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[ROLE OF AEROBIC BACTERIA IN DOLOMITE FORMATION IN THE

EVAPORITIC ENVIRONMENTS OF QATAR SABKHAS

BY

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ABSTRACT

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Title: ROLE OF AEROBIC BACTERIA IN DOLOMITE FORMATION IN THE EVAPORITIC ENVIRONMENTS OF QATAR SABKHAS

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Sabkha (salt flats) are extreme evaporitic environments inhabited by a large diversity of halophilic microorganisms. Sabkhas have been studied to understand the microbe-mineral interactions that lead to the formation of dolomite, a carbonate mineral with the formula $CaMg(CO_3)_2$. Dolomite is abundant in ancient sedimentary sequences and constitutes economically important oil and gas reservoirs. However, because it is virtually impossible to form abiotically at Earth's surface conditions, its mechanism of formation remains unclear and debated.

Most of the previous studies on microbial dolomite formation in evaporitic environments focused on anaerobic sulfate-reducing bacteria. A first goal of this thesis was to clarify if also some aerobic species possess the same ability. Aerobic bacteria were, therefore, isolated from the sediments of a Qatari sabkha –the Dohat Faishakh sabkha– that are known to contain dolomite. The strains, identified by 16S rRNA gene sequencing, resulted to belong to the genera *Bacillus*, *Staphylococcus, Salinivibrio* and, primarily, *Virgibacillus*. Subsequent experiments conducted in the laboratory under conditions that simulate the sabkha environment showed that *Virgibacillus* strains are capable of mediating the formation of non-ordered dolomite with up to 48 mol% Mg. This result

shows that not only sulfate-reducing bacteria but also aerobic microorganisms may play an important role in dolomite formation in sabkha environments.

To evaluate how the high temperature and salinity that are typical of sabkhas concur and affect the studied biomineralization process, we conducted precipitation experiments at variable temperatures, salinity and Mg^{+2}: Ca^{+2} ratios. The results showed that temperature is the most important among the tested factors and that it is positively correlated with the amount of Mg that is incorporated into the carbonate mineral during the biomineralization process.

Finally, to test the hypothesis the extracellular polymeric substances (EPS) play a key role in the mineralization process, we characterized how temperature and salinity affect the production and chemistry of EPS and compared EPS of bacterial strains capable and noncapable of mediating mineral formation. We found that high temperatures (i.e., 40°C) cause microbes to produce EPS that contain a large proportion of carbohydrates. This finding is consistent with the hypothesis that carboxyl groups play a crucial role in the formation of dolomite at low temperature. We have also reinforced this conclusion performing experiments with organic molecules that simulate different types of natural EPS.

Together, our results provide new insights into microbial diversity and biomineralization mechanisms that lead to the formation of dolomite in sabkha environments.

DEDICATION

This work is dedicated to all members of my family, especially to the ones who no longer with us.

May God bless their souls.

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LIST OF ABBREVIATIONS

AOM	Anoxygenic Oxidation of Methane
HMC	High Magnesium Calcite
EDS	Energy- dispersive X-ray spectroscopy
EPS	Extracellular Polymeric Substances
FTIR	Fourier Transform Infrared Spectrometry
SEM	Scan Electron Microscopy
SRB	Sulfur Reducing Bacteria
TCHO	Total Carbohydrates
TP	Total Proteins
XRD	X-ray Diffraction

CHAPTER 1 : INTRODUCTION

The long-standing so-called “Dolomite problem” arose from the abundance of dolomite in the ancient sedimentary rocks and its rare occurrence in modern environments, and numerous unsuccessful attempts to synthesize dolomite in the laboratory at room temperature under sterile conditions (Arvidson & Mackenzie, 1999).

The interest of studying dolomite formation is also of economic importance. Indeed, dolomite rocks represent the world’s primary oil and gas reservoirs. The largest natural gas condensate field, South Pars/North Dome is located in the Arabian Gulf and is mainly formed of dolomite, limestone and anhydrite (Rahimpour-Bonab et al., 2010). Understanding the mode of dolomite formation would contribute to the basic research on mineral formation and to the improvements of oil and gas extraction technologies.

Evaporitic environments represent appropriate settings to study the mineralization process. Interestingly, it was recognized since the 1960s that the Arabian Gulf evaporitic environments (named sabkhas) as rare places on Earth where dolomite form at ambient temperature. Dohat Faishakh sabkha located north-west of Qatar was the first site to be studied (Wells, 1962; Illing, Wells, & Taylor, 1965). Sabkha is characterized by extreme environmental conditions (Taher, 2014). Many dolomite formation models in sabkha were proposed and revisited, but still could not be reproduced in the laboratory. The extensive evaporation in sabkha leads to precipitation of gypsum, which removes Ca^{+2} and increases the Mg^{+2}: Ca^{+2} ratio. Subsequent evaporation results in the precipitation of calcite and aragonite causing an additional increase in the Mg^{+2}: Ca^{+2} ratio, this

progressive mg; ration increase was thought ‘according to earliest models’ as a critical factor for the replacement of aragonite by dolomite (Illing et al., 1965).

More recently, biomineralization by microorganisms was evidenced, it was shown that microorganisms including sulfur reducers, methanogens, phototrophs and aerobes exhibit the potentiality to form minerals, suggesting that mineral mediation may not be related to a particular microbial activity (Petrash et al., 2017). Microorganisms could create microenvironments characterized by specific pH and redox conditions suitable for the primary mineral formation. Furthermore, the observed close association between dolomite nucleation sites and microbially secreted Extracellular Polymeric Substances (EPS) led to the formulation of a microbial sabkha-model, suggesting that, EPS within living and decaying mats play a crucial role in the incorporation of Mg^{+2} into the carbonate minerals. (Bontognali et al., 2014; Tourney & Ngwenya, 2014; Decho & Gutierrez, 2017; Petrash et al., 2017).

Several changes of physicochemical factors, such as temperature, salinity and pH that can concur in several ways in the evaporitic area influence the parameters of mineral precipitation and concomitantly the microbial activity (Babel & Schreiber, 2014). As a consequence, microorganisms have the tools to adapt to such changes considered as stress and employ mechanisms intracellularly and extracellularly to survive, including EPS secretion (Dang & Lovell, 2016). The adaptations routes would lead to a biodiversity of such microorganisms in relation to their ecology (Malik et al., 2017). These factors affecting the saturation index, the microbial growth and the excreted EPS, are considered as major factors in the mineral mediation process (Dupraz et al., 2009; Zhu & Dittrich, 2016).

Most of the studies on the microbial mediation of dolomite formation focused on anaerobes (sulfur reducing bacteria (SRB) and methanogens), while fewer studies were carried on aerobic bacteria. Actually, there is an increasing interest in evidencing the involvement of aerobic bacteria and understanding their role in dolomite formation.

Dohat Faishakh sabkha would provide a set of answers in studying the biodiversity of aerobic bacteria and their biological activities employed in dolomite formation, being in a continuous process at the natural conditions. This would contribute to better understanding the overall process of microbial dolomite formation.

The goal of this work is to:

1. Evidence the role of halophilic, heterotrophic, aerobic bacteria in High magnesium calcite (HMC) Formation in Dohat Faishakh Sabkha's evaporitic environment; construction of a local collection of dolomite-forming bacteria.
2. Study the influence of the main physical and chemical parameters (temperature, salinity and $Mg^{2+:}$ Ca^{2+} ratio) on microbial mediation of Mg-rich carbonates at aerobic conditions
3. Study the effect of the main physical and chemical factors on EPS produced by the aerobic bacteria; evidencing their role in mediating dolomite formation
4. Contribute to understanding the role of the bacterial EPS, by simulation of its main components (carbohydrates and proteins).

