assist in maintaining a near neutral internal pH when the external pH is highly alkaline.

4.1. INTRODUCTION

The biological membrane is where the cell meets its environment, forming the boundary between the cytoplasm and the outside world, and also at the point where the cell senses environmental changes. They are composed of proteins, lipids, lipid-like molecules and sugars. The way in which the cell senses these changes are through membrane embedded and membrane associated proteins [1]. Proteins in the membrane have diverse functions, from the obvious bioenergetics processes, such as facilitating active transport of useful solutes into the cell, through to broader regulatory functions. Dual function is also common, such as for an enzyme found in the membrane-bound pathway for cellular respiration, an enzyme known as the F_1F_o ATP synthase. On one hand its purpose is to harness both a proton or sodium gradient alongside membrane potential to regenerate ATP, a form of chemical cellular energy, on another hand it is also thought to regulate cytoplasmic pH [2].

The membranes of extremophiles are even more important as a physical barrier, as they have a much more protective role from the adverse environment outside the cell. For example, conditions such as high concentrations or high temperatures, high salinity, extreme acidity or alkalinity are common in extreme environments. Thermophiles adapt by producing more saturated lipids or by branching fatty acids and sometimes lipids may have complex structures [3–5], increasing their rigidity [4]. With alkaliphiles the defining environmental pressure is a lack of environmental proton availability. This leads to a situation where there is a lower pH inside the cell, than outside the cell – a so-called 'inverted pH gradient'. In aerobic alkaliphiles this is a mountainous challenge and a complete bioenergetics conundrum, because cellular energy generation is coupled to this inverted proton gradient [6–8]. When these two environmental pressures are combined a given organism the membrane is theoretically very sensitive to leakage, so it must prevent the loss of protons to the outside environment [8–11]. Adaptations to minimize proton leakage are localised to the membrane [6, 12]. The architecture of the lipid bilayer is important for the protective properties, but for all other membrane processes, proteins are involved. Adaptations to cope with this environment are proposed to include a highly saturated and branched lipid composition [13], making solubilisation of such membranes to extract membrane proteins for downstream analysis a challenging process. This situation is a 'nightmare scenario' in terms of a proteomics approach to understand environmental adaptation at the cell membrane.

Proteomics of membrane proteins is challenging in general. The typical protocol for membrane protein detection and quantification relies on proteolytic digestion of soluble or solubilised proteins, followed by liquid-chromatography linked to mass-spectrometry (MS) detection. Subsequently, processing of the data obtained is delineated using various database search tools. This yields a tremendous amount of information, but invariably underrepresents the membrane proteins [14]. Most of the transmembrane proteins harbour a large amount of hydrophobic TMH's. The hydrophobic nature of these proteins

makes them insoluble in aqueous environments, a particular challenge in MS detection. Moreover, TMH's are protected from proteolytic digestion, a key requirement to generate peptide fragments for MS detection. With these proteins, only the short loops connecting such helices and soluble domains are accessible for proteolytic cleavage. To add to these challenges, membrane proteins suffer from low absolute expression levels, because membrane 'real estate' is severely limited [15, 16]. Solubilisation of membrane extracts is a potential approach for the extraction of integral membrane proteins, yet defining a universal protocol for that solubilisation is seemingly impossible [17, 18]. A key reason for the problems in building such a protocol is that every organism constructs its membrane to suit the condition under which it grows – i.e. an organism will adapt the membrane composition to avoid compromising on membrane fluidity or polarity [19–21]. In general, literature on solubilizing membranes for proteomics focuses on well-studied model organisms, such as *Escherichia coli* [18]. The field of membrane proteomics in extremophiles is poorly developed. For thermophiles, also few studies focus on this subject. Two published works detail whole-cell studies in *Clostridium thermocellum* [22, 23], and membrane solubilisation of the archaeon *Sulfolobus solfataricus* is summarised [24]. For alkaliphiles, two studies have been reported. One investigates membrane solubilisation with urea in *Alkalihalobacillus marmarensis* [25], while the other focuses on a few other well-known, yet ultimately ineffective detergents for *Alkalimonas amylolytica* [26]. None of these studies rely on a comparative approach that assesses contribution of each step to the protocol. Here, we attempted to address this shortcoming and advance the field of membrane proteomics for polyextremophiles.

In this study, we focus on a polyextremophile, the thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1. This microorganism is highly adapted to the harsh conditions that it lives in, pH 9.5 at 65°C [27] and has a high degree of variability in its ETC [28].We reveal the membrane proteome by implementing different solubilisation techniques while considering the organism's physiological growth temperature. Specifically, we present an overview of the detected membrane proteome of *C. thermarum* TA2.A1, focusing particularly on its transporters and ETC. Our findings also resulted in a noteworthy increase in the detectable proteins and peptides.

4.2. MATERIALS AND METHODS

4.2.1. CULTIVATION AND HARVESTING OF BIOMASS

Caldalkalibacillus thermarum TA2.A1 was grown in 5 L flat bottom shake flasks at 65°C with a working volume of 1 L rich medium, the composition of which was described previously [29]. A smaller pre-inoculum was grown beforehand in a 500 mL round bottom shake flask with a 100 mL working volume. The larger shake flasks were inoculated at 1% from the pre-inoculum. Incubations lasted for approximately 18 hours. Cells were harvested by centrifugation

at 8,000 × *g* and subsequently washed. The fraction appropriated to whole cell proteomics – ±100 mg cell wet weight – was washed in ice-cold Phosphate-Buffered Saline (PBS and thereafter flash frozen with liquid nitrogen and stored at -80°C. The fraction used for membrane proteomics was washed in 50 mM TRIS·HCl buffer, pH 8.0, with 10% (w/v) glycerol and 2 mM $MgCl_2$, and thereafter also flash frozen and stored at -80°C.

4.2.2. MEMBRANE EXTRACTION AND SOLUBILISATION

Membrane preparation was performed as described earlier [8], with modifications. Briefly, samples were thawed gradually by placing them on top of a layer of ice. Extraction started with a lysis step in 50 mM Tris·HCl buffer, pH 8.0, with 10% (w/v) glycerol, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Lysis was initiated by addition of 1.2 mg ml^{-1} lysozyme and 0.2 mg DNaseI. Lysed cells were subsequently passed through a cell disruptor twice at 20,000 psi. As a trial the amount of passages through the cell disruptor was decreased to a single passage, but that yielded no discernible difference (Supplementary Figure I). The resulting liquid fraction – discard denatured fraction – was centrifuged at 8,000 × *g* for 10 minutes. The resulting supernatant was ultracentrifuged at 180,000 × *g* for 45 minutes. The supernatant was discarded and the pellet resuspended in approximately 2 mL 50 mM Tris·HCl buffer, pH 8.0, with 10% (w/v) glycerol and 2 mM $MgCl_2$. When required, extracted membranes were flash frozen and stored at -80°C. *C. thermarum* TA2.A1 membranes were solubilised in 200 µM Ammonium-Bicarbonate (ABC) buffer with either 2% (w/v) n-Tetradecylphosphocholine (FOS-choline-12, Anatrace) or 0.01% (w/v) sodium dodecyl sulfate (SDS) for 30 minutes at 50°C, 65°C or 80°C, whilst gently shaking at 300 rpm. Extracted membrane samples were diluted to a final concentration of 4 mg mL^{-1} protein. After solubilisation, samples were frozen at -20°C until further preparation.

4.2.3. WHOLE CELL LYSATE PREPARATION

The following protocols are based on extensive work done before [30, 31], to optimize the protocols. For whole cell proteomics, the pellet stored after washing with PBS was thawed and 29 mg of sample was used and transferred to a LoBind Eppendorf tube. The pellet was dissolved in 0.175 mL 1 M Triethylammonium bicarbonate buffer and 0.15 g glass beads (105 – 212 µm, Sigma Aldrich) were added. The sample subjected to a vortexing regime that was repeated thrice; the sample was vortexed at max speed for 90 seconds, then placed on ice for 30 seconds. Afterwards, the sample was incubated at 80°C for 3 minutes at 1000 rpm and subsequently ultrasonicated for 10 minutes. The sonicated sample was centrifuged and the resulting supernatant (250 µL) was carefully pipetted in a new LoBind Eppendorf tube. The proteins were then precipitated by adding 62.5 µL 6.1 N tricholoacetic acid by vortexing and subsequent incubation at 4°C for 30 minutes. After centrifugation the

resulting pellet was washed with 200 µL ice cold acetone and then centrifuged again. The washed pellet was redissolved in 50 µL 6 M urea. The sample was then subjected to the proteolytic digestion protocol as described below.

4.2.4. PROTEOLYTIC DIGESTION

After thawing, in case of the membrane extract, samples were transferred to a LoBind Eppendorf tube, vortexed and an additional 50 µL 200 mM ABC buffer was added. 100 µL (≈ 100 µg protein) of that mix was used to reduction and alkylation. For the whole cell sample, proteolytic digestion occurred straight after lysate preparation. In this case, the preceding protocol yielded 30 µg protein. For the following part, two volumes are given for every step, the lower volume corresponds to the whole cell sample, the higher volume to the membrane extracts. Reduction was performed by incubation with 8.5 or 30 µL 10 mM dithiothreitol at 37°C for 60 minutes at 300 rpm. Alkylation subsequently was performed by adding 8.5 or 30 µL 20 mM iodoacetamide and incubating at 20°C for 30 minutes in the dark. Samples were then further diluted with 285 or 400 µL ABC and 5 or 20 µL of 0.1 µg μL^{-1} trypsin was added for digestion. Digestion occurred for approximately 18 hours at 37°C and 300 rpm. After digestion, all samples were treated equally again. After digestion, peptides were extracted by solid phase extraction in an Oasis HLB 96-well µElution Plate (Waters) according to manufacturer specifications. Peptides were eluted in two phases: (1) with 200 µL 2% formic acid in 80% methanol and (2) with 200 µL 1 mM ABC in 80% methanol. The peptides were then collected in an Integrated SpeedVacTM System (Thermo Scientific) at 50°C for 2 hours. Samples were stored at -20°C until analysis.

4.2.5. SHOTGUN PROTEOMICS AND RAW DATA PROCESSING

Shotgun proteomic analysis was performed as described earlier [32, 33]. Speed vac dried samples were dissolved in 20 µL 3% acetonitrile plus 0.1% trifluoroacetic acid. Protein concentrations were estimated by NanoDrop ND-1000 spectrophotometry (Thermo Scientific). An aliquot corresponding to approximately 0.5 µg protein were analysed using a one dimensional shotgun proteomics approach, using a nano-liquid-chromatography system consisting of an EASY nano-LC 1200 (Acclaim PepMap RSLC RP C18 separation column, 50 µm x 150 mm, 2 µm, Cat. No. 164568) and a QE plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany). The flow rate was maintained at 350 nL/min over a linear gradient from 5% to 25% solvent B over 88 min, then from 25% to 55% over 60 min, followed by back equilibration to starting conditions. Data were acquired from 5 to 240 min. Solvent A was H_2O containing 0.1% formic acid and solvent B consisted of 80% ACN in H_2O and 0.1% FA. The Orbitrap was operated in data-dependent acquisition (DDA) mode acquiring peptide signals from 385–1250 *m*/*z* at 70 K resolution in full MS mode with a maximum ion injection time (IT) of 75 ms and an automatic gain control (AGC) target of 3E6. The top 10 precursors were selected for

MS/MS analysis and subjected to fragmentation using higher-energy collisional dissociation (HCD). MS/MS scans were acquired at 17.5 K resolution with AGC target of 2E5 and IT of 75 ms, 2 *m*/*z* isolation width and normalised collision energy (NCE) of 28. Processing of mass spectrometric raw data. Data were analysed against the proteome database from *C. thermarum* (GenBank: CP082237.1, strain *Caldalkalibacillus thermarum* TA2.A1 complete genome, Tax ID: 559292) [28] using PEAKS Studio X (Bioinformatics Solutions Inc., Waterloo, Canada), allowing for 20 ppm parent ion and 0.02 *m*/*z* fragment ion mass error, 3 missed cleavages, carbamidomethylation and methionine oxidation and N/Q deamidation as variable modifications. Peptide spectrum matches were filtered against 1% false discovery rates (FDR) and identifications with ≥2 unique peptides were considered as significant. Results from PEAKS were exported to 'proteins.csv', listing the identified proteins.

Proteins from database searching files (Supplementary Table 1) were further processed using Python (version 3.9). Files were converted to 'Pandas' dataframes and counted based on the following two criteria: 'Area' > 0 and 'Unique Peptides' ≥ 2. Venns were then visualised using the 'Matplotlib' functions '*venn2_unweighted*' or '*venn3_unweighted*'. For the Venn diagrams concerning only membrane proteins, an additional criterion was added: TMH's ≥1. For the heat map, the raw absolute area were used directly, after filtering using Supplementary Table 2. The heat maps were the visualised using the function '*vizus.gene_exp_hmap*' from the package 'Bioinfokit'.

4.3. RESULTS AND DISCUSSION

4.3.1. PROTEOMICS PROTOCOL OPTIMIZATION FOR DETECTION OF C. thermarum TA2.A1 MEMBRANE PROTEINS

We first performed a conventional whole cell lysate shotgun proteomics experiment to detect the soluble protein fraction. The full genome of *C. thermarum* TA2.A1 [28] contains 3,085 genes (NCBI; accession: CP082237), of which 1,244 (Figure 4.1A) were detected in this experiment (approx. 40% of genome). DTU's TMHMM tool was used to distinguish which of these proteins were likely integral membrane proteins, using a single TMH as the cut-off [34]. The *C. thermarum* TA2.A1 genome contains 754 proteins of the total 3,085 that contain a single predicted TMH. It should be noted that proteins associated with the membrane (peripheral membrane proteins), but lacking a TMH are not 'soluble proteins' and are also included in our analysis. Peripheral membrane proteins are membrane associated via hydrophobic patches, lipid anchors or lateral helices [1]. However, these proteins cannot be identified from genetic information, nor be unequivocally identified or in a whole cell proteome analysis without supporting biochemical data. Our analysis confirmed that only 124 of the 1,244 proteins detected contain a TMH. Considering that roughly a quarter of all proteins of the complete genome will be either integral or peripheral membrane proteins [35, 36], this reinforces the notion that the

membrane proteome are generally underrepresented in conventional whole cell lysate proteomics experiments. Nevertheless, the whole cell proteomics approach yields a membrane proteome with a considerable proportion of membrane proteins. For instance, the majority of the aerobic ETC proteins were detected. The integral membrane protein, NADH dehydrogenase type I (Ndh1) was detectable and so was the peripheral membrane protein NADH dehydrogenase type II (Ndh2) [37–39]. Other integral membrane proteins, succinate dehydrogenase (Sdh) and the cytochrome b_6c_1 complex (a hybrid complex III) were also detectable, as well as the cytochrome oxidase aa_3 complex. The cytochrome oxidase aa_3 complex is expected to be the primary terminal oxidase under fully aerobic conditions. However, we also detected a single subunit pertaining to the microaerophilic cytochrome oxidase ba_3 complex. Surprisingly, the subunit detected contains 13 TMH's, whilst the soluble subunits were undetectable. However, caution should be taken with such a result because to confidently state that *C. thermarum* TA2.A1 expressed two terminal oxidases under the growth conditions we imposed, detecting the additional subunits provides greater clarity towards the notion of functionality. Lastly, the ATP synthase was also detected, revealing that we can get very close to detecting a fully functional *C. thermarum* TA2.A1 pathway for oxidative phosphorylation from the whole cell proteomics.

To better evaluate the possible expression of the cytochrome oxidase ba_3, as well many more functional proteins, an increased detection is paramount. Unfortunately, membrane proteins are lesser in number in a cell, so a whole-cell proteome analysis including soluble proteins dwarfs the lower signals of membrane proteins [40]. Isolating the membrane provides an initial step of enrichment. Secondly, detergent solubilisation can singularly isolate proteins and is an easily accessible method to purify proteins for this reason [8, 41, 42]. Proteins are in a crowded space in the biological membrane and this crowding can prevent access by the proteases necessary for proteomics. Lastly, as previously mentioned, for peripheral membrane proteins lacking biochemical description we cannot rely on genetic information and cannot be unequivocally identified or in a whole cell proteome analysis [1].

In order to obtain a better coverage of the membrane proteome, a modified membrane extraction protocol was followed. While extracting the membrane fraction from the rest of the cell constituents added a very modest increase protein hits, adding a membrane solubilisation step significantly increased the detection of membrane proteins (Figure 4.1A and 4.1B). The breadth of solubilisation methods available in literature [17, 18, 22–26], leaves a plethora of options available, but it is well noted that there is no common optimal method. Given our knowledge of membrane proteins, we opted for the use of detergents. Most detergents are not compatible with mass spectrometry, limiting options. Fortunately, two extremely effective detergents for membrane protein solubilisation were feasible, FOS-choline-12 and SDS. Additionally, the effect of the temperature (50°C, 65°C and 80°C) used for solubilisation was assessed. For solubilisation with FOS-choline-12, using 65°C or 80°C yielded

optimal results, whilst for SDS 50°C was clearly optimal (Figure 4.1C and 4.1D). Additionally, cross-comparison between the two detergents showed that each detergent appears to solubilise the membrane protein fraction selectively (Figure 4.1B). Finally, we tested whether changing the digestion enzyme made a difference in protein detection. An *in silico* digestion (Supplementary Figure II) showed potential improvement in the optimal peptide length range using both LysC and chymotrypsin as digestion enzyme. Contrary to expectations, neither increased the detection of membrane proteins significantly, having only a modest effect (Figure 4.2 and 4.3). Combining all proteomics experiments allowed for constructing an overview of all detected proteins containing a TMH, as well as zooming in on the ETC and known transporters.

4.3.2. AN OVERVIEW OF THE DETECTED C. thermarum TA2.A1 MEMBRANE PROTEOME

The main goal of our study was to identify as many membrane proteins as possible by optimization of membrane proteomics. In total 158 membrane

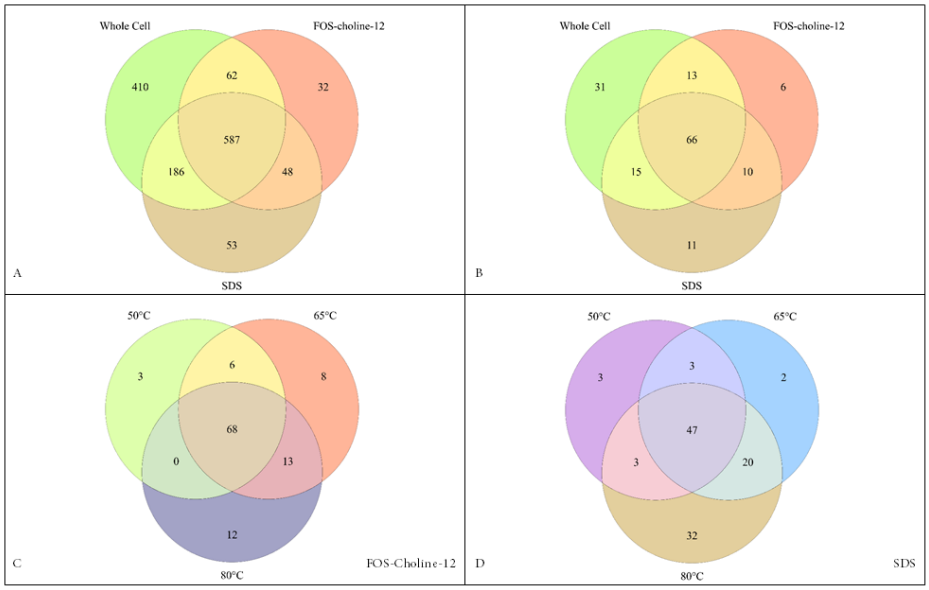

Figure 4.1: Detection of proteins by proteomics methods with different solubilisation methods. **A** covers the entire potential proteome, whilst **B**, **C** and **D** zoom in on solely the membrane proteins. **A** and **B** compare the detection using whole cell proteomics versus that with solubilisation with FOS-choline-12 at 65°C and SDS at 80°C. (**C**) and D show the effect of solubilisation at different temperatures (50°C, 65°C and 80°C) using either FOS-choline-12 (**C**) or SDS(**D**).

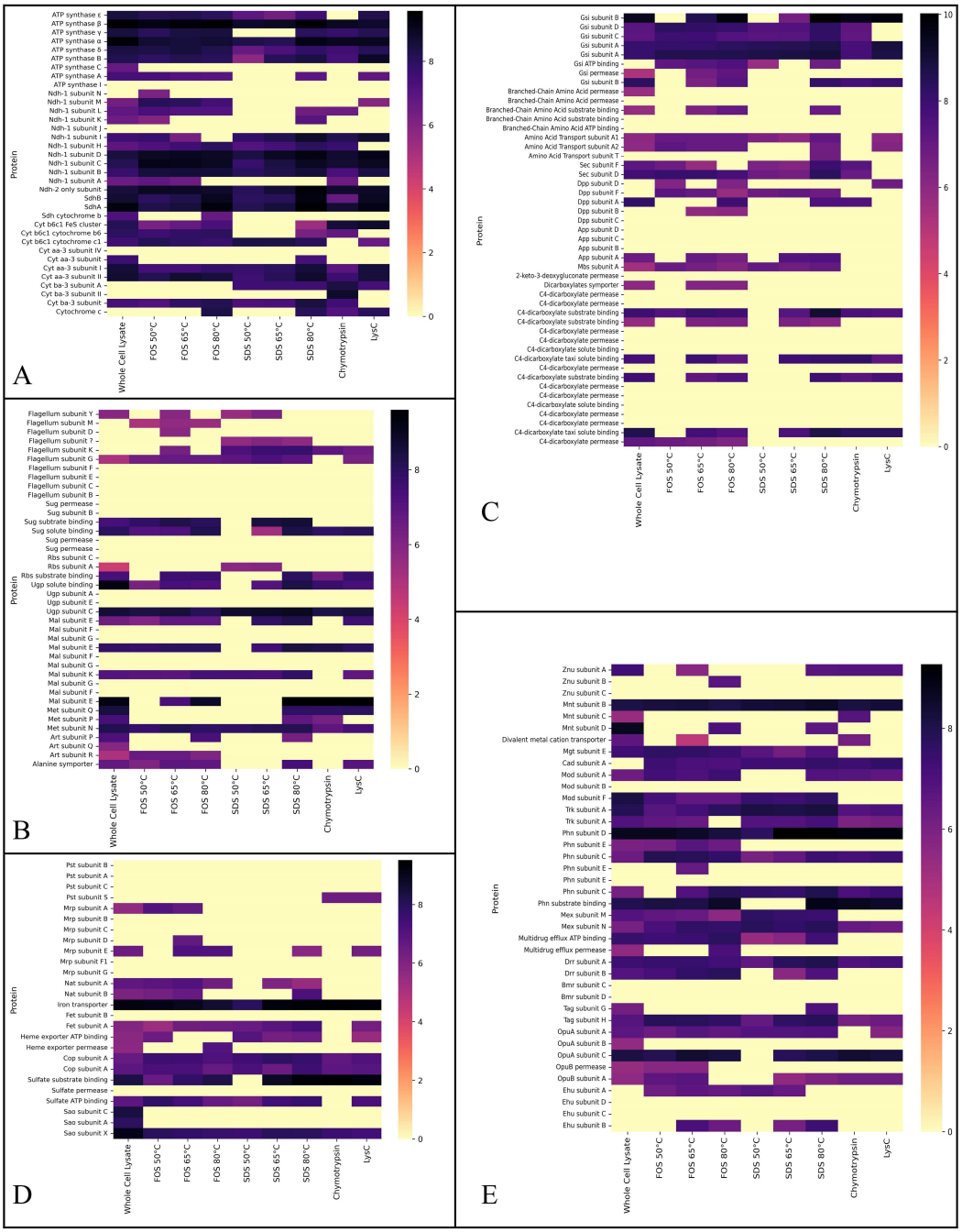

Figure 4.2: Captions continues on next page.

Figure 4.2: Proteomics data showing the logarithm (base 10) of absolute abundances detected in the whole cell lysate experiment and the experiments in which membrane extracts were solubilised with either FOS-choline-12 or SDS. For FOS-choline-12 and SDS, the temperature of solubilisation is given. Experiments with the alternative digestions enzymes; Chymotrypsin and LysC are accrued together. **A**: Heat map shows proteins of the ETC. **B**: Heat map shows proteins of the flagellum and transporters of sugars amino acids. **C**: Heat map shows transporters of primarily secondary metabolites; **A**, **B** and **C** together resemble heat maps corresponding to Figure 4.3. **D** & **E**: Heat maps selectively show transporters of metals and other inorganic cations and anions, as in Figure 4. Heat maps show all subunits pertaining to a certain protein complex if at least a single subunit was detected in one of the conditions. Note that proteins absent here, but shown in both Figures 3 and 4, were not detected by any method.

proteins were detected using the standard whole cell proteome method, although a full aerobic ETC was detected, not all subunits each complex were found. Solubilisation enabled detection of the other two remaining subunits pertaining to the microaerophilic cytochrome oxidase ba_3 (Figure 4.2A). The aerobic and most efficient ETC *C. thermarum* TA2.A1 can possibly express consists of Ndh1, Sdh, cytochrome *c*omplex b_6c_1, cytochrome *c*, and cytochrome oxidase aa_3. Expression of the F_1F_o ATP synthase completes an aerobic oxidative phosphorylation pathway. All these complexes were detected (Figure 4.3).

Beside the expected aerobic ETC, somewhat surprisingly ETC components that have been proposed to be more tuned towards microaerophilic lifestyle, Ndh2 and cytochrome oxidase ba_3, were expressed under aerobic conditions. The ba_3 complex proteins were among the few extra proteins detected after digestion with chymotrypsin or LysC instead of using the standard trypsin digestion. Detecting of the Ndh2 is in line with earlier research on this enzyme in *C. thermarum* TA2.A1, where it has been studied as a potential analogue for drug targets [37, 38, 43]. Finding the presence of cytochrome oxidase ba_3 was more surprising, as it is only half as efficient as aa_3 [44]. The expression could be due to the culturing conditions for this trial. A simple shake flask batch was used, which could have led to some oxygen limitation at the end of the batch phase, forcing *C. thermarum* TA2.A1 to adopt a more austere lifestyle. Such questions could be solved by culturing under more controlled conditions such as a chemostat. We would expect that at culturing at lower dissolved oxygen would also trigger the expression of the other two terminal oxidases, namely cytochromes bb_3 and *bd*.