CHAPTER 2 : LITERATURE REVIEW

2.1 Evaporitic minerals and formations

The broad definition of evaporites is referred to sedimentary deposits that usually occur as result of evaporation (Twenhofel, 1950; Babel & Schreiber, 2014). Evaporites are commonly composed of water-soluble salts (Goldschmidt, 1937), which accumulate in ocean water and natural water basins. These salts accumulate in substantial quantities only through evaporation of water, that is the fundamental characteristic of evaporites (Sonnenfeld & Finetti, 1985). Evaporation, the vital driving force in the evaporite system works in two ways, 1) bring the water-soluble salts to a state of saturation and supersaturation, hence, 2) promote continuous precipitation of these salts in the evaporite basin (Babel & Schreiber, 2014). The variation in the physiochemical parameters such as temperature, salinity and pH is the main characteristic of the evaporitic environments and brines. Halite, anhydrate and gypsum are common evaporite minerals, though other minerals could form due to continental flux (Schreiber & Tabakh, 2000). Evaporitic environments and brines are characterized by fluctuating –within some limits- underlying physicochemical features and parameters, such as salinity, temperature and pH. In such evaporites complex systems, one or more salts may precipitate simultaneously when the saturation or supersaturation with respect to these salts is reached (Babel & Schreiber, 2014).

Three main evaporite minerals formulate the major rocks and the largest volumes of deposits, which are gypsum ($CaSO_4 \cdot 2H_2O$), anhydrite ($CaSO_4$) and less frequently halite (NaCl). They are estimated to account for more than 90–95% of ancient and modern precipitates (Warren, 2006). Dolomite ($CaMg(CO_3)_2$), magnesite ($MgCO_3$) and calcite

($CaCO_3$) are usually associated with evaporites; but they are not considered as classic evaporite minerals (Warren, 2006). The accumulation of outstanding hydrocarbons often occurs in various evaporite- related basins. In many cases, the traps and seals of such accumulations are controlled by the transitions of stratigraphic distribution of evaporite-carbonate facies (Dolson, 2016).

2.2 Sabkha, Arabic term for evaporitic mudflat

Sabkhas provide a possible environment to study the formation of evaporitic minerals, one of the few modern ones. The term "Sabkha" is the Arabic name for the salt flat. Sabkhas occur as a result of the periodic accumulation of evaporites mainly carbonates and sulfates. A recent description by Warren (2016), defines sabkha as a hydrological setting where raising groundwater form, within a host sediment (e.g., aragonite mud, dolomite mud) replacive and displacive evaporite minerals such as halite, gypsum, anhydrite, glauberite, polyhalite, etc.This formation occurs in brine-saturated muds immediately below the saline water table or in the capillary fringe above.

Sabkhas commonly occur along the coasts of the Arabian Gulf, which is a marginal sea with depth ranging from 35 m to maximum of 100 m (Purser & Seibold, 1973). It is the warmest sea in the world with mean surface temperatures in the open Gulf varying between 20 °C and 34 °C from winter to summer and in the inner lagoons, temperatures reach up to 40 °C, with daily variations of 10 °C. The mudflats temperatures vary between 5 °C and 50 °C and sediment surface temperatures in the sabkha may reach up to 60 °C. The sabkha is extremely humid in summer, especially at night, when humidity may reach 100% (Kinsman, 1969; Warren, 2016), while it was reported that salinity

might reach up to 30% in the summer seasons (Al-Youssef et al., 2006; Al-Youssef, 2015).

2.2.1 Types of Sabkha

Sabkhas develop either inland or on the coast, in response to the decrease of sediment surface or accumulation of sediments in a lagoon or both (Evans, 1970; El-Omla & Aboulela, 2012). Because of different primary depositional systems, Sabkhas exhibit significant variations based on the physical processes controlling their development and the sedimentary responses to those processes. Based on dominant physical processes, three end types of sabkhas are recognized: 1) fluvial-lacustrine, 2) marine and 3) eolian-dominated systems. Playas (mudflats) are present in fluvial-lacustrine dominated field, while coastal sabkhas are usually controlled by marine processes and interdune sabkhas are eolian-dominated (Handford, 1981).

There are also three subclasses, 1) permanently wet sabkha which contains lakes and ponds where the groundwater table is always within a few centimeters of the ground surface. 2) Periodically flooded sabkha which refers to ephemeral surface water brought about by heavy rains or windblown tides and 3) permanently dry sabkha (Barth & Böer, 2002).

Studies showed that the origin and evolution of sabkhas in Qatar are mostly influenced by the following environmental factors: 1) Surface topography and morphology; 2) level of Shallowness; 3) Desert climate; 4) Quaternary changes in sea level; 5) Groundwater; 6) Geological conditions and; 7) Anthropogenic interventions (Ashour et al. 1991).

2.2.2 Microbiology of Sabkhas, occurrence of microbial mats

Microbial mats commonly occur in sabkhas environments and, according to recent hypotheses, they could have an important and highly underestimated involvement in the formation of evaporitic minerals. A variety of environments provide a habitat for microbial mats, including hypersaline ponds, hot and deserts, hot springs, alkaline lakes and coastal intertidal sediments (Stal, 1994). They appear to have a remarkable role in the development of organosedimentary deposits. In the evaporitic environments of a sabkha, microbial mats exhibit an incredible diversity of microbial species and make use of an expansive range of metabolic activities (Wong et al., 2016; Decho & Gutierrez, 2017). This is supported by variable resources of nutrients available in sabkhas, making the mat a dynamic ecosystem (Wong et al., 2016), that exhibit unique microbially induced sedimentary assemblies (Dupraz et al., 2009). The simplicity of recognition and sampling makes sabkha a suitable model for evaporitic disposition for the study of potential preservation of life signatures (Taher, 2014).

Microbial mats are multilayered structures of microorganisms, mainly archaea, bacteria, fungi and occasionally enriched with protozoans, which could reach centimeter-thick (Reitner, 2011). The activities of numerous microorganisms, primarily, aerobic, heterotrophic, photosynthetic and sulfate-reducing bacteria are associated with limitations in mass transfer, causing the formation of particular microenvironments characterized by high spatial and temporal variations, these microenvironments lead to heterogeneous environments with diverse microbial assemblages (Al-Thani et al., 2014).

The microbial populations of the sabkha in Qatar demonstrate an apparent horizontal and vertical distribution. The upper intertidal zone is occupied by cyanobacterial mats while

the archaebacterium *Halobacterium salinarum* and the microgreen alga *Dunaliella salina* dominate the middle intertidal region. The lower intertidal zone is characterized by a muddy black substrate due to large quantities of sulfur and methane that are produced by particular bacteria (Mahasneh et al., 2006).

In a study conducted by Al-Thani et al. (2014) the microbial community structure of Um Alhool-Qatar microbial mats was studied on a four layers' basis. It was revealed that all layers were dominated by different classes, belonging to *Proteobacteria* including different classes (*alpha*, *beta* and *gamma*); members of *Alphaproteobacteria* were most abundant in layer 2. *Gammaproteobacteria* belonging to two main groups; obligate anaerobes represented by purple sulfur bacteria and aerobic or facultative anaerobic bacteria were found in all layers, but with high occurrence in the upper layer. The third upper layer was characterized by anoxygenic photosynthetic bacteria (dominated by *Chloroflexus*), whereas the abundance of *Deltaproteobacteria* increased with the increase of the depth of the dark black layer 4. *Deltaproteobacteria* comprise most of the known anaerobic -sulfate and sulfur-reducing bacteria, which are responsible for high sulfate reduction rates. Members of domain Archaea had less rate of recurrence, lower diversity, with a distribution less stratified than in Bacteria (Al-Thani et al., 2014).