In terms of carbon import machinery, a range of different transporters was detected, pertaining to the SugABC, RbsABC, MalEFGK, DctPQM and UgpABC complexes (Figures 2A and 4.3). Considering the usage of sucrose

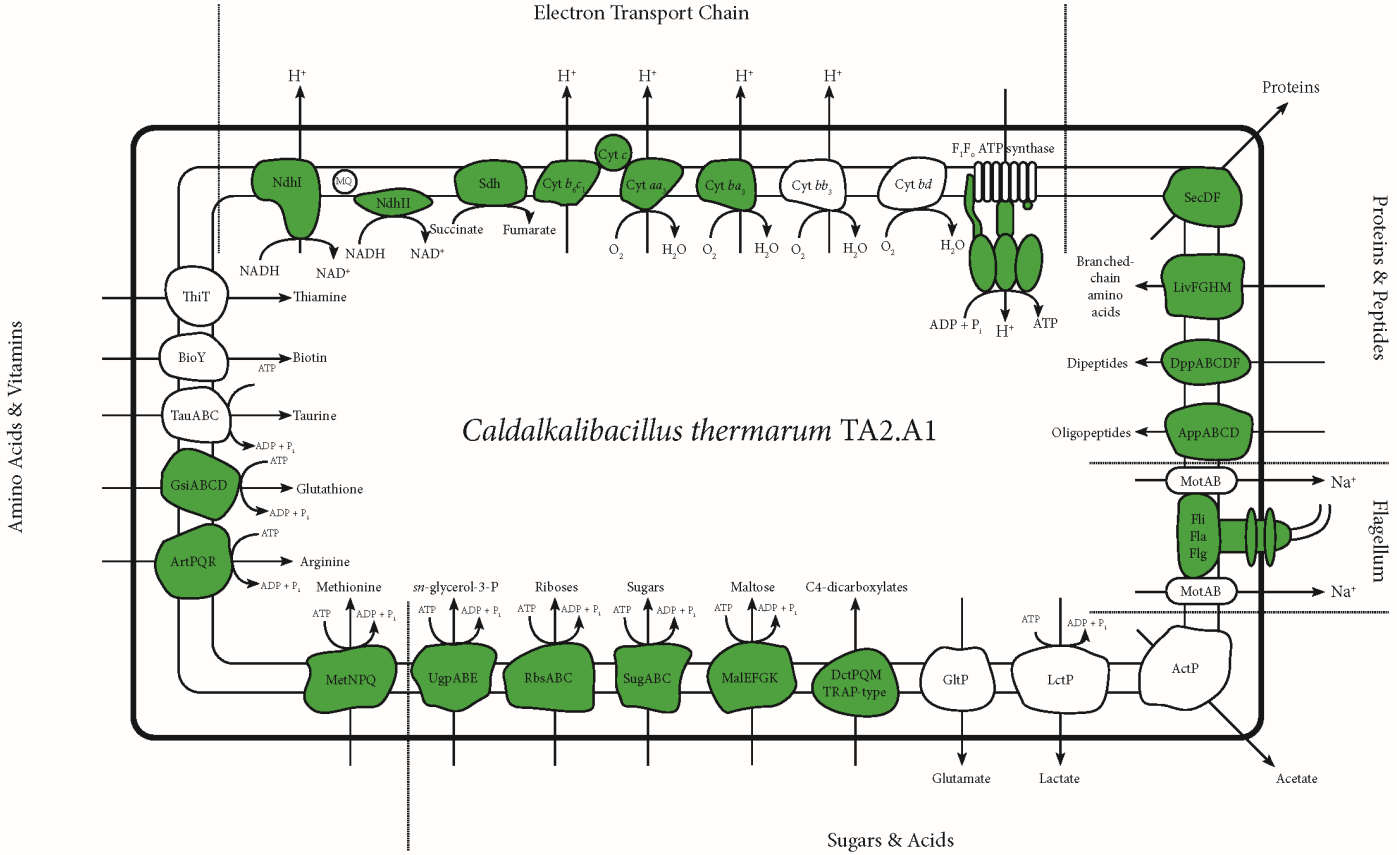

Figure 4.3: An overview of membrane proteins detected with any of the abovementioned protocols. This figure selectively shows all membrane proteins concerning the ETC and transporters concerning central carbon metabolism and anabolism. It also shows the flagellum. Detected proteins are coloured green. For the ATP synthase and the flagellum, this figure is more detailed, and only the subunits detected are coloured green.

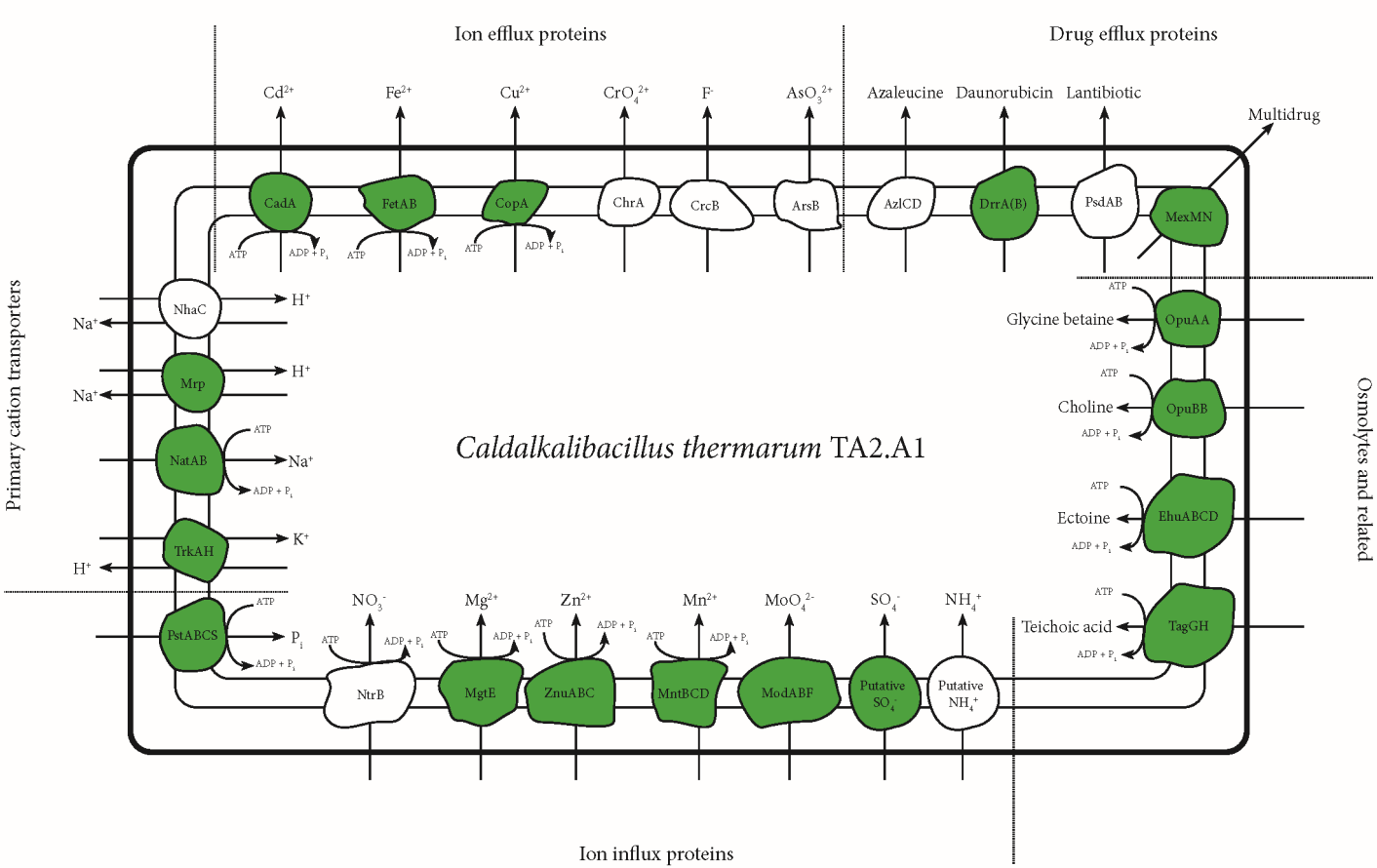

Figure 4.4: An overview of transporters detected with any of the abovementioned protocols. This figure selectively shows transporters of metals and inorganic cations and anions. Transporters related to drug efflux and to osmolyte import are also shown. The teichoic acid importer is displayed, being regarded as an important protein to alkaliphiles [45–47], even though previous tests showed teichoic acid presence in *C. thermarum* TA2.A1 [32]. Detected proteins are coloured green.

as carbon source, the detection of multiple subunits of SugABC was expected (Figure 4.2B). MalEFGK is annotated as a maltose importer, but might transport sucrose as well, this dual specificity is described in Streptococcus mutans [48]. The detection of possibly two sucrose importers means this research failed to pinpoint the genes encoding for the sucrose:sodium symporter that originally sparked the interest in *C. thermarum* TA2.A1 [27, 49]. Detection of the TRAP-type C4-dicarboxylate importer DctPQM, RbsABC complex and UgpABC complex (Figures 2A and 4.3) may be due to their scavenging functions rather than favoured presence under our culturing conditions. In line with other alkaliphiles, no defined medium for *C. thermarum* TA2.A1 exists, and it still requires the presence tryptic peptone extract to grow, which could contain substrate for all of these transporters. In agreement with this proposition, several amino acid importers, oligopeptide importers and importers of related compounds, like glutathione, were also detected. Lastly, the proteins translocase SecDF and the flagellum proteins were also detected.

Homeostasis of monovalent cations is crucial for any alkaliphile, and generally this is coupled to proton transport in an antiport protein [50]. *C. thermarum* TA2.A1 has two types of sodium:proton antiporters, a few subunits of the multisubunit Mrp were detected under the conditions used. The active ATP powered NatABC was also expressed. *C. thermarum* TA2.A1 relies on the TrkAH system for potassium uptake. The low affinity TrkAH system found is in most bacteria, but has been highlighted as a strategy to combat saline stress [51, 52]. *C. thermarum* TA2.A1 is capable of growth up to 6% NaCl [53], perhaps in part due to its reliance on this potassium uptake system. Two proteins pertaining to the A subunit of the potassium:proton antiporter TrkAH were detected (Figures 4.2B and 4.4); the *trkA* genes encoding for expression of these proteins are not part of a single operon, nor are they close to any of the other *trkA* or *trkH* genes. As importer for inorganic phosphate, the subunit S of the Pts complex was detected, and only in the experiments using alternative digestion enzymes (Figures 4.2B and 4.4). For import of (trace) metals, transporters for zinc, molybdate, magnesium and manganese were detected (Figure 4.4).

Other metals transporters, for cadmium, copper and iron, were also detected. Notably these were all described as exporters [54, 55]. The ferrous exporter FetAB was previously described as an exporter and implied as an important protein in avoiding problems with radical formation [56]. Several drug exporters were also detected; further shedding light on the possible mechanisms *C. thermarum* TA2.A1 might use to remain dominant in a given environment. Lastly, several importers related to osmolytes were detected. The incorporation of transporters of glycine betaine and ectoine could be another method used by *C. thermarum* TA2.A1 to combat the challenges posed by the high pH at which it lives. Especially glycine betaine was abundantly available in the rich medium used; the strategy of utilizing compatible solutes should be perfectly viable.

This study is the first report on the proteome of *C. thermarum* TA2.A1, in

which 1,398 proteins (45.3% of all potential proteins) were detected in at least one of the experiments. Of this, 158 contain at least a single TMH (11.3% of all identified proteins) and are therefore considered membrane proteins. The total coverage of the membrane proteome is 20.9%. *C. thermarum* TA2.A1 is an alkaliphile and therefore this study focuses primarily on proteins identified in the ETC and on transporters. For an alkaliphile, homeostasis of protons, sodium and potassium is crucial and this study detected transporters for all of these compounds. Importers of various compounds related to central carbon metabolism and to anabolism were also detected, as well as a complete ETC. Combined, this study gives a comprehensive overview of the membrane proteins required by an alkaliphile to survive; it provides a valuable benchmark for further research on this organism. The authors specifically note its adaptability to a broad pH range as an area of further research. The conditions at which *C. thermarum* TA2.A1 employs its alternative terminal oxidases are also yet to be revealed. In that regard, performing solubilisation of membrane extracts, as an addition to whole cell lysate proteomics will increase the chances of detecting these alternative terminal oxidases.

4.4. CONCLUSION

Here we report on a proteomic study where we investigated several sample preparation appraoches to maximise the coverage for membrane proteins for *C. thermarum* TA2.A1. The whole cell proteomics experiment combined with proteomics of solubilised membrane extracts yielded in 1398 proteins (45.3% of total proteome), of which 158 (11.3% of the discovered proteins; 20.9% of the membrane proteome) were membrane proteins. Interestingly, all different membrane solubilisation methods provided a unique set of membrane proteins. In terms of solubilisation of membrane proteins, we conclude that treatment with SDS at 50°C with the analysis of a sample treated with FOS-choline-12 at 65°C or 80°C proved most effective. The entire optimal ETC was detected using the combined methods for analysis, as well as two less efficient proteins – the Ndh2 as an 'alternative Complex I' [57, 58] and the cytochrome oxidase ba_3 as an alternative Complex IV. Additionally, we detected all proteins required for the transport of protons, sodium and potassium, homeostasis of which is crucial to an alkaliphile. Lastly, the expression of the glycine betaine and ectoine importers implies *C. thermarum* TA2.A1 relies in part on osmolytes for its survival, something that has not been reported previously in literature for this organism. Overall, this research has given a comprehensive overview of proteins that we expect to be expressed, and shed light on some additional potentially interesting proteins. In short, this research provides a valuable benchmark for further research into alkaliphiles, and *C. thermarum* TA2.A1 in specific.

4.5. ACKNOWLEDGMENTS

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5

QUANTITATIVE PROTEOMICS REVEALS OXYGEN-INDUCED ADAPTATIONS IN *Caldalkalibacillus thermarum* TA2.A1 MICROAEROBIC CHEMOSTAT CULTURES

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Raw proteomics data can be found on ProteomeXchange consortium database with the identifier PXD047466

ABSTRACT

Caldalkalibacillus thermarum possesses a highly branched respiratory chain. In model organisms, these primarily facilitate growth at a wide range of dissolved oxygen levels. The aim of this study was to investigate whether the thermoalkaliphilic *C. thermarum* is regulated similarly. *C. thermarum* was cultivated in chemostat bioreactors with a range of oxygen levels in the inlet gas (0.25% O_2 – 4.2% O_2). Proteomic analysis unexpectedly showed both the type I and the type II NADH dehydrogenase present in all conditions. Moreover, two different terminal oxidases were present. The cytochrome *c*:oxygen aa_3 complex abundance was highest at 4.2% O_2. The cytochrome *c*:oxygen ba_3 complex exhibited higher abundance at most other O_2 levels, but its abundance started to decline below 0.42% O_2. We expected this would result in emergence of the cytochrome *c*:oxygen bb_3 complex or the menaquinol:oxygen *bd* complex, the other two terminal oxidases of *C. thermarum*; but neither was detected. Furthermore, the sodium-proton antiporter complex Mrp was downregulated under the lower oxygen levels. Normally, in alkaliphiles, this enzyme is considered crucial for sodium homeostasis. We propose that the existence of a sodium:acetate exporter decreases the requirement for Mrp under strong oxygen limitation, introducing a novel perspective to the field of alkaliphiles.

5.1. INTRODUCTION

Branched respiratory chains are a common feature in the microbial world. Equipped with alternatives for canonical ETC complexes, microbes can effectively manage fluctuating oxygen conditions encountered within their native environments [1]. As example, the gut microbe *Escherichia coli* has a terminal bo_3 oxidase for aerobic conditions and two bd type terminal oxidases for anaerobic conditions [2, 3]; the latter condition would be encountered more often by *E. coli* [4]. While prior research under controlled conditions predominantly focused on model organisms [5–10], much remains unknown about the regulation of respiratory enzymes of microorganisms from more extreme origins. For instance, the oxic layer of an exotic environment such as a hot spring is confined to the uppermost 1 – 2 cm [11, 12], and can undergo diurnal variations [13]. This environment selects for microbes with a branched respiratory chain to adapt to fluctuating oxygen levels. In the case of microbes from hot springs, a compounding challenge is the combined effects of high temperature and extreme pH. The natural habitat of the obligate aerobic thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1 is an alkaline hot spring with a pH of 10 and 65°C, Mount Te Aroha, New Zealand [14]. To cope with the broad spectrum of oxygen concentrations it faces in its native environment, *C. thermarum* has a highly branched proton-mediated ETC [15].

C. thermarum has a putative type I (Ndh1) and a type II (Ndh2) NADH dehydrogenase [15]. Ndh1 from aerobic bacteria are large proton-pumping multi-subunit integral membrane proteins that regenerate NADH from NAD^+ (**Figure 5.1**) [16]. In contrast, Ndh2 are small single-subunit peripheral membrane proteins with the same regeneration function, but without pumping protons (**Figure 5.1**) [17]. The *C. thermarum* Ndh2 is biochemically well described, and is capable of rapid NADH turnover in a membrane environment [18]. *C. thermarum* also harbours a succinate dehydrogenase (Sdh) and a putative fumarate reductase (**Figure 5.1**; fumarate reductase not depicted) [19, 20]. Little is known about either enzyme in *C. thermarum*, however Sdh activity has been measured in purified *C. thermarum* membranes [21].

There is a putative hybrid electron-pair splitting Complex III menaquinol:cytochrome *c* oxidoreductase b_6c_1 complex (Cyt. b_6c_1) [15]. Cyt. b_6c_1 is a hybrid cytochrome with high homology between the cytochromes bc1 and b6f, integral membrane protein complexes found in mitochondrial and cyanobacterial electron transport chains, respectively. Cyt. b_6c_1 serves to transfer electrons from menaquinone to cytochrome *c* (**Figure 5.1**) [22, 23]. *C. thermarum* also boasts four putative terminal oxidases, all integral membrane proteins [15]. Cytochrome aa3 (Cyt. aa_3), cytochrome ba3 (Cyt. ba_3) and cytochrome bb3 (Cyt. bb_3) rely on cytochrome *c* as electron source (see **Figure 5.1**). Cyt. aa_3 is known as an 'aerobic oxidase', typically being expressed under aerobic growth conditions [24]. A model Cyt. aa_3 from Rhodobacter sphaeroides pumps protons at an efficiency of 0.7 H^+/electron [25]. Cyt. ba_3 and Cyt. bb_3 are not as biochemically well described as Cyt. aa_3. The Thermus thermophilus Cyt. ba_3 and bb3 both pump protons, but

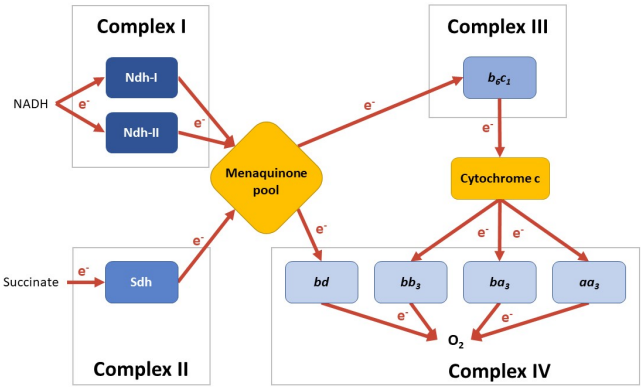

Figure 5.1: Schematic of the menaquinone mediated *C. thermarum* TA2.A1 ETC. Enzymes are coloured per reaction type and the electron flow is visualized with arrows. Note that currently no condition is known under which all options are expressed simultaneously.

with a lesser efficiency of 0.5 proton per electron transferred to the catalytic site [24, 26]. This leads to speculation that they may differ in oxygen affinity in *C. thermarum*. In support of this, Cyt. bb_3 oxidase allows human pathogens to colonize anoxic tissues and agriculturally important microbes involved in nitrogen fixation [26]. An *Escherichia coli* homologue of the final terminal oxidase, Cytochrome *bd* (Cyt. *bd*), takes its electrons straight from the quinone pool rather than through cytochrome *c* (**Figure 5.1**) [27], so it is likely the same occurs in *C. thermarum*. Additionally, in contrast to the other three oxidases, Cyt. *bd* does not pump any protons.

Lastly, completing the ensemble is a proton-coupled F_1F_O-ATP synthase [28, 29]. The *C. thermarum* enzyme is a large multisubunit integral membrane protein complex that natively unidirectionally synthesizes ATP [29, 30]. The enzyme is highly adapted to function in alkaline pH conditions to be able to recapture protons for ATP synthesis and to be irreversible [29–32].

Previous investigations into the membrane proteome of *C. thermarum* in the stationary phase of batch cultivation found that Ndh1, Ndh2, Sdh, Cyt. b_6c_1, Cyt. aa_3, Cyt. ba_3 and the ATP synthase were expressed [33]. However, the regulation of the *C. thermarum* TA2.A1 ETC as a response to differing oxygen availability has never been researched before. To study respiratory regulation as a function of oxygen availability in *C. thermarum* TA2.A1, cultivation in chemostat cultures is required. The use of chemostat bioreactor operation is preferred over other methods as it provides the most replicable results and because it allows precise dosing of oxygen. The term ‘aerobiosis’ was initially coined for *E. coli* to quantify the microaerobic range using acetate production as a benchmark [6]. Presumably, in *C. thermarum* the Ndh1, Sdh, Cyt.

b_6c_1, Cyt. aa_3 respire the bulk of the electron potential under fully aerobic conditions, as this is the most energy-efficient combination available. The primary aim of this study assesses whether that expectation is correct and to test the hypothesis that Ndh2 and the alternative terminal oxidases replace the most energy efficient respiratory proteins under oxygen limitation. Whole cell proteomics can then be used to pinpoint the enzymes within the ETC responsible for respiration at every level of aerobiosis.

Distinctive to alkaliphiles is their inverted proton gradient, whereby the internal pH is lower than that of the external environment. This makes proton homeostasis in alkaliphiles a subject that has been studied intensively before [34–38]. As such, a secondary aim of this study is determining if the response to oxygen scarcity is comparable to model organisms. If our hypothesis that *C. thermarum* switches to its alternative ETC proteins proves correct, pH homeostasis must adapt as result of that. Potential knock-on effects of decreased proton translocation on pH homeostasis in *C. thermarum* TA2.A1 might occur and are an additional area of interest. Finally, this study also aims to pinpoint overarching metabolic changes, and to benchmark them against model organisms. The multifaceted approach will result in a comprehensive insight into the response of *C. thermarum* to varying oxygen availabilities.

5.2. MATERIALS AND METHODS

5.2.1. CULTIVATION MEDIUM

C. thermarum TA2.A1 was cultivated in a medium adapted from earlier work on the same organism [21]. The medium composition in this work was (in g L^{-1}): Tryptone Peptone (Difco), 10.0; Sucrose, 10; $NaHCO_3$, 9.0; Na_2SO_4, 0.5; K_2HPO_4, 0.2; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7\ H_2O$, 0.1; $MnCl_2 \cdot 4\ H_2O$, $5.0 \cdot 10^{-5}$; $ZnSO_4$, $1.4 \cdot 10^{-5}$; $Na_2MoO_4 \cdot 2\ H_2O$, $1.2 \cdot 10^{-5}$. In case of cultivation in a bioreactor, 0.25 mL L^{-1} of Antifoam C (Sigma-Aldrich, Missouri, USA) was added to the medium. Medium without sucrose was autoclaved at 121°C for 15 minutes. pH of the medium was set to 9.5 before autoclaving. Sucrose was autoclaved separately at 110°C in a 50% (w/v) concentrate and added aseptically afterwards; basal medium was concentrated during preparation accordingly.

5.2.2. BIOREACTOR OPERATION

A 3.0 L jacketed bioreactor (Applikon Biotechnology, the Netherlands) was used with two Rushton impellers for stirring at 800 rpm, controlled by an ADI 1012 (Applikon Biotechnology, the Netherlands). Working volume inside the reactor was kept at 1.0 L by continuous addition of medium and by continuous removal of effluent. After an initial batch phase (see below), reactors in this study were operated in chemostat, with a dilution rate of D = 0.1 h^{-1}. Temperature was kept at 65°C by a thermostat, an Ecoline Staredition E 300 (Lauda, Germany). The reactor was sparged with an air-N_2 mix; to reach

desired aerobiosis level, this mix was altered each time, see **Table 5.1**. Off-gas was cooled using a RM6S Refrigerated Circulating Bath (Lauda, Germany) as cryostat and the composition thereof was subsequently measured using an NGA 2000 off gas multiplexer (Rosemount Inc., Minnesota, USA). pH was kept stable at 9.5 by automatic addition of either 2 M H_2SO_4 or 2 M NaOH, controlled by an ADI 1030 Bio Controller (Applikon Biotechnology, the Netherlands). Dissolved oxygen was measured with an AppliSens DO probe, (Applikon Biotechnology, the Netherlands) and pH was measured using an AppliSens pH+ probe (Applikon Biotechnology, the Netherlands), both with a length of 235 mm.

The inoculum for each bioreactor replicate was started by thawing a fresh

Table 5.1: Required mix of compressed air and nitrogen gas to sparge with desired O_2 concentrations in the bioreactor. Five conditions were used and can be identified by their final O_2 concentration in the gas inlet. Note these are only the O_2 concentrations in the gas inlet. Regardless of the O_2 concentrations in the inlet gas, the dissolved oxygen concentration was always 0%.

Oxygen level in gas inlet (%)	Air (L_n min^{-1})	Nitrogen Gas (L_n min^{-1})
4.2	0.200	0.800
2.05	0.100	0.900
1.05	0.050	0.950
0.42	0.020	0.980
0.25	0.012	0.988

±1.6 mL glycerol stock of *C. thermarum* TA2.A1 (frozen at 80°C). All stocks used in this research line originate from the same batch. Thawed cells were reconstituted in a 500 mL round bottom shake flask, with 100 mL working volume, at 65°C and 140 rpm for 18 hours. 2% of this culture was transferred to a fresh shake flask containing 100 mL medium and again cultivated for 18 hours. The resulting culture was used entirely as reactor inoculum. In the reactor, a batch phase of roughly 8 hours followed inoculation, after which the reactor was set to chemostat. Samples were taken periodically for optical density and dry weight measurements; supernatant was kept at -20°C for acetate and sucrose concentration determination at a later stage. Steady state was assumed when off-gas profiles, OD_{600} and dry weight measurements were stable for 3 consecutive retention times. Aseptic conditions were maintained throughout the cultivation procedure. All aeration levels tested were biologically duplicated.