Microbial mats are defined based on different fields of interest, i.e., sediment stabilization agents, laminated sediment ecosystems (Gemerden, 1993), producers of paleoenvironmental proxies, or recent analogs of stromatolites, demonstrating them as the oldest recognized ecosystems on Earth (Seckbach & Oren, 2010). Substitution assemblies resulting from the interaction between microbial mats and sediment are particularly common and well conserved in Precambrian rocks (Altermann et al., 2006).

2.2.3 Sabkha, a model of mineralization process

Since 1960s sabkhas of the Arabian Gulf received increasing attention as modern environments where evaporitic minerals --and in particular the enigmatic mineral dolomite-- form in a continuous process (Illing et al., 1965). The intense evaporation in the supratidal zone of sabkha leads to increase of salinity and subsequent supersaturation with respect to anhydrite and gypsum. The continuous precipitation of aragonite combined with gypsum and anhydrite removes the Ca^{+2} from the solution causing an increase in Mg^{+2}: Ca^{+2}, which was thought –according to earliest models as a key factor for the replacement of aragonite by dolomite (Illing et al., 1965).

With the recent development of the microbial model for dolomite formation, new insights into the sabkha model were proposed. Based on several studies conducted on microbial mats of Abu Dhabi sabkhas (i.e., Bontognali et al., 2010; Strohmenger et al., 2011; Bontognali et al., 2014) and Dohat Faishakh sabkha in Qatar (Brauchli et al., 2015). It was shown that microbial activities could create microenvironments characterized by pH and redox conditions that are highly variable from those of lagoon's and sabkha's pore-waters (Bontognali et al., 2010). Moreover, the observed close association between dolomite nucleation sites and microbially secreted Exopolymeric Substances (EPS) led to the formulation of microbial sabkha-model, suggesting that EPS within living and decaying mats play a crucial role in the incorporation of Mg^{+2} into the carbonate minerals (Petrash et al., 2017).

Laboratory experiments evidencing the formation of poorly ordered dolomite and high magnesium calcite using microorganisms, isolated from sabkha, suggest that the subsequent increase in salinity and supersaturation caused by the intense evaporation may

promote an environmental stress. The environmental stress stimulates the extensive production of EPS by microorganisms aiming to protect microbial cells from salinity, desiccation and intense UV radiation stress (Petrash et al., 2017; Al Disi et al. 2017). The combination of EPS production, supersaturated pore-waters, high alkalinity and specific redox conditions maintained by microbial activities may create the appropriate conditions required for low-temperature dolomite formation in the buried microbial mats (Petrash et al., 2015: Petrash et al., 2017).

2.3 Importance of Dolomite

Dolomite ($MgCa(CO_3)_2$) is rarely available in modern environments, as it is a common carbonate mineral found in ancient sedimentary rocks (Arvidson & Mackenzie, 1999; Burns et al., 2000). It makes up around 50% of the world's hydrocarbon basins (Mazzullo, 2004). Interestingly, about 15%–20% of the world's gas reservoirs is estimated to be contained in the Khuff Permian-Triassic Formation in the Middle East (Adam et al., 2014) and is of economic importance for Qatar and Gulf countries. Indeed, it is considered a leading example of petroleum reservoirs formed in an arid and evaporitic environment.

In the ideal ordered dolomite, equal proportions magnesium and calcium are arranged in planes containing Ca cations alternating with planes containing Mg cations and separated by layers of CO_3 (Warren, 2000). Dolomite is found in a wide variety of geological settings including hydrothermal veins, lakes, shallow ocean, lagoons and evaporative basins. Theories proposing the origin of dolomite continue to evolve, among controversy and speculation and many modes-of-origin have been proposed over the years (Al-Awadi et al., 2009).

Dolomite is defined as primary mineral if precipitated directly from different fluids, whereas dolomitization of thick stratigraphic units of limestone causes the formation of a diagenetic product defined as secondary dolomite (Warren, 2000). Thus, dolomite can be divided into two main families: *Penecontemporaneous* and *Postdepositional* dolomites. *Penecontemporaneous* dolomites form soon after deposition of carbonate precursors as a result of geochemical circumstances that conquer within the precursor's environment of deposition (Al-Awadi et al., 2009). The majority of *Penecontemporaneous* dolomites are from the Holocene age and are limited to particular evaporite lagoonal of lacustrine settings. *Postdepositional* dolomites form after the deposition of carbonate sediment and then removed from the sedimentation active zone. This may occur through progradation of the sedimentary surface, burial and subsidence, elevation and emergence of eustatic sea-level fluctuations. Almost all examples of massive regionally extensive dolostone are postdepositional (Al-Awadi et al., 2009).

2.3.1 Dolomite Formation

Many dolomite models were suggested to understand the mechanism of formation of this mineral and its variable abundance in the geologic history (i.e. Badiozamani, 1973; Von Der Borch & Jones, 1976; Zenger & and Dunham, 1980; Baker & Kastner, 1981; Kelts & McKenzie, 1982; Hardie, 1987; Shatkay & Magaritz, 1987; Friedman, 1988 ;Arvidson & Mackenzie, 1999). All of these models compared suggests that dolomite can form in various chemical and physical conditions (Purse et al., 2009).

Dolomite formation is considered to be the outcome of the net effect of various kinetic and thermodynamic factors influencing dolomite nucleation, which may vary in different geological structures (Morrow, 1982; Machel & Mountjoy, 1986). Sediments are exposed

to various physical, chemical and biological forces that define what type of rocks they become. Diagenesis is known as the collective effects of bioturbation, burial, compaction and chemical reactions between the rock, fluid and organic matter (Ali et al., 2010), that will eventually determine the commercial feasibility of the reservoir (Dillinger et al., 2014). Although initial porosity and permeability of the reservoir are controlled by sedimentary settings at the period of deposition, they are subsequently altered by diagenesis (Ali et al., 2010). Carbonate sediments that are mainly composed of calcite, aragonite and magnesian calcite (dolomite), are made of minerals that are highly susceptible to chemical alterations (Maliva, 2016). The impact of biological and physical dispositional processes, in combination with the diagenetic overprint of metastable chemical deposits, can make the distribution of porosity and permeability in carbonates much more heterogeneous than the sandstones (Ali et al., 2010).

2.3.2 Dolomite Enigma

Up-to-date, the formation of Dolomite is still not resolved (Arvidson & Mackenzie, 1999). Despite numerous attempts, the synthesis of dolomite in the laboratory at low temperatures has not been successful yet, an issue often discussed in the literature as "the dolomite problem" (Van Tuyl, 1916; Fairbridge, 1957; Jinghwa & Siegenthaler, 1969; Land, 1988; Holland & Zimmermann, 2000; Mckenzie & Vasconcelos, 2009).

The primary dolomite formation at Earth surface temperatures and the general non-linear occurrence of both primary dolomite and diagenetic substitutions of limestones are the main essence of dolomite enigma (Fairbridge, 1957). Fundamental questions are raised dolomite not being forming in modern oceans despite their supersaturation with regard to dolomite and what are the catalyzer(s) and physiochemical conditions that promote

dolomite formation, but no longer exist in the modern oceans (Sass & Katz, 1982; Brady et al., 1996).

2.4 Role of Microorganisms in carbonate minerals formation

The emergence of biogeochemical studies supported the role of various microbial populations in carbonate precipitation in various natural environments. Many multidisciplinary studies, including biogeochemistry, ecology and genetics had established the involvement of microorganisms in precipitation of several minerals, in terms of biomineralization that is "*biologically induced"* and/or" *biologically influenced"* (Dupraz et al., 2009).

All living organisms impact the formation of carbonate minerals through the interchange of CO_2 or bicarbonate that affects the carbonate equilibrium (Cao et al., 2016). Biomineralization, the processes by which organisms form minerals (Weiner and Dove, 2003) play an essential role in the global biogeochemical cycles (Cappellen, 2003). Biominerals, the products of biomineralization, besides being important biosignatures for studying Earth history, have many important commercial applications (Lin et al., 2014).