5.2.3. ANALYTICAL METHODS

Optical density measurements were performed at a wavelength of 600 nm (OD_{600}) with a 1 cm light path length in a Biochrom Ultrospec 2100 Pro UV Vis

Spectrometer (Amersham, United Kingdom). For dry weights, 10 mL sample was filtered over a pre-dried 0.20 µm Supor® PES Membrane Disc Filter (Pall, New York, USA), and dried at 105°C; filter was weighed before sample addition and after drying for > 1 hour. Acetate was measured using high performance liquid chromatography (HPLC). HPLC analysis was performed with 1.5 mmol L^{-1} H_3PO_4 as eluent at a flow of 0.75 mL min^{-1}. Compounds were separated over an Aminex HPX-87H column (BioRad, California, USA) at 59°C and thereafter detected by a refractive ERC RefractoMax 520 (ERC Inc., Japan). Sucrose was measured using the GOPOD D-Glucose Assay Kit (Megazyme, Ireland), with addition of 160 mg L^{-1} Grade VII Invertase (300 U mg^{-1} from *Saccharomyces cerevisiae*; Sigma Aldrich, Missouri, USA) to the reaction mix. Except for the addition of the invertase enzyme to convert sucrose into glucose, manufacturers specifications were followed; the same UV Vis detector was used as for optical density measurements. Analytical measurements were conducted in duplicate.

5.2.4. PROTEIN EXTRACTION AND PROTEOMICS

For proteomics, at the end of each condition, a 100 mg sample (wet weight) was taken and washed in ice-cold phosphate-buffered saline. This sample was flash frozen in liquid nitrogen and stored thereafter at 80°C until analysis. For analysis, the protocol of which was based on extensive prior work [39, 40], the samples were thawed. 22.2 ± 0.5 mg sample was dissolved in 0.175 mL 1M triethylammonium bicarbonate buffer in a LoBind tube (Eppendorf, Germany), and thereafter treated exactly according to the 'whole-cell' procedure in our previous work [33]. This is also true for shotgun proteomic analysis up to the point where results were exported as 'proteins.csv' files [41, 42].

5.2.5. DATA PROCESSING AND VISUALIZATION TECHNIQUES

The proteomic data in this study were categorised in two different ways: by Kyoto Encyclopedia of Genes and Genomes (KEGG) modules and the membrane found in our previous work [33]; with some proteins falling in both categories. All analysis was done in Python. Modules were retrieved from the KEGG database using the '*requests*' library; the identifier for *C. thermarum* TA2.A1 genome is '*cthu*'. From the absolute abundances in 'proteins.csv', $\log_2$ ratios were calculated in order to equally assess up- and downregulation under the various conditions; 4.2% O_2 was used as reference. The resulting data was collected in a single Excel file. This Excel file, containing the proteomics data of each condition and replicate was subsequently loaded in a '*pandas*' dataframe and script automatically checked which proteins of a certain module were discovered in the shotgun proteomics analysis. In the case of this study, if 6 or more proteins were found for a specific module in each condition, data was stored in a separate excel file to make a boxplot. The boxplot was made using the '*matplotlib.pyplot*' library and settings were adjusted to show the average, whiskers and outliers. Module names were added manually. For the

heatmaps of the short-chain fatty acid module (Supplementary Figure I) or of membrane proteins of interest, the '*visuz*' package of the '*bioinfokit*' library was used. Fake data was added in order to ensure a $\log_2$ ratio of zero was always the centre.

5.3. RESULTS AND DISCUSSION

5.3.1. AN OVERVIEW OF THE ADAPTATION TO OXYGEN LIMITATION BY C. thermarum TA2.A1

The goal of this study was to assess the regulation of the *C. thermarum* ETC, uncover potentially alkaliphile-specific adaptation, and assess overarching metabolic changes under varying oxygen availabilities. The first objective was to find the required oxygen level in the gas inlet at which oxygen would become limiting (i.e. dissolved oxygen = 0 at steady state). Preparatory chemostat cultivations were performed at a broader range than reported here; the highest aeration level at which oxygen limitation was detected was at 4.2% O_2 in the gas inlet; the lowest oxygen level at which growth was detected was 0.25% O_2 (Supplementary Figures II & III). In all of these conditions, the dissolved oxygen concentration was zero, meaning consumed oxygen was limited by the oxygen transfer capacity. For the purpose of this report, *C. thermarum* was grown with five different oxygen levels in the gas mix: 4.2%, 2.05%, 1.05%. 0.42% and 0.25%. As described above, N_2 gas was added to keep gas flow at 1.0 L_n min^1. The biomass specific substrate consumption rate qs barely varied at 0.42% O_2 and above: 29.3 $mmol_s$ $Cmol_x^{-1}$ h^{-1} ≤ q_s ≤ 38.5 $mmol_s$ $Cmol_x^{-1}$ h^{-1}. At the 0.25% O_2, an increase was measured: q_s = 51.0 $mmol_s$ $Cmol_x^{-1}$ h^{-1}. While all bioreactors operated in oxygen limited conditions, acetate production only occurred at 2.05% O_2 and lower mole fractions. The biomass specific acetate production rate q_{ac} varied between 44.2 $mmol_{ac}$ $Cmol_x^{-1}$ h^{-1} and 123.6 $mmol_{ac}$ $Cmol_x^{-1}$ h^{-1}. Although acetate production generally increased with decreasing oxygen, no linear trend was observed, unlike what is described for *E. coli* [6]. From a basic physiological perspective, *C. thermarum* follows the same strategy to combat oxygen limitation, which is to supplement its flailing respiration with increasing amounts of substrate level phosphorylation provided by partial acetate fermentation.

Whole cell proteomics was performed for both replicates of each condition. To determine on a more detailed level whether the regulation under oxygen limited conditions is comparable to model organisms, proteomics of the highest (4.2% O_2) and lowest (0.25% O_2) levels were compared. **Figure 5.2** shows this regulation per pathway, calculated as $\log_2$ ratios. As stated above, *C. thermarum* uses a different strategy compared to model organisms such as *E. coli* and *S. cerevisiae* to move from respiration to fermentation. The discrepancy between this study and model organism studies stem from the aforementioned stable q_s in this study, and the fact that *C. thermarum* cannot grow under absolute anaerobic conditions. Together with the q_s, glycolysis

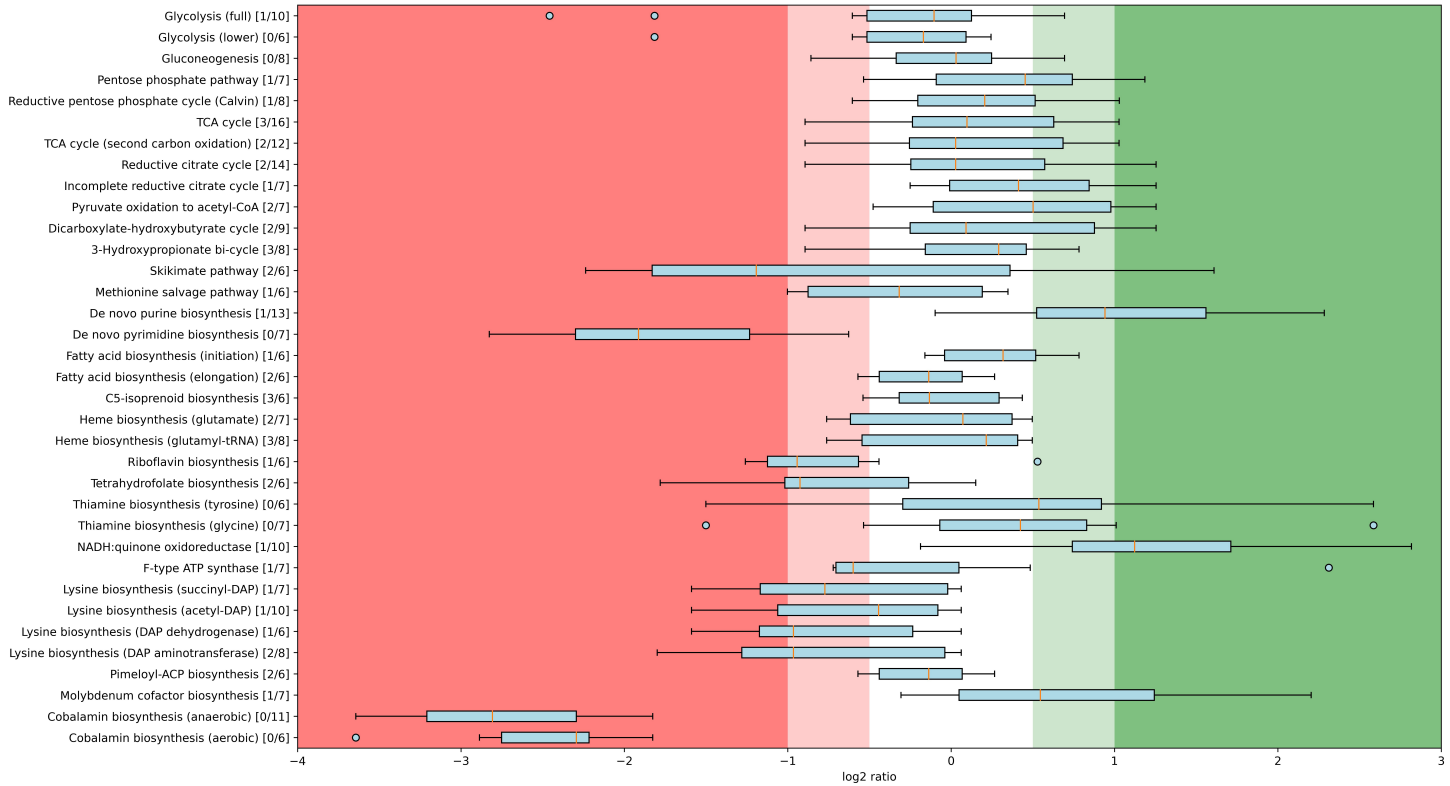

Figure 5.2: Box plot with whisker and outliers comparing proteomics results of 4.2% O_2 (highest level) and 0.25% O_2 (lowest level) chemostats per pathway. Regulation is calculated as log2 ratios; 0.25% O_2 relative to 4.2% O_2. Displayed pathways are KEGG modules of which at least 2 unique peptides of 6 or more proteins are detected in both conditions. Module names are as descriptive as possible, underlying KEGG identifiers can be found in textbfSupplementary Table I, and the underlying proteomics data is publicly available (see title page).

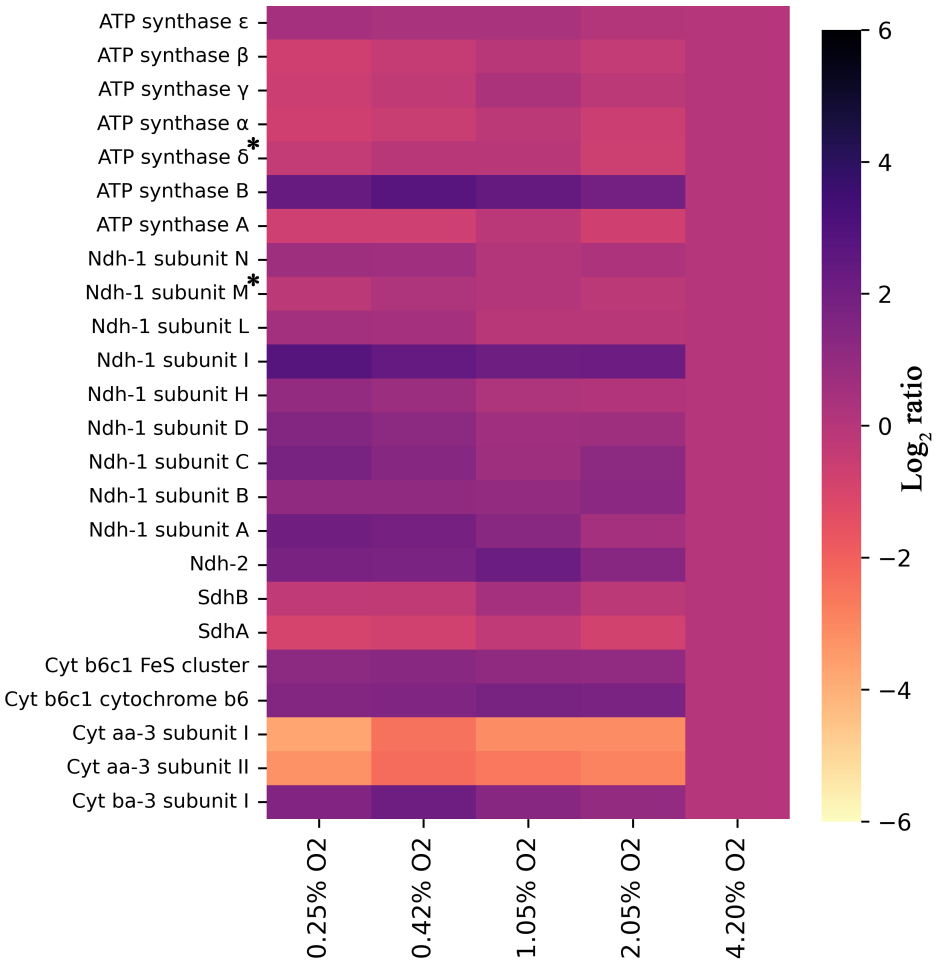

Figure 5.3: Heat map showing $\log_2$ ratios of all detected subunits of the ETC for each condition, provided 2 or more unique peptides were detected, relative to 4.2% O_2. All trends have a significance of $p < 0.05$, except those denoted with an asterisk. For ATP synthase subunit δ the $p = 0.058$, and for Ndh1 subunit M, the $p = 0.150$. For all others, the exact significances and the corresponding protein number can be found in Supplementary File I, and the locus tags in textbfSupplementary Table I.