Two different primary processes are proposed to be included in the biotically induced precipitation (1) Microbially induced carbonate precipitation (MICP); by metabolic activities of bacteria that mediate pH and pCO_2 causing an increase in saturation state which favors the precipitation of a particular mineral phase (see section 2.4). And, (2) microbially catalyzed carbonate precipitation (MCCP), where bacteria act as a catalyzer in decreasing the kinetic barrier, hence, promoting the precipitation of a mineral from a supersaturated solution with respect to that mineral (Bosak & Newman, 2003).

Microorganisms, basically, produce negatively charged organic molecules that may serve as nucleation sites through adsorbing positively charged anions (i.e., Ca^{+2}, Mg^{+2}) by creating a supersaturated local environment or by reducing the activation energy needed for crystal growth initiation (Bontognali et al., 2014; Cao et al., 2016).

2.5 Role of microorganisms in Mg-Calcite formation

Among different microbially mediated processes that lead to the formation of carbonate minerals, those that lead to the formation of Mg-rich carbonate are of particular interest. Indeed, as explained before, the incorporation of Mg into carbonate minerals at low temperature seems to be kinetically inhibited. Microbes seem to have the capability to overcome such kinetic inhibition. The highly hydrated Mg^{2+} ions favor the formation of aragonite, instead of calcite, making the formation of high-magnesium calcites exceptionally challenging (Lenders et al., 2012; Yu et al., 2017). High magnesium calcite (HMC), containing about (50 mol% $MgCO_3$) with a nearly- stoichiometric dolomite is commonly referred to in the literature as 'disordered dolomite' or 'proto-dolomite' ((Loy et al., 2010; Gregg et al., 2015). HMC occurs in natural environments and can be formed in the laboratory, either by chemical synthesis at high temperatures, or microbially at low temperature (Long et al., 2011, 2014). Mg-rich carbonates are often proposed to contain potential precursors for ordered dolomite (Baker & Kastner, 1981; Kelleher and Redfern, 2002; Sánchez-Román et al., 2011; Rodriguez-Blanco et al., 2015). However, it is to be determined whether or not the formation of very high-magnesium calcite (VHMC) is a first step to the ordered dolomite formation.

The sulfate inhibition model was proposed based on high-temperature laboratory experiments performed by Baker & Kastner (1981) at 200 ºC that showed that the

occurrence of sulfate in very low concentrations inhibits the calcite conversion to dolomite, suggesting that dolomite may only precipitate from waters upon depletion of sulfate. Therefore, the microbial sulfate reduction was the earliest mechanism proposed for dolomite formation (Vasconcelos et al., 1995; Vasconcelos and McKenzie, 1997; Wright, 1999; Warthmann et al., 2000; van Lith et al., 2003). It was suggested that sulfate-reducing bacteria were able to overcome the kinetic barriers for dolomite formation by consuming and removing the dissolved sulfate, which raises the pore water alkalinity and causes a subsequent increase of dolomite saturation index (Vasconcelos and McKenzie, 1997).

However, the results from Sánchez-Román et al. (2006-2009) showed that dolomite forms in the presence or absence of sulfate ions in the media under aerobic conditions, concluding that microbial activity overcomes the sulfate inhibition factor (Sánchez-Román, 2006; Sánchez-Román et al., 2009). Moreover, the examined sediments containing dolomite forming in the supratidal zone, the measured sulfate concentrations were equal or significantly higher than the sulfate concentrations of the region's lagoonal seawater, suggesting that the sulfate ions kinetic inhibition effect can be overcome under particular sedimentary conditions (Wood et al., 2002). These results imply that sulfate inhibition model suggested by Baker and Kastner (1981) may be only applied to the formation of inorganic dolomite at high temperature (Morrow and Ricketts, 1988).

Methanogenic Archaea may also play a role in mediating dolomite formation, particularly in environments characterized with low Mg^{+2}: Ca^{+2} ratio and low ionic strength (Roberts et al., 2004; Kenward et al., 2009). Methanogens can collectively promote saturation of carbonates through employing different metabolic pathways (Roberts et al., 2004;

Madigan et al., 2015). Indeed, acetoclastic methanogens generate dissolved organic carbon, while autotrophic methanogens increase the solution pH by consuming CO_2. Reverse methanogenesis or anoxygenic oxidation of methane (AOM) is a reaction driven by the association of methanogens and communities depending on any given electron acceptor (Timmers et al., 2017).The observations of Petrash et al., (2015) proposed a model demonstrating that anaerobic biogeochemical processes, which couple intermediate Mn and S cycling with suboxic organic matter respiration seem to promote the generation of alkalinity thus, catalyze the stabilization of the dolomite mineral, maybe more efficiently than bacterial sulfate reduction alone.

The research work of Rivadeneyra et al., (1993- 2004) revealed that halophilic aerobic bacteria facilitate primary precipitation of dolomite concomitantly with calcite and high-magnesium calcite (Rivadeneyra et al., 1993, 1998, 2000, 2004). These studies demonstrate that heterotrophic microorganisms use oxygen as an electron acceptor to enable dolomite precipitation at low temperatures; hence, they may have a key role in modern environments, in addition to their role in geological history (Sánchez-Román et al., 2008).

Moreover, recently published work (Al Disi et al., 2017) showed that pure cultures of aerobic bacterial strains -specially *Virgibacillus*- isolated from the Dohat Faishakh sabkha (Qatar) were able to mediate the formation of a mixture of carbonate minerals including very-high-magnesium calcite (VHMC).

2.6 The link between microbial activities and carbonate bioprecipitation

Microbial activities such as ammonification, denitrification, methane oxidation, photosynthesis, ureolysis and sulfate reduction, are common microbial activities that promote carbonate precipitation as a by-product of their metabolism (Muynck et al., 2010; Zhu & Dittrich, 2016). (Table 2-1) shows examples of reported microbial activities known to be involved in carbonate bioprecipitation.

Table 2-1: Microbial activities reported to be involved in carbonate bioprecipitation.

Metabolism	**Organisms identified (examples)**	**Reactions**	**Reference**
Photosynthesis	Many species of Cyanobacteria: i.e., *Synechococcus* strain PCC 7942 Algae	$2HCO3 + Ca^{2+} \rightarrow CH_2O + CaCO_3 + O_2$	(Kamennaya et al.,. 2012) (Dupraz, et al., 2009) (Kosamu & Obst, 2009)
Ureolysis	Ureolytic bacteria: i.e. *Bacillus licheniformis* *Bacillus sphaericus* *Sporosarcina pasteurii*	$CO(NH_2)_2 + 2H_2O + Ca^{2+} \rightarrow 2NH4^{+} + CaCO_3$	(Helmi et al., 2010) (Hammes et al., 2003) (Mortensen, et al., 2011)
Denitrification	Nitrate-reducing bacteria *Alcaligenes, Bacillus, Pseudomonas, Spirillum, Micrococcus, Achromobacter*	$CH_2COO^{-} + 4H^{+} + 2NO3 \rightarrow 2CO_2 + N_2 + 3H_2O$ $Ca^{2+} + CO_2(aq) + 2OH^{-} \rightarrow CaCO_3(s) + H_2O$	(Karatas, 2008)
Ammonification	Myxobacteria: *Myxococcus xanthus*	NHCO (Nitrogen containing matter) + $H_2O \rightarrow CO_2 + NH_3$ $NH_3 + H_2O \rightarrow NH_4^{+} + OH^{-}$	(Jimenez-Lopez et al., 2008)

Metabolism	Organisms identified (examples)	Reactions	Reference
Sulfate reduction	Sulfate reduction bacteria *Desulfostipes saporovans* *Desulfosarcina spp.* *Desulfovibriae*	$SO2^{-4} + 2[CH_2O] + O$ $H^- + Ca^{2+} \rightarrow CaCO_3 +$ $CO_2 + 2H_2O + HS^-$	(Vasconcelos and McKenzie, 1997; Warthmann et al., 2000; Wright & Wacey, 2005) (Baumgartner, et al., 2006)
Methane oxidation	Methanogens	Anaerobic oxidation: $CH_4 + SO2{-}4 + Ca^{2+}$ $\rightarrow CaCO_3 + H_2S + H_2$ O Aerobic oxidation: $CH_4 + 2O_2 \rightarrow CO_2 + 2$ H_2O	(Moore et al., 2004) (Reeburgh, 2007)

In conclusion, various bacterial activities produce local supersaturation in the microenvironment that favors precipitation of carbonate minerals. However, to overcome the low-temperature kinetic barriers to dolomite precipitation, it is possible that the chemistry of the cell walls and associated EPS also play a crucial role (Roberts et al., 2013; Voegerl, 2014; Qiu et al. 2107).