remains stable in *C. thermarum*, whilst upregulation of glycolysis is generally observed in *E. coli* and *S. cerevisiae* [7, 8, 43] to ensure enough carbon is available to ferment. The partial fermentation of *C. thermarum* to acetate likely stems from the carbon flux being diverted from the TCA cycle to acetate production, without the requirement of significant proteomics reallocations over a whole pathway. Proteomics data show an increase in enzymes responsible for acetate production, Pta and Ack (Supplementary Figure I), to facilitate the increased carbon flux through that route. Few previous studies tried to also assess anabolic proteomics changes, yet some interesting observations deserve discussion. A few pathways are entirely up- or down-regulated more than twofold under 0.25% O_2 relative to 4.2% O_2 (i.e. having a log2 ratio greater than -1 or +1). Three pathways stand out as being multifold downregulated as a whole. *De novo* pyrimidine biosynthesis and both cobalamin biosynthesis pathways. Enzymes of pyrimidine biosynthesis are known to be 6–20 times more active under anaerobic conditions, as was first reported in *Staphylococcus aureus* [44]. A similar increase in enzymatic activity in *C. thermarum* under diminished oxygen conditions should decrease production requirement of pyrimidine synthesis proteins, causing the observed down-regulation. Cobalamin can be produced both aerobically and anaerobically, and *C. thermarum* contains both pathways. Both of these pathways are down-regulated more than fourfold. A possible reason for decreased cobalamin production is that if it were involved in protection against reactive oxygen species (ROS) as has been demonstrated in the acidophile *Leptospirillum* Group II CF-I [45]. Having this function in *C. thermarum* next to its various regular functions would explain the decreased requirement when oxygen becomes limiting [46, 47]. Two modules are completely up-regulated more than twofold: NADH:menaquinone oxidoreductases, which will be discussed later, and *de novo* purine biosynthesis.

The upregulation of purine biosynthesis in anaerobiosis has been previously shown in *S. cerevisiae* [7]. A plausible cause for upregulation of purine biosynthesis proteins is that its regulatory enzyme, a glutamine 5 phoshophoribosyl 1-pyrophosphate amidotransferase, is oxygen sensitive [48]. Whether any post-translational modifications exist to prevent intracellular buildup of purines is unknown, however it is highly likely.

5.3.2. UNRAVELLING THE ROLE OF THE MANY RESPIRATORY ENZYMES

As mentioned in the previous section, and contrary to expectations, a higher abundance of NADH:menaquinone oxidoreductases was detected at 0.25% O_2 compared to 4.2 % O_2. If assessing per subunit though, as detailed in **Figure 5.3**, the upregulation is not as stark for Ndh1. Rather, a few subunits, subunits A, C and I, have $\log_2$ ratio's exceeding 1.5, whilst the other are closer to 0.5 – 1.0. It is curious that the subunits are differentially regulated to an extent. Conclusions are somewhat more definitive with Ndh2,

which has a log2 ratio of 1.8 at 1.05% O_2 (**Figure 5.3**). When compared to *E. coli*, in which solely Ndh2 has been shown to be *upregulated* and Ndh1 *downregulated* when oxygen becomes limiting, this result is surprising [8]. This result puzzles even more considering the type II NADH dehydrogenase of *C. thermarum* has a higher maximum specific activity than its homolog in *E. coli* [18, 49–51]. Normally, in *E. coli*, the switch to acetate during decreased aerobiosis is coupled to concomitant ethanol fermentation [6]. This is because whilst acetate production provides ATP, ethanol is required to regenerate NAD^+. *C. thermarum* is unable to make ethanol. The inability of *C. thermarum* to produce ethanol could explain the higher Ndh1 abundance at lower oxygen levels, though the authors would reason completely switching to Ndh2 would be more efficient. More research is required to fully explain this counterintuitive behaviour. The succinate dehydrogenase (Sdh) and the F_1F_o-ATP synthase were downregulated, following the same protein presence patterns detailed for *E. coli* (**Figure 5.3**) [43]. Down-regulation of Sdh is another contributing factor to the observed acetate production. The inability to fully utilize the TCA cycle dictates that pyruvate should be consumed by another pathway. *E. coli* does not have any genes encoding for a Complex III, but *S. cerevisiae* does. In a previous yeast study [7], its cytochrome bc1 complex is reported to be downregulated, but sadly not by what extent. In this study, *C. thermarum*'s Complex III, the cytochrome b_6c_1 complex, is slightly upregulated at lower O_2 levels. This observation could be related to the aforementioned upregulation of Ndh1. Alternatively, Babauta and coworkers suggested intracellular pH could modify redox potential [52]. Since low oxygen concentration decreases intracellular ROS concentration [53] and increases NADPH content [54], another potential explanation for Ndh1 upregulation and F_1F_o-ATP synthase repression at low O_2 is that the intracellular pH may be increased to keep the redox homeostasis.

Two types of terminal oxidases were detected in this experiment, in fact in all conditions: Cyt. aa_3 and Cyt. ba_3. As expected, Cyt. aa_3 is downregulated when oxygen becomes limiting, whilst Cyt. ba_3 is upregulated (**Figure 5.3**). The main difference between these two enzymes is that Cyt. aa_3 allows for the translocation of 2 H^+ per 2 e^-, while Cyt. ba_3 only translocates 1 H^+ per 2 e^-. As described in the introduction, a similar pattern is observed in *E. coli*. This organism switches from its cytochrome bo_3 (pumping $1H^+$/electron), through cytochrome *bd* (pumping $0H^+$/electron) and finally to cytochrome *bd*-II (pumping $0H^+$/electron) under increasing anaerobiosis [8, 9]. Though the abundance of Cyt. ba_3 in this study starts to tail of below 0.42% O_2, no other terminal oxidase was detected. This is like due to the hydrophobicity of both proteins and accessibility to proteolytic digestion, a major challenge in membrane proteomics. Regardless, based on our findings of aa_3 and ba_3, it seems most likely that Cyt. bb_3 or Cyt. *bd* takes over responsibility for oxygen electron transfer at oxygen concentrations less than 0.25%. Cyt. bb_3, as described in the introduction, allows human pathogens to colonize anoxic tissues, translocates 1 H^+ per electron pair, but possibly with a higher affinity

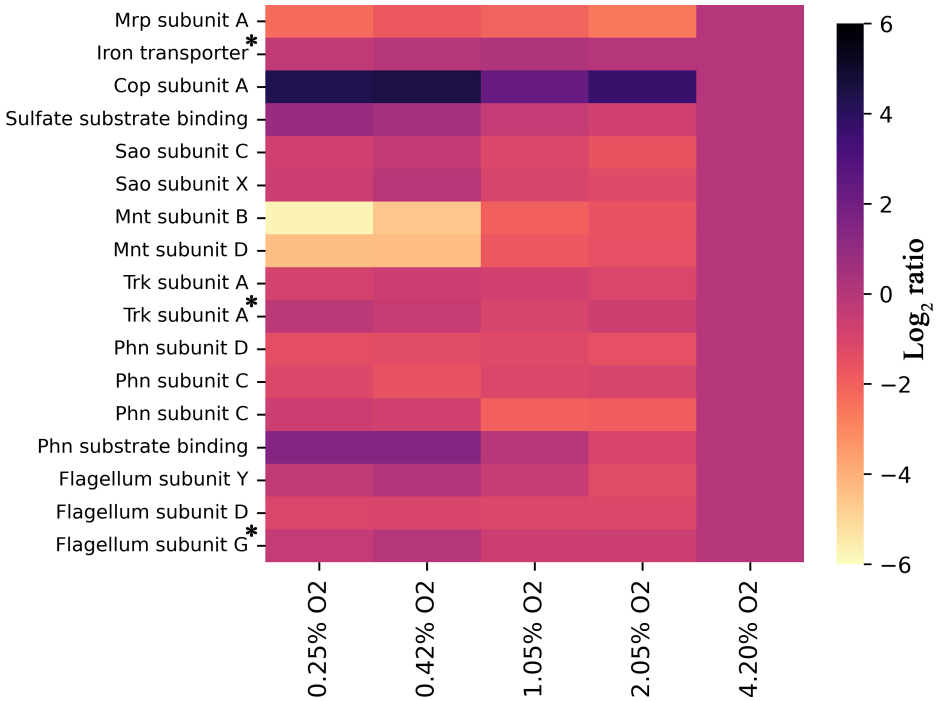

Figure 5.4: Heat map showing log2 ratios of all detected subunits of known transporters of inorganic compounds for each condition, provided 2 or more unique peptides were detected, relative to 4.2% O_2. All trends have a significance of $p < 0.05$, except those denoted with an asterisk. For the Iron transporter $p = 0.255$, for the Trk subunit A $p = 0.053$ and for the Flagellum subunit G $p = 0.101$. For all others, the exact significances and the corresponding protein number can be found in Supplementary File I, and the locus tags in textbfSupplementary Table I.

since it is expressed at lower O_2 concentrations. Cyt. *bd* is also known to be used at low oxygen conditions in *E. coli* [8, 43].

5.3.3. A LOW-OXYGEN ADAPTATION SPECIFIC FOR ALKALIPHILES?

In addition to the respiratory chain which is obligately coupled to oxygen, other membrane processes also adapt at different oxygen availabilities (**Figure 5.4**). **Figure 5.4** shows the log2 ratios for transporters detected in this study and our previous work on unravelling the membrane proteome of this organism [33]. There is a striking 6-fold upregulation of CopA, the ATP-coupled copper export mechanism linearly coupled to oxygen limitation (**Figure 5.4**) Copper can be toxic to cells and in Rubrivivax gelatinosus, it has been demonstrated that CopA mutants have decreased cytochrome *c* oxidase activity [55]. The solubility of copper is lower at high pH than under the conditions used in R. gelatinosus [56], perhaps further exacerbating this issue. Besides this, copper efflux might be induced in *C. thermarum* to protect Iron-Sulfur Cluster Enzymes from copper mismetallation [57, 58]. This is because the intracellular labile Fe^{2+} pool is higher under oxygen limitation, which leads to a net increase of Fe^{2+}-Fur activity with concomitant iron-protein expression [59]. This gains feasibility especially considering the ionic radii of Fe^{2+} is 0.076 nm vs Cu^{2+} 0.073nm and Cu^{1+} at 0.077 nm [60]. Lastly, another possible scenario is that the copper requirement of the cell decreases at lower oxygen levels, due to the decreased Cyt aa3 production, for which copper is required. Whilst Cyt. ba_3, also requiring copper, is produced in its stead, the absolute amount of each individual enzyme can differ quite starkly; data in this study only shows regulation of proteins relative to themselves. A decreased content of heme-copper oxidases could increase the need for copper export and thus explain the observed pattern. In contrast, the manganese transport complex Mnt is down-regulated 6-fold. Generally, manganese is required for living with oxygen [61], explaining the lower requirement at low O_2.

Aside from Mnt, the transporter Mrp is also significantly down regulated at lower O_2 concentrations. The Mrp antiporter facilitates the export of Na^+ coupled to the import of H^+. This antiporter is considered a vital protein for alkaliphiles, as it is one of the primary methods to regulate internal sodium *and* proton levels [36, 37, 62]. Data on the ETC shows that proton export does not decrease to the extent that Mrp becomes useless. Another reason must drive the decreased need for sodium:proton antiport through Mrp. Since all other medium components remained the same, we especially expect sodium stress to remain as compelling for an alkaliphile like *C. thermarum* TA2.A1 under low O_2. Interestingly enough, the very first studies into *C. thermarum* TA2.A1 centred on the fact that substrate import (glutamate and sucrose) is mediated using sodium ions, not protons [14, 63]. Though no *in vivo* nor *in vitro* data is reported regarding acetate export, the expectation is that this is also mediated by sodium ions [15]. Considering the high level of acetate production at low O_2 levels, we hypothesize that the function of Mrp is – at least partly – replaced by

the acetate exporter under these conditions. This would constitute a valuable addition to the field of alkaliphiles, as no such regulation of Mrp, to the best of our knowledge, has been found previously.