2.7 Role of EPS in carbonate minerals formation

The chemistry of the cell walls and associated EPS is progressively considered as an essential aspect of the precipitation of calcium carbonates (Riding 2000; Dupraz et al. 2004; Bontognali et al., 2008; Braissant et al. 2009). The chemically responsive EPS matrix is of vital ecological importance because it constitutes the physical barrier between the cell and other organisms as well as organic and inorganic metabolic substrates (Freeman & Lock, 1995). Bacteria and microalgae produce polysaccharide

matrix with a molecular weight that ranges between 8-1000 kDa and may contain proteins, extracellular DNA, non-carbohydrate acidic functional groups (i.e., pyruvate or succinate) and inorganic compounds such as sulfates and phosphates (Sutherland, 2001).

Particular groups of genes control the production and secretion of EPS that are differentially regulated using chemical signaling known as quorum sensing (Miller & Bassler, 2001). Quorum sensing facilitates coordination of activities between groups of bacteria within mats and increases metabolic efficacies (e.g., nutrients' consumption).

A wide range of microorganisms can produce EPS, both photoautotrophic and heterotrophic bacteria (Stal, 2003; De Philippis et al., 2001; Richert et al., 2005; Pereira et al., 2009). EPS matrix inside microbial mats works as a 'sponge'; that absorbs cations from the surrounding liquids (Braissant et al., 2007). Initially, carbonate formation is inhibited by the presence of EPS, then, this cation-binding characteristic could be reduced through various mechanisms resulting in the precipitation of carbonates within the EPS matrix (Bontognali et al*.,* 2010). Such mechanisms include: (1) increase of external cation concentrations (e.g., Ca^{2+} and Mg^{2+}) until EPS acidic bonds are saturated; (2) cations are released as result of EPS hydrolytic destruction; and (3) Organization of EPS to form acidic template for biomineralization (Dupraz et al., 2004). (Figure 2.1) illustrates a theoretical model for microbial mediation of carbonate mineral formation in the EPS matrix proposed by Braissant et al., (2008)

Trichet and Défarge's (1995, 2001) proposed that the acidic macromolecules are distributed randomly throughout the EPS-matrix in microbial biofilms. Reorganization of these acidic sites through diagenetic processes provides a pre-arranged nucleation

templates for complete biofilm biomineralization. Thus, the biomineralization process can be driven either intrinsically through microbial metabolisms, or extrinsically through degassing and evaporation. Biomineralization can be either biologically-induced (active) or biologically-influenced (passive) processes (Trichet & Defarge, 1995; Trichet et al., 2001).

Besides the effect of active metabolism, the surfaces of microbial cells and the excreted EPS can bind ions due to their net negative charge, that has been suggested as primary sites for dolomite nucleation (Lith et al., 2003; Bontognali et al., 2008; Sanchez-Roman et al., 2008). Thus, both active microbial activities and the presence of EPS within the sediments have been estimated to be critically important for dolomite formation (Bontognali et al., 2010, 2014). These examples advocate that the presence of EPS favor the formation of Mg-rich mineral phases.

The role of microorganisms in the precipitation of various types of carbonate minerals has been widely discussed and progressively acknowledged in the literature (Riding, 2000; Sanchez-Roman et al., 2007; Bontognali et al., 2014; McCutcheon, 2013; Zhu & Dittrich, 2016). However, the mechanisms of biomineralization are still controversial (not commonly agreed). Several researchers speculated a role of living/ non-limiting bacterial cells in mediating the formation of carbonates, i.e. (Douglas & Beveridge, 1998; Sánchez-Román et al., 2015; Silva-Castro et al., 2015). While others emphasized the role of specific macromolecules – Exopolymeric Substances (EPS) in providing nucleation sites that facilitate the bioprecipitation in of carbonates and other types of minerals (Bontognali et al., 2014; Tourney & Ngwenya, 2014; Petrash et al., 2017).

Based on lifestyle choices, EPS produced by a species may vary. Secreted EPS may exhibit changed or modified properties due to geochemical, enzymatic and photochemical processes. Hence, more molecules are possibly trapped or sorbed, which results in major alterations of their physiochemical properties. In natural environments, EPS exist in a 'dynamic state' in terms of compositional and partial degradation rates (Decho & Gutierrez, 2017). Extreme environments provide novel microbial biodiversity that produces diverse and promising EPS (Nichols et al., 2005)

In most modern deposits, a growing association is found between the occurrence of authigenic dolomite and microbial mats, composed of a heterogeneous population of microorganisms and their EPS (Decho, Visscher, & Reid, 2005; Bontognali et al., 2010). EPS account for more than 50% of the sedimentary organic matter (Flemming and Wingender, 2001). Upon the decay of EPS at differential degradation rates into sugars and amino acids, the negatively charged acidic functional groups are self-rearranged with diverse affinities towards Ca^{+2} and Mg^{+2} cations, may act as nucleation templates (Trichet et al., 2001; Braissant et al., 2007, 2008)

The variable composition of EPS is known to be dependent on the species of bacteria, growth phase, growth medium (mainly, carbon source), pH, temperature and many other environmental factors (Omoike & Chorover, 2004; Wingender, Neu, & Flemming, 2012; Tourney & Ngwenya, 2014). The changes in the EPS composition in response to various factors may result in favoring specific mineral formation.

The combined microbial metabolism by microbial community acts as an "alkalinity engine" through changing the saturation index (SI), or Ca^{2+} and CO_3^{2-} concentrations,

while, the EPS matrix, influences the mineral formation through cation release/binding and by providing nucleation sites. This matrix can strongly impact the morphology and composition of the formed minerals (Dupraz et al., 2008).

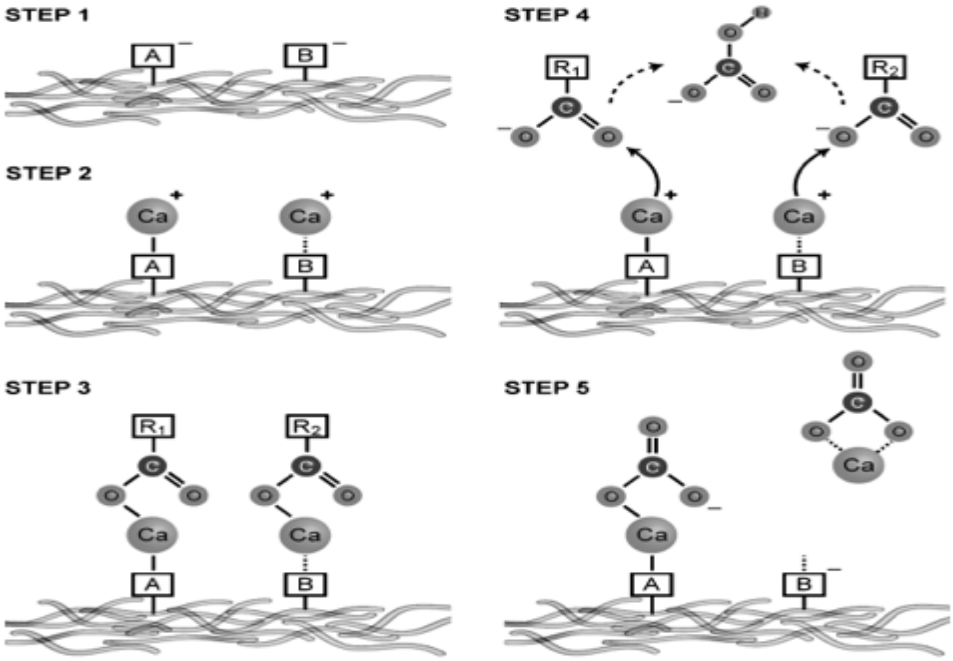

Figure 2.1: Theoretical model for microbial mediation of $CaCO_3$ formation in the EPS matrix (Braissant et al., 2008).

1. Existence of (A, B) functional groups on EPS with different pK values and dissociation constant (Kd) for calcium;
2. Calcium ions bind (more or less) tightly to A and B;

3. Low Molecular Weight (LMW) organics form a bidentate complex with carbon and calcium;
4. LMW organic carbon is removed by microbial activity (solid arrow), which is successively oxidized to bicarbonate (dashed arrow);
5. EPS–Ca complex binds carbonate.

The formed $CaCO_3$ may remain in association with the EPS matrix (as EPS-A-$CaCO_3$) or occurs freely within the EPS matrix.

Braissant et al. (2003) investigated the effects of exopolysaccharides and amino acids on the morphology and mineralogy of $CaCO_3$ crystals produced by bacteria and concluded that it is likely that spherical crystals indicate the presence of bacteria or biofilms associated with mineral formation. Much remains to be resolved about the mechanisms by which different acidic organic molecules impact the composition, size and morphology of the formed carbonate minerals (Kröger, 2015; Cao et al., 2016).

In conclusion, it is clear that bacteria can create the suitable conditions for mineral formation at low temperature through exerting specific exopolymeric substances (EPS) within the supersaturated pore-waters while maintaining high alkalinity and specific redox conditions by microbial respiration (Bontognali et al., 2014; Petrash et al., 2017).

2.8 Abiotic factors affecting biomineralization

Although it seems that in sabkhas and other modern environments, a microbial factor is essential for the incorporation of Mg^{+2} into the formed carbonate phase, it is important not to underestimate more traditional abiotic factors. These factors can be equally fundamental for directly determine what type of mineral can form in a given setting, but

also for controlling the ecology of the environment hosting the microbes involved in the mineralization process.

Many abiotic factors influence microbial growth and the production of EPS and, in turn, the bioprecipitation of minerals. However, their impact is still not well understood and therefore the optimal conditions for microbially mediated precipitation are still not reported. Chemical and physical parameters such as fluid chemistry, viscosity, ionic composition, salt concentration, pH and temperature are the most important factors (Buczynski & Chafetz, 1991; Silva-Castro et al., 2015; Balci & Demirel, 2016).

For example, Qiu et al. (2017) reported an increase of carboxyl groups on the cell surface of *Haloferax volcanii* as a response to high salinity levels. Under such conditions, the formation of dolomite was prompted. However, a further increase in salinity inhibited the bacterial growth and caused cell lysis. This suggests that high salinity (if not exceed the threshold that inhibits microbial growth) may promote the formation of Mg-rich carbonates (Rivadeneyra et al., 2004; Rivadeneyra et al., 2016; Qiu et al., 2017).

CHAPTER 3 : MATERIALS AND METHODS

3.1 Culture media

3.1.1 Culture media for isolation and mineral precipitation

To isolate halophilic, heterotrophic, aerobic bacteria, liquid MEC2 media was used (Sánchez-Román et al., 2007). MEC2 is composed of (per liter): yeast extract 10 g, peptone 5 g, glucose 1g, NaCl 75 g and supplemented with calcium acetate monohydrate ($Ca(CH_3CO_2)_2 \cdot H_2O$) 1 g and magnesium acetate tetrahydrate ($(CH_3COO)_2Mg \cdot 4H_2O$) 8 g, (corresponding to Mg^{2+}: Ca^2 molar ratio 6.0). 18 of agarose were added to the liquid medium to obtain MEC2 solid media. The pH was adjusted to 7.0 with 0.1 M KOH. All culture media were autoclaved for 20 min at 121°C.

3.1.2 Culture media to study the role of media components in biological activity (mineral precipitation)

The culture media used for investigating the role of media components in biological activity are shown in (Table 3-1).

Table 3-1: Concentrations (g/l) of media component in the different culture media

Media	Yeast Extract	Peptone	Glucose	NaCl	$Ca(CH_3CO_2)_2 \cdot H_2O$	$CH_3COO)_2Mg. 4H_2O$	Mg^{+2}/Ca^{+2} molar ratio
M1	10	5	1	75	0.5	4	6
MEC2	10	5	1	75	1	8	6
M2	10	5	1	75	1.5	12	6
M3	10	5	1	75	2	16	6
M4	10	5	1	75	3	24	6
MR1	10	5	1	75	1	16	12.3
MR2	10	5	1	75	1.5	16	8.3
MR3	10	5	1	75	3	16	4.1
MR4	10	5	1	75	4	16	2.96

3.1.3 Culture media to study the impact of salinity, temperature and Mg^{2+}: Ca^{2+} ratios on morphology and composition of minerals

The culture media used to study the effect of salinity, temperature and Mg^{2+}: Ca^{2+} ratios on morphology and composition of crystals are labeled MD1 through MD9. The media are composed of (g/l): yeast extract 10, peptone 5, glucose 1, agarose 18 and supplemented with different concentrations of acetate salts. Additionally, the salinity was adjusted with different concentrations of Sodium Chloride (Table 3-2).

Table 3-2: Concentrations (mM) of Acetate salts and Sodium Chloride (%) in the different culture media.

Medium	Mg^{+2} (mM)	Ca^{+2} (mM)	Mg^{+2}: Ca^{+2}	NaCl (%)
MD1	9	9	1	3.5
MD2	9	9	1	7.5
MD3	9	9	1	10
MD4	56	9	6	3.5
MD5	56	9	6	7.5
MD6	56	9	6	10
MD7	108	9	12	3.5
MD8	108	9	12	7.5
MD9	108	9	12	10

3.1.4 Culture media for screening urease activity

The urea media contained (g/l); peptone 1, glucose 1, sodium chloride 5, potassium dihydrogen phosphate 0.8, disodium phosphate 1.2, phenol red 0.012 and agar 15.0. (4%, w/v) urea solution was separately prepared by filtration using 0.45 μm membrane filter, 10 ml of sterilized urea solution was aseptically added to 990 ml of media. (Omoregie et al., 2011).

3.1.5 Culture media for EPS production

The MD 4, MD5 and MD6 liquid culture media (as described in 0) were used for EPS production. The media were supplemented with 56 mM Mg^{+2} and 9.0 mM Ca^{+2} to obtain 6:1 Mg^{+2}: Ca^{+2} molar ratio. Additionally, the salinity was amended with three different concentrations of Sodium Chloride (3.5%, 7.5% and 10%) as shown in (Table 3-2), the pH was adjusted to 7 with 0.1 M KOH.