5.4. CONCLUSION

C. thermarum TA2.A1 was successfully cultivated within a microaerobic range in chemostats, demonstrating its low oxygen requirement. Oxygen limitation became evident near 4.2% O_2 in the gas inlet while the microorganism grew even at 0.25% O_2 in the gas inlet. Acetate is produced concurrent with respiration in order to complement energy generation. Surprisingly, proteomics data showed that both NADH dehydrogenases are constitutively expressed under the conditions tested. It makes little sense that an Ndh1 is upregulated at low O_2, pumping out protons, when the F_1F_o-ATP synthase that takes the protons back into the cell is downregulated under the same conditions. One potential explanation is that the intracellular pH should be increased at low O_2 to keep the redox homeostasis functional.

Cyt. aa_3 is the predominant contributor to the terminal oxidase pool at the highest oxygen level, whilst Cyt. ba_3 takes over below 4.2% O_2. Detection of either Cyt. bb_3 or Cyt. *bd* was expected at 0.25% O_2, given that the abundance of Cyt. ba_3 started to decline. Neither Cyt. bb_3 nor Cyt. *bd* was detected, but there is a probability that either or both are present at low oxygen levels. Proteomics analysis of membrane proteins needs to further developed in order to fully use this methodology for the study of respiratory systems. Lastly, a notable finding was a decreased abundance of the Mrp complex, considered a crucial cog in alkaliphilic sodium homeostasis. We hypothesized that this could be due to the presence of an acetate:sodium symporter, responsible for facilitating acetate export and thereby decreasing sodium stress.

5.5. ACKNOWLEDGMENTS

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CONCLUSION AND OUTLOOK

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Inside, an alkaliphilic bacterium closely resembles any neutrophilic microorganism. Alkaliphiles set themselves apart through specific adaptations in the cell wall, cell membrane, and proteins within these structures. Studying these components will further our understanding of how alkaliphiles maintain an internal pH close to neutral despite the scarcity of protons in their environment. Previous research into alkaliphiles highlighted the use of acidic components in the cell wall [1–6]. In those studies, the high degree of acidic residues is implicated in enabling more favourable respiratory conditions by aiding sodium homeostasis, though the mechanism behind this remains unclear. We concur that alkaliphiles that use proton-based ATP synthases require a mechanism to induce local proton motive force [7]. However, whether this is solely due to the mechanism resulting from the elevated levels of acidic residues in the cell wall, another mechanism, or a combination of mechanisms cannot be concluded based on the identification of only two components, as done by the labs of Horikoshi and Krulwich. Further research, specifically focusing on the untargeted characterization of the cell wall is required, though this was not part of the aim of this research.

This thesis focused on two components of alkaliphilic adaptations in gram positive bacteria: the cell membrane and the membrane proteins. The cell membrane must be highly adapted to minimize proton leakage; any lost protons must be actively transported back in, which is energetically costly. In addition to various other functions, such as respiration, membrane proteins transport charged components across the cell membrane. Alkaliphiles must especially adapt two membrane transport mechanisms. The first is the import of protons, which need to be strongly concentrated relative to the external environment. Where protons are scarce in alkaline conditions, sodium is often present at elevated concentrations. Therefore, efficiently handling sodium is another key requirement for alkaliphiles. This thesis revealed specific adaptations in the membrane of an alkaliphile, *Caldalkalibacillus thermarum* TA2.A1. It laid the groundwork for more detailed studies of (alkaliphilic) membrane proteins and provided one initial insight on that front. Additionally, this thesis delved into the respiration in alkaliphiles.

6.1. MEMBRANE PROTEINS OF ALKALIPHILES

6.1.1. MEMBRANE PROTEOMICS

As stated above, the study of membrane proteins in alkaliphiles should elucidate how these microbes concentrate protons relative to the external environment while preventing intracellular sodium buildup. The use of proteomics is quite commonplace presently and it is optimized for soluble proteins. Since predominantly membrane proteins are interesting when studying alkaliphiles, maximizing the detection of the membrane proteome was a focal point within this thesis. **Chapter 4** concentrated on identifying the maximum number of membrane proteins in *C. thermarum* TA2.A1. Based on genomic information from **Chapter 2**, which identified a total of 3,085 genes

encoding for proteins, we determined that *C. thermarum* TA2.A1 contains 754 proteins with a single transmembrane helix; as detected by DTU's TMHMM tool [8]. This means that roughly a quarter of all the proteins in *C. thermarum* are membrane proteins, in line with earlier theories [9, 10]. In **Chapter 4**, we combined whole cell proteomics with dedicated membrane proteomics. Additionally, we subjected the membrane extracts to solubilization, significantly improving detection compared to untreated extracts. The optimal conditions for *C. thermarum* TA2.A1 were found to be a combination of whole cell proteomics, membrane proteomics of extracts solubilized at 65°C or 80°C with FOS-choline-12, and membrane proteomics of extracts solubilized at 80°C with SDS. Using a single transmembrane helix as a cutoff, this workflow detected 158 membrane proteins. Particularly noteworthy was our ability to detect a broad range of proteins responsible for respiration and transporters of organic and inorganic solutes. In the future, the insights from **Chapter 4** should form the basis for a proteomics study using samples obtained under the same conditions as described in **Chapter 3**; these samples exist in fact. Conducting this proteomics study should provide further insights into alkaliphile-specific adaptations related to membrane proteins. Sadly, this thesis had to be completed before this study was carried out.

In **Chapter 4**, we used a single transmembrane helix as a criterion to distinguish between membrane proteins and other proteins, but this is an imperfect cutoff for determining the efficiency of a membrane proteomics pipeline. Many membrane proteins, such as the F_1F_o ATP synthase, consist of multiple subunits. In the case of the F_1F_o ATP synthase, three of these subunits are embedded in the membrane and are correctly categorized by the TMHMM tool. The remaining subunits are incorrectly identified as soluble proteins. Conversely, some proteins might contain a highly hydrophobic helix domain and be incorrectly categorized as a membrane protein, thus leading to inclusion in our analysis. The categorization of proteins has no effect on the identification of the individual proteins. It is solely useful to determine the efficiency of a membrane proteomics pipeline. Further development of bioinformatics analysis tools like Toot-M should enhance our ability to correctly categorize all membrane protein, including peripheral membrane proteins [11]. We recommend that future research addressing the role of the membrane proteome takes note of our observations. For any other organism or consortium, finding the optimal detergent and temperature for solubilization will be necessary.

6.1.2. O_2 RELATED ADAPTATIONS

In **Chapter 5**, we cultivated *C. thermarum* TA2.A1 under microaerobic conditions and subjected steady-state samples to whole cell proteomics, though with a keen interest in the membrane proteome. Based purely on the cultivations, a notable finding in this study was the ability of *C. thermarum* TA2.A1 to replicate at the set dilution rate even at low oxygen partial pressures.

At the lowest aerobic level of cultivation, only 0.25% O_2 was present in the gas inlet stream, marking the first report of *C. thermarum* TA2.A1's capability to grow under such low oxygen partial pressure.

The genome of *C. thermarum* TA2.A1 encodes for four terminal oxidases (**Chapter 2**). As expected, whole cell proteomics revealed that the cytochrome *c*:oxygen *aa*$_3$ complex was most abundant at the highest oxygen level. This terminal oxidase translocates one proton per electron, whilst the other terminal oxidases are less efficient. At lower oxygen levels, the abundance of the cytochrome *c*:oxygen *ba*$_3$ complex increased. This terminal oxidase translocates half a proton per electron. Interestingly, at 0.25% O_2, the abundance of the cytochrome *c*:oxygen *ba*$_3$ complex began to decrease, yet neither of the other two terminal oxidases was detected. This underscores the importance of dedicated membrane proteomics. We hypothesize that either the cytochrome *c*:oxygen *bb*$_3$ complex or the menaquinol:oxygen *bd* complex plays a role at 0.25% O_2, but we are unsure of our ability to measure these complexes. In **Chapter 4**, which focused on membrane proteomics specifically, neither complex was detected. However, in that case proteomics was conducted on an aerobic batch shake flask culture, making it incomparable to a chemostat with 0.25% O_2 in the gas inlet. The laborious membrane proteomics workflow, as well as spectroscopic methods to determine cytochrome *c*ontent, were omitted in **Chapter 5** due to time constraints. Subjecting membrane extracts to the workflow of **Chapter 4** might therefore still detect either the cytochrome *c*:oxygen *bb*$_3$ complex or the menaquinol:oxygen *bd* complex. Such an experiment will help in elucidating whether *C. thermarum* TA2.A1 indeed harbours four terminal oxidases that are actively used under a certain condition. Answering that question is more of fundamental relevance for further characterization of *C. thermarum* TA2.A1 than of consequence to the broader study of alkaliphiles.

6.1.3. THE ROLE OF MRP IN ALKALIPHILES

The Mrp complex, a Na^+:H^+ antiporter found in most microbes but considered essential for alkaliphiles [12–14], was downregulated in as oxygen availability decreased (**Chapter 5**). The exact role of this enzyme in pH and Na^+ homeostasis under oxygen limitation warrants further exploration. We hypothesized that the sodium aspect of its function might be complemented by an acetate exporter, which is indeed produced in increasing amount as oxygen decreased. Like other *C. thermarum* TA2.A1 organic solute transporters, the acetate exported likely concomitantly exports sodium ions [15, 16]. If the downregulation of Mrp at low O_2 is solely due to the complementation of sodium export resulting from acetate production, it suggests that pH homeostasis is less challenging at these conditions or at the very least not influenced greatly by Mrp. At lower O_2 availabilities, the cytochrome *c*:oxygen *ba*$_3$ complex becomes more abundant and this translocates half the amount of protons compared to the cytochrome *c*:oxygen *aa*$_3$ complex. Perhaps pH

homeostasis is less challenging because the level of proton translocation is lowered? This intriguing question warrants further research.

6.1.4. CYTOCHROME b_6c_1

Whole genome sequencing in **Chapter 2** detected the presence of a cytochrome b_6c_1 complex. Given the homology of its iron-sulfur cluster to that of cytochrome b_6f, we presumed that this complex functions as a hybrid version of Complex III. The iron sulfur cluster containing protein and the b_6 subunit likely originate from the b_6f complex, while the c_1 subunit originates from the cytochrome bc_1 complex. A cytochrome b_6c_1 complex was previously identified in *Geobacillus stearothermophilus* [17], and was shown to function as a Complex III replacement in *Rhodobacter capsulatus* [18]. This enzyme was detected in **Chapter 4** and in all conditions of **Chapter 5**, suggesting some vital activity at the very least. However, if the protein truly is a hybrid cytochrome b_6c_1 complex remains unknown. Efforts to overexpress this protein in another organism or to natively purify this protein were unsuccessful thus far, leaving its characterization pending.

6.1.5. FURTHER STUDIES ON THE F_1F_o ATP SYNTHASE OF C. thermarum TA2.A1

The F_1F_o ATP synthase of *C. thermarum* TA2.A 1 is the best studied protein of the organism [19–27]. Previous work on this enzyme showed that it is functional at pH 9.5 but not at pH 7.5 [24]. This specificity for high pH is due to the presence of a lysine residue at position 180 in the a subunit, which is responsible for proton capture. Lysine is a basic amino acid with a side-chain pK_a of 10.54 at 25°C. Although optimal cultivation temperature of *C. thermarum* TA2.A1 (65°C) [15, 28] shifts side-chain pK_a, it remains basic. In the pH range of 8.5 to 10.5, where *C. thermarum* TA2.A1 stabilized quickest (**Chapter 3**), the lysine residue can exist in its protonated and deprotonated state. At pH 7.5 however, the side-chain is always deprotonated, therefore blocking ATP synthesis activity. In **Chapter 3**, oxygen consumption at steady state remained stable throughout the whole pH range. Clearly, *C. thermarum* TA2.A1 adapted to respire at these conditions. We hypothesized that the adaptation required is to modify the residue at position 180 in the a subunit of the F_1F_o ATP synthase to another amino acid, most likely cysteine. That might explain why the adaptation time of *C. thermarum* TA2.A1 at pH 7.5 was three times longer than at in the pH range of 8.5 to 10.5. The adaptation time at pH 11 was similar to that of pH 7.5. Whether that indicates the need for a similar in the a subunit of the ATP synthase, this time exchanging the lysine residue for arginine, is unknown. At pH 11, the extended adaptation time could also stem from counterintuitive need to upregulate menaquinone expression. Sequencing the *a* subunit at steady state in all conditions, as well as other subunits of the F_1F_o ATP synthase, should answer the question whether this was part of reason for the long adaptation time. If confirmed, it would further

highlight the importance of the lysine residue in the functioning of alkaliphilic F_1F_o ATP synthases.

6.2. MEMBRANE LIPIDS OF ALKALIPHILES

Chapter 3 of this thesis explored adaptations in the lipid membrane of *C. thermarum* TA2.A1 over a pH range of 7.5 to 11. As mentioned in Chapter 1 and **Chapter 3**, mitigating proton leakage is a key challenge for alkaliphiles. In **Chapter 3**, we observed higher levels of acylphosphatidylglycerols in the membrane at pH 9.5 and above. Additionally, at pH 11, menaquinone concentrations significantly increased. Acylphosphatidylglycerols serve to decrease the surface area of the membrane. **Chapter 3** proposes that these lipids are incorporated into the outer leaflet in the curved regions of the membrane, opposite to cardiolipin. Incorporating acylphosphatidylglycerols in this manner decreases 'roughness' of the cell curvature, which helps in minimizing proton leakage across the membrane.