3.2 Isolation and purification of halophilic, heterotrophic, aerobic bacteria

MEC2 liquid and solid media were used for the isolation and purification steps. Starting from the top layer, 1.0 g of sediment from equally sectioned layers were mixed in 25 ml liquid medium and then incubated in a shaker at 200 rpm for 48 h at 30°C. An enrichment step was performed by inoculating 2ml of each culture in 25 ml fresh medium and incubated at conditions similar to the first cultures. The enrichment steps were repeated twice. Then, 100 µl aliquots of each culture were spread on solid medium and kept in an incubator set at 30°C. Distinct colonies formed after two days were isolated. The purification steps were performed several times using the streak plate method till pure isolates were obtained. The differentiation of isolates was performed based on colony color and shape using an optical microscope with 40x and 100x magnification to detect distinct colonies from each plate. Finally, the selected isolates from each layer were coded according to the numbers of core, layer and colony respectively.

3.3 Molecular identification of bacterial isolates

The DNA was extracted from cells grown on LB solid media overnight at 30°C. Pure colonies were inoculated into 0.2 ml of sterile distilled water, incubated for 10 min in a water bath set at 100°C and then centrifuged 12,300 rpm for 30 seconds. The supernatant (containing total DNA) was transferred to a fresh Eppendorf tube. The amplification of 16S rRNA gene fragments of ~1.5 kb was carried out using the universal primers; RibS74sp 5'-AAGGAGGTGATCCAGCCGCA-3' and RibS73sp 5'-AGAGTTTGATCCTGGCTCA-3' (Lane, 1991). Each of the PCR reactions was performed with the total volume of 25µl including $MgCl_2$ 1.5µM, dNTP 0.8 µM,

forward primer 1.35 µM, reverse primer 1.35 µMand 0.5 IU Taq DNA polymerase. 2 µl genomic DNA from the isolates was used as template for PCR reactions.

For each PCR reaction, the thermocycler program started with a 3 minutes initial denaturation step at 94°C, followed by 35 cycles of denaturation step at 94°C for 45 seconds each, annealing step at 50°C for 45 seconds and elongation step at 72°C for 45 seconds, then one final 2-minute extension step at 72°C. The PCR products were purified using a Qiagen- QIAquick PCR Purification Kit.

A second PCR was carried out for the purified amplicons. The final purification steps were carried out using the BigDye X-Terminator Purification Kit. The sequencing was performed using a Genetic Analyzer-Applied Biosystems 3500 Series. The sequences of 16S rRNA gene fragments were used to determine the most closely related species available in the database of GenBank using the NCBI Blast server.

3.4 Screening for urease activity

To test for the production of urease, urea agar plates were inoculated and incubated under aerobic conditions at 30°C for 48 h. The production of urease production was detected by visual observation upon color changes from yellow to pink.

3.5 Screening for mineral-precipitating isolates

MEC2 plates were inoculated with each isolate and incubated at 30°C for 30 days. The plates were regularly observed using light microscope at 40x and 100x magnification to detect the occurrence of solid phase (crystals). Minerals were harvested from plates and observed under 100x magnification, using the “Acid test” method (King, 2016); a drop of 1M HCl was allowed to leak under the cover slide. Carbonate minerals were dissolved

and disappeared, causing visible bubbles of CO_2 gas, resulting from an acid/carbonate reaction.

Experiments were performed in triplicate and four sets controls were used.

- Control 1: Two of the isolates (DF141 and DF2101) which were unable to form mineral crystals were used interchangeably as internal negative controls.
- Control 2: The 2nd control was performed using uninoculated plates.
- Control 3: MEC modified medium adjusted to lower concentrations of acetate salts (3 mM calcium acetate and 18 mM magnesium acetate) was used as a 3rd control.
- Control 4: 100 µl of 1M $NaCO_3$ were spread on the surface of MEC2 plates, to prompt mineral formation by reaching supersaturation. This last control was performed to verify if abiotic minerals formation would occur in MEC2 plates.

3.6 EPS production conditions

DF112 and DF2141 bacterial strains were grown in MD solid media, then pure colonies were from each strain were suspended in 1ml of MD liquid media to prepare a suspension with an optical density of 0.2 at 600 nm. 100 µl of each suspension were transferred into 50 ml falcon tubes containing 25ml of MD liquid media; all cultures were incubated for five days at 30°C with 200 rpm agitation.

3.7 EPS extraction and purification

At the end of incubation period, the cultures were centrifuged at 6,000 g for 20 min at 4° C. The supernatant was placed into 250 ml flask and two volumes of cold ethanol to precipitate the EPS, then kept overnight at 4°C. The precipitated EPS was re-suspended

in distilled water dialyzed to remove metabolites and low molecular weight salts using a cellulose dialysis membrane (molecular weight cut-off = 12 kDa) and kept for 24 h, at 4°C in a volume of 20 ml distilled water. Purified EPS were freeze-dried and stored at -20°C for further analysis.

3.8 Total Carbohydrates and proteins estimation

5 mg of freeze-dried EPS were hydrolyzed with 0.5 ml of 2N H_2SO_4 at 100°C for 16 h and then neutralized with 1M NaOH. The phenol-sulfuric acid method (DuBois et al., 1956; Nielsen et al., 2010) was used to determine total polysaccharide content. 100 µl of each sample was made up to 2 ml of distilled water, then 50 µl of (80 % (w/v) phenol were added and mixed. Finally, five mL of concentrated sulfuric acid (95–97 %, Sigma-Aldrich) was added rapidly. Solutions were mixed via gentle vortexing and let to stand for 10 min. The absorbance was measured at 490 nm after allowing the samples to cool down to room temperature in a water bath for 30 min. Glucose standard solution was used to perform the calibration curve.

The Bradford method was performed for protein quantification (Bradford, 1976; Kruger, 1996), 5 mg of freeze-dried EPS were suspended in 200 µl distilled water, then 5 ml of Bradford dye reagent was added to the solutions and kept to stand at room temperature for 5 min. The absorbance was measured at 595 nm. The standard calibration curve was constructed using Bovine Serum Albumin (BSA).

3.9 Fourier Transform Infrared Spectroscopy FTIR

2 mg of freeze-dried EPS were homogenized and powdered with 200 mg of KBr and pressed to a pellet in a 13-mm diameter mold. Fourier Transform Infrared (FTIR) spectra were acquired by (PerkinElmer, Spectrum BXII). The absorbance range was 4000–400 cm^{-1}.

3.10 Crystal formation in the presence of xanthan and amino acids

Calcium Chloride $CaCl_2$ 0.1 M and 0.025 M and Magnesium Chloride $MgCl_2$ 0.1 M and 0.350 M solutions were prepared, then 0% or 0.1% (w/ v) of xanthan were added, the solutions were sterilized through autoclaving for 20 min at 121 °C. Stock solutions of three amino acids (L-glutamine, L-glutamic acid or L-Aspartic acid) were prepared. The amino acids solutions were sterilized using membrane filter 0.2 μm.

The precipitation experiments were performed using the NH_4CO_3 free-drift method, Petri dishes filled with solutions were placed in enclosed large desiccator sterilized by ethanol. Each Petri dish contains a total of 20 mL (10 ml of $CaCl_2$ and 10 ml $MgCl_2$), to which 1.0% (w/v) amino acid (either L-glutamine or L-glutamic acid or L-Aspartic acid) was added. The Petri dishes were kept for 14 days in a sterilized desiccator filled with $(NH_4)_2CO_3$. The NH_4CO_3 decomposition produces NH_3, CO_2 and H_2O. The dissolution of NH_3 into experimental solutions cause the increase of pH and CO_2 dissolution provides CO_3^{2-} source, which results in carbonate precipitation. At the end of incubation period, the solution containing precipitates were centrifuged at 5000 g, washed several times with distilled water and then air-dried at 40ºC. Crystals were analyzed by SEM/EDS and XRD.

3.11 Scanning electron microscopy, Energy- dispersive X-ray spectroscopy (SEM/EDS) analyses

Sediments from each layer (1 g) of sampled cores were homogenized and manually powdered using a pestle and mortar and then air-dried overnight at 40°C. The dry sediment samples were used for SEM/EDS and XRD analyses.