Chapter 3 introduced that in other alkaliphiles, triterpenes are incorporated into the centre of the lipid bilayer to act as an insulating layer – again with the aim of decreasing proton leakage. *C. thermarum* TA2.A1 lacks the capacity to produce triterpenes. We hypothesized that it increased menaquinone concentrations instead, as that is the only neutral lipids it is capable of producing. Like triterpenes, menaquinone resides within the centre of the lipid bilayer. **Chapter 3** confirmed that this is also true at highly elevated menaquinone levels. Therefore, menaquinone could serve as insulating layer, similar to triterpenes. **Chapter 3** concluded that neutral lipids are crucial in alkaliphilic membranes, but that the exact nature of the neutral lipid is less important.

Utilizing quinones to counteract proton leakage in gram positive bacteria was previously proposed [29], and our observations support this hypothesis. However, *in vitro* data confirming the ability of quinones to reduce proton leakage across lipid membranes is currently lacking. A prior study on ubiquinone indicated that if Haines' hypothesis is correct, it would likely apply only to oxidized quinone. Reduced quinol might actually aid proton leakage [30]. Although both Roche and Haines discuss ubiquinone specifically, their observations and theories likely extend to menaquinone. Since oxygen consumption of *C. thermarum* TA2.A1 at pH 11 remained stable (**Chapter 3**), most menaquinone at pH 11 would likely be present in its oxidized form, thus capable of fulfilling its insulating role. Ideally, future *in vitro* membrane leakage experiments should include both menaquinone and menaquinol in the lipid membranes to further explore this phenomenon. In any case, *in vitro* data on the effect of quinones on proton leakage would offer valuable insights into this proposed additional role of quinones.

The discovery of acylphosphatidylglycerols was surprising and their presence in alkaliphiles remains largely unexplored. We suggest that identifying of this lipid should be included into the standard lipids analysis pipelines.

Additionally, future research should focus on identifying the genes responsible for acylphosphatidylglycerol production. Understanding the pathway for acylphosphatidylglycerol production will enable a comprehensive study of its prevalence in alkaliphiles or other organisms.

6.3. THE FUTURE OF ALKALIPHILIC RESEARCH

This thesis advanced our fundamental understanding of gram positive alkaliphiles, using the facultative alkaliphile *C. thermarum* TA2.A1 as a model organism. Intracellularly, alkaliphiles resemble neutralophilic microbes, so the key adaptations to study are those related to the cell wall, cell membrane and the proteins therein. Although the cell wall is an intriguing aspect of alkaliphilic survival, it was outside the scope of this research.

Chapter 3, which explored the membrane of *C. thermarum* TA2.A1 grown over a pH range from 7.5 to 11, led to two conclusions. The first aligned with past research: alkaliphiles incorporate a higher than normal amount of neutral lipids into the centre of the lipid bilayer, which helps with limiting proton leakage [31–33]. In **Chapter 3**, we detected an unusually high concentration of menaquinone at pH 11, positioned mainly in the centre of the lipid bilayer, as confirmed by molecular dynamics simulations. This lends further support to the hypothesis that neutral lipids are crucial for alkaliphiles. This study adds the possibility of using menaquinone, primarily involved in respiration, when the genetic machinery for synthesizing traditional neutral lipids is absent. Future research on alkaliphiles should focus on analysing the membrane composition at the highest possible pH level for cultivation, where finding a substantial presence of neutral lipids is most likely. Such research may uncover additional neutral lipid options incorporated by alkaliphiles. Additionally, as stated above as well, we recommend conducting membrane leakage assays to examine the ability of squalene and menaquinone to limit proton leakage compared to a non-neutral lipid membrane.

A completely novel and unexpected observation in **Chapter 3** was the increased biosynthesis of acyl-PGs, a lipid with three aliphatic tails. Molecular dynamics simulations in a membrane-only system indicated that this lipid lowers the membrane's surface area. It would be interesting to test whether this would still be true if a membrane protein is included in the simulation. We hypothesized that acyl-PGs contribute to minimising proton leakage particularly in the curved membrane regions. This hypothesis is based on previous research, which hypothesized that acyl-PGs primarily reside in the outer leaflet of the membrane, opposite to cardiolipin, helping plug small gaps in the membrane surface [34, 35]. The absence of prior observations of acyl-PGs in alkaliphiles may be attributed to limited awareness of the existence of such lipids. To facilitate the detection of acyl-PG production capacity in alkaliphiles, it is crucial to uncover the biosynthetic pathway for this lipid. Some previous studies biochemically confirmed certain pathways but did not identify the corresponding genes [36–38]. These studies provide an excellent starting point

for future research.

Lastly, this research places significant emphasis on membrane proteomics, as evident in **Chapter 4** and **Chapter 5**. As mentioned earlier, future research should delve deeper into understanding the influence of Mrp on proton and sodium homeostasis across various conditions, especially considering its reduced abundance under low oxygen conditions, which is noteworthy. Furthermore, subjecting *C. thermarum* TA2.A1 samples grown under the same pH range as in **Chapter 3** to the proteomics pipeline detailed in **Chapter 4** may reveal novel insights into the adaptation of alkaliphilic membrane proteomes. This kind of study has not been conducted to date, and there remains a wealth of unexplored territory in the realm of alkaliphiles. We wish future researchers success in their efforts.

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There are two people whom I would like to thank first and foremost: my supervisors **Prof. dr. ir Mark van Loosdrecht** and **Dr. Duncan McMillan**. Now I have never called you by those extensive names during my PhD, so I will revert back to **Mark** and **Duncan** from here on out. I remember my interview at EBT with you quite well **Mark**. I think we were halfway through my interview when you asked me what I *really* wanted to do. The answer to that had very little to do with the position I was applying for, so I wasn't surprised you hired **Stefan** for that position. Afterwards though, my overeager honesty was rewarded with a e-mail saying I should contact a guy named **Duncan**. I also remember this interview quite well. I think we talked about the success of Ajax for wayyy to long (the times...) and also a bit about science. The overarching theme of the talks with both of you was to do some real microbiology, preferably about something a bit 'out there'. In the end, that bit of outlandish microbiology turned out to be a project about Alkaliphiles. On top of this 'weird' topic, both of you gave me almost complete freedom in how to structure my PhD. The result of this freedom was of course that I felt very lost in the first year. Looking back, I think this feeling was only natural. In fact, I am very grateful for the supervision I received from both of you, being never directive, but always supportive. I wouldn't have chosen anything else! Now aside from my official TU Delft supervisory team, I was also blessed with having an unofficial advisor, or adwiser in his own words: **Dimitry**. Having someone around who always who always provided me with honest and fair feedback was the best addition to my supervisory team I could have, thank you for that.

Having both **Mark** and **Duncan** as supervisor meant that I was also part of two groups: EBT and BOC. I will try to name everyone in the coming part, but I might forget someone, apologies in advance. You see, being part of two groups wasn't (and thus still isn't) always easy. It asked a lot of my time, both for official duties as for social activities. In the end though, I always felt welcome in both groups. At the BOC side, I started when a lot of people who were finishing up: **Marine**, **Sébastien**, **Fabio**, **Morten**, **Hanna**, **Luuk** and **Stefan**. The short time I knew you guys was pre-covid luckily! Especially drinks with **Luuk** and **Stefan** never failed to disappoint. After the defence of **Stefan**, the 'Membrane Bioenergetics Unit' only had one other PhD: **Albert**. When I started, you quickly bombarded yourself to the status of my scientific older brother and you taught me all about being part of 'The **Duncan** experience'. I was also very honoured to be part of your defence as paranimph. Later, the BOC stocks got replenished and BOC had a great squad of PhD's and PostDocs during my time: **Aster**, **Hugo**, **Thomas**, **Ewald**, **Yinqi**, **Xiaomin**, **Angelique**, **Allison** and **Christian**.

EBT was extremely crowded and entertaining. I think what really set our cohort apart was the fact that we preferred to resolve each question with a competition, not in the least place because of the enthusiastic organization skills of **Timmy** and the chosen victim of the moment. Somehow, as long as **David** hadn't defended yet, I would always lose. Luckily I returned the favor during the Masterchef, together with **Venda** and **Beatrix**. In terms of competition, especially the beer tasting competition, won by **Rodoula**, was a low point on my front. I would like to say I learnt to stop bragging prematurely, but this is of course not true, and I would be reminded of my 'losses' daily during lunch (or breakfast). The lunch table was never quiet with our squad of radios (**David**, **Timmy**, **Kiko** and **Sergio**) and with interrogator-in-chief **Maxim**. Our roadtrip to the north of Denmark, with **Maxim**, **Nina**, **Timmy**, **Stefan**, **Kiko** and **Lemin** was also amazing, although I remember feeling way to young in the one party street of Aalborg. Also I will always remember our countless Friday afternoon drinks. So thanks also to (people inside EBT and outside): **Ingrid**, **Marta**, **Jules**, **Chris**, **Hugo**, **Morez**, **Gonçalo**, **Ali**, **Claudia**, **Jitske**, **Ramon**, **Siem**, **Marit**, **Puck**, **Gerben**, **Jure**, **Samarpita**, **Philipp**, **Roxana**, **Marina**, **Daphne**, **Tiago**, **Jan**, **Angelos**, **Matteo**, **Gabriel**. Thanks also to **Jonna** and **Charlotte** for the many coffee breaks and supportive words during my PhD and it was also a pleasure being your paranimph **Jonna**. Also many thanks to the students that trusted me to supervise them: **Glenn**, **Judith**, **Martijn**, **Javi**, **Andrea**, **Maud**, **Kadir** and **Daphne**.

In the second year of my PhD, I was also part of the BT PhD committee. The team we had, **Timmy**, **Mariana**, **Céline** and **Susan**, was the best I could ask for. Starting during covid times, we had to improvise. The strength of our team ensured we could think of countless online activities to entertain everyone. That's also where I learnt that my counting skills in not what I think they were. To this day I still don't know whether **David** and **Sergio** won the office battle legitimately. Luckily, by the end of our tenure, we could actually do some in-person activities, with the BT symposium (with unasked additional entertainment by the location itself) and the BT sports day, which we almost won ourselves by accident. Big thanks also to my SIAM TU Delft squad, **Timmy**, **Stefan**, at times **Hugo**, and later **Samarpita** and **Yubo**. For the SIAM network, I was also part of the PhD council (instead of committee, just to have some variation in name). That council was also very productive with a plethory of online meetings, a few symposia and at the end some in-person stuff. Thanks **Jolanda**, **Merve**, **Linnea**, **Maider**, **Maaike** and lastly **Stefan** for eventually taking over my TU Delft spot.

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CURRICULUM VITAE

Samuel Ilan de Jong

Sam de Jong was born in Delft on March 19^{th}, 1993. He was raised primarily in The Hague, and attended the Gymnasium Haganum high school there. In the last few years of high school, Sam became especially interested in microbiology and the workings of DNA. The logical next step therefore was to enroll in the still relatively novel Bachelor Life Science & Technology. This Bachelor had classes at both Leiden University and Delft University of Technology. Sam did his Bachelor thesis in the Industrial Microbiology group at TU Delft and decided in that period to enroll in the eponymous Master program at TU Delft. During the Master Life Science and Technology at TU Delft, Sam followed the tracks Cell Factory and Biochemical Engineering. His Master thesis was a collaborative project between the Industrial Microbiology group and the Environmental Microbiology group, under the supervision of Laura Valk. To finalize his Master program, Sam emigrated to Lausanne, Switzerland to become an Intern at Nestlé Research. At Nestlé Research, Sam was mainly working in the lab, which wasn't quite to his taste. He therefore decided to remigrate to the Netherlands to follow a PhD program at Delft University of Technology. He found another collaborative project, this time between Dr. Duncan McMillan of the Biocatalysis group and Prof. dr. ir. Mark van Loosdrecht at the Environmental Microbiology group, to pursue the next step of his carreer. This dissertation is the end result of that. In his spare time, Sam like to play hockey, read books, play games, cook and go on hikes.

LIST OF PUBLICATIONS

3. S. I. de Jong, D. Y. Sorokin, M. C. M. van Loosdrecht, M. Pabst en D. G. G. Mcmillan. 'Membrane proteome of the thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1'. In: *Frontiers in Microbiology* 14 (2023), p. 1228266. doi: 10.3389/fmicb.2023.1228266

2. A. Krah, T. Vogelaar, S. I. de Jong, J. K. Claridge, P. J. Bond en D. G. McMillan. 'ATP binding by an F1Fo ATP synthase ϵ subunit is pH dependent, suggesting a diversity of ϵ subunit functional regulation in bacteria'. In: *Frontiers in Molecular Biosciences* 10.February (2023), p. 1–11. issn: 2296889X. doi: 10.3389/fmolb.2023.1059673

1. S. I. de Jong, M. A. van den Broek, A. Y. Merkel, P. de la Torre Cortes, F. Kalamorz, G. M. Cook, M. C. van Loosdrecht en D. G. McMillan. 'Genomic analysis of *Caldalkalibacillus thermarum* TA2 . A1 reveals aerobic alkaliphilic metabolism and evolutionary hallmarks linking alkaliphilic bacteria and plant life'. In: *Extremophiles* 24.6 (2020), p. 923–935. issn: 1433-4909. doi: 10.1007/s00792-020-01205-w. url: https://doi.org/10.1007/s00792-020-01205-w