For examination of crystals formed in the pure cultures, bacterial biomass and associated minerals were carefully removed from plates using a scalpel to scrap the upper layer. Solid media containing crystals were smashed into small pieces, placed into 50-ml centrifuge tubes. To remove saline and impurities, samples were washed several times with distilled water and then centrifuged at 5000 g for 20 min. The minerals were harvested from the bottom of the centrifuge tubes and then air-dried at 40°C. This method did not affect the morphology of the crystals, as observed by optical microscopy inspection before and after the recovery steps. Dried samples were used for XRD and SEM/EDS analyses.

SEM images and EDS analysis were obtained using a Scanning Electron Microscope - FEI Quanta 200, with a 5 nm resolution an x200,000 magnification, equipped with energy dispersive X-ray microanalysis system (EDS, model-2011).

3.12 X-ray diffraction (XRD) analyses

Dried samples were prepared as described in section (3.11). To determine their mineralogical composition, selected targeted particles were allocated for X-ray analysis. Rigaku model-Miniflex II Desktop X-ray Diffractometer, equipped with a Scintillator NaI (TI) detector was used to determine the bulk mineralogical composition of the analyzed samples.

3.13 Analyses of brine

The artificial growth media used for the experiments were designed considering the composition and natural variability of some sabkha water samples, collected in the Dohat Faishakh sabkha –the modern dolomite-forming environment from which the strains used for the experiments have been isolated– at different locations and in different seasons. The temperature range during sample collection was 23–40°C. Chemical analyses of the sabkha waters were performed by inductively coupled plasma (ICP)-mass spectroscopy was performed for Na^{+}, Mg^{2+} and Ca^{2+} cations and Cl^{-}, Br^{-} and $SO4^{-2}$ anions using the ICP-mass spectrometer Agilent 7500CX-United States.

3.14 Statistical Analysis

Three replicates were used for all the experiments. The significance of differences between conditions was analyzed using ANOVA. Multilinear regression analysis was performed to estimate the association between the three independent variables, (temperature, salinity and Mg^{2+}:Ca^{2+} ratios) and the % mole Mg, and to determine which predictor variable(s) are the most important. Statistical analyses were carried out at the 95% confidence level using IBM SPSS Statistics.

CHAPTER 4 : EVIDENCE OF A ROLE OF AEROBIC BACTERIA IN HIGH MAGNESIUM CALCITE FORMATION IN THE EVAPORITIC ENVIRONMENT OF DOHAT FAISHAKH SABKHA

4.1 Introduction

A wide range of organisms, including bacteria, are involved in the biotic formation of calcium carbonates. Described as biologically induced or influenced, the biotic formation has been reported extensively in the literature (Chahal, Rajor, & Siddique, 2011; Dhami et al., 2013; Wei et al., 2015; Anbu et al., 2016; Zhu and Dittrich, 2016). However, the highly hydrated Mg^{2+} ions favor the formation of aragonite, instead of calcite, making the formation of high-magnesium calcites exceptionally challenging (Lenders et al., 2013). HMC is frequently referred to in the literature as 'disordered dolomite' or'proto-dolomite' (Gregg et al., 2015) and considered to constitute potential dolomite precursors (Baker & Kastner, 1981; Arvidson & Mackenzie, 1999; Kelleher & Redfern, 2002; Kaczmarek & Duncan, 2011; Rodriguez-Blanco et al., 2015).

Sabkha, the Arabic name for inland, or coastal mudflats, are characterized by extreme environmental conditions such as high temperature, salinity

and light intensity (Dong and Yu, 2007) and occupied by diverse communities of extremophilic and halophilic microorganisms. Precipitation of minerals in sabkha is crucially dependent on its unique dynamic evaporitic systems (Edwards et al., 2010). Proto-dolomite - poorly ordered non-stoichiometric dolomite- usually precipitates in evaporitic environments (Warren, 2000). Successive diageneses processes, such as multiple inorganic recrystallizations, are suggested to lead to the development of ordered dolomite (Morse & Mackenzie, 1990; Burns et al., 2000; Machel, 2005; Baldermann et al., 2015).

In the evaporitic environments of a sabkha, microbial mats exhibit an incredible diversity of microbial species and make use of a broad range of metabolic activities (Baumgartner et al., 2009; Wong et al., 2016). This is supported by variable resources of nutrients available in sabkhas, making the mat a dynamic ecosystem. The activities of various microorganisms, especially, aerobic, heterotrophic, photosynthetic and sulfate-reducing bacteria lead to the creation of specific microenvironments classified by high spatial and temporal variations (Al-Thani et al., 2014).

First microbial models proposed for dolomite formation discussed the role of sulfate-reducing bacteria through rising pH and alkalinity and removal of sulfate (Vasconcelos et al., 1995; Wright, 1999). However, laboratory experiments showed that dolomite forms in the presence or absence of sulfate ions in the media under aerobic conditions (Sanchez-Roman et al. 2006, 2009). There is cumulative evidence suggesting a role for aerobic microorganisms in mediating formation of dolomite (Sanchez-Roman et al., 2006, 2007, 2008, 2009; Rivadeneyra et al., 2016).

A close association between microbial mats and dolomite formation has been recently reported in sabkhas in Abu Dhabi and Qatar, proposing a microbial role in the mineralization process (Bontognali et al., 2010, 2012; Brauchli et al., 2015).

Dohat Faishakh, a hypersaline coastal sabkha located in the north-west of Qatar, was considerably recognized since the 1960s as one of the rare modern environments where dolomite forms ((Wells, 1962; Illing et al., 1965). Recent research work on Dohat Faishakh sabkha emphasized the importance of microbial mats - living and non-living- in authigenic dolomite formation (Brauchli et al., 2015). Therefore, the sabkha exemplifies a unique environment to study the involvement of aerobic bacteria in mineral formation.

The goal of this study is to determine whether halophilic, heterotrophic, aerobic bacteria, demonstrating biological activities that may potentially lead to the formation of dolomite in the evaporitic environment of Dohat Faishakh sabkha and the diversity of such microorganisms. Aerobic bacterial strains were isolated from different layers of Dohat Faishakh sabkha sediments and their role in mineral formation investigated.

4.2 Selection of the sampling sites

Dohat Faishakh hypersaline coastal sabkha is located in the north-west of Qatar. The choice of sampling points was based on former studies citing the occurrence of various forms of sediments including dolomite (Illing et al., 1965; Brauchli et al., 2015). Four cores were sampled at different seasonal intervals (March and October 2015-2016) as shown in (Table 4-1).

Table 4-1: Description of the collected core samples.

Sample No.	**Core 1**	**Core 2**	**Core 3**	**Core 4**
Collection date	12-March-2015	21-Oct-2015	22-Mar-2016	Oct-2016
GPS Coordinates	25°37'49.1"N 50°57'52.8"E	25°38'13.4"N 50°57'31.9"E	24°38'44.0"N 51°20'48.4"E	24°38'17.0"N 50°57'31.7"E
Temperature (°C)	25	40.6	27	40
Core length (cm)	24	52	20	55

To eliminate possible contamination, core samples were harvested aseptically and appropriately sealed, to avoid any further exposure to light. All cores were, labeled and enfolded with foil; they were temporarily stored in an icebox at 4°C and then transported to the laboratory for analysis.

4.3 Occurrence of dolomite and HMC in Dohat Faishakh sabkha

The presence of HMC and dolomite in Dohat Faishakh sabkha was evidenced in the sediments along the sampled cores, using SEM/EDS and XRD analyses. (Figure 4.1) Shows SEM/EDS images of bulk sediments sampled from layers of cores 1 and core 2, the EDS analysis indicate the abundance of carbonate minerals with various Mg^{+2}: Ca^{+2} ratios.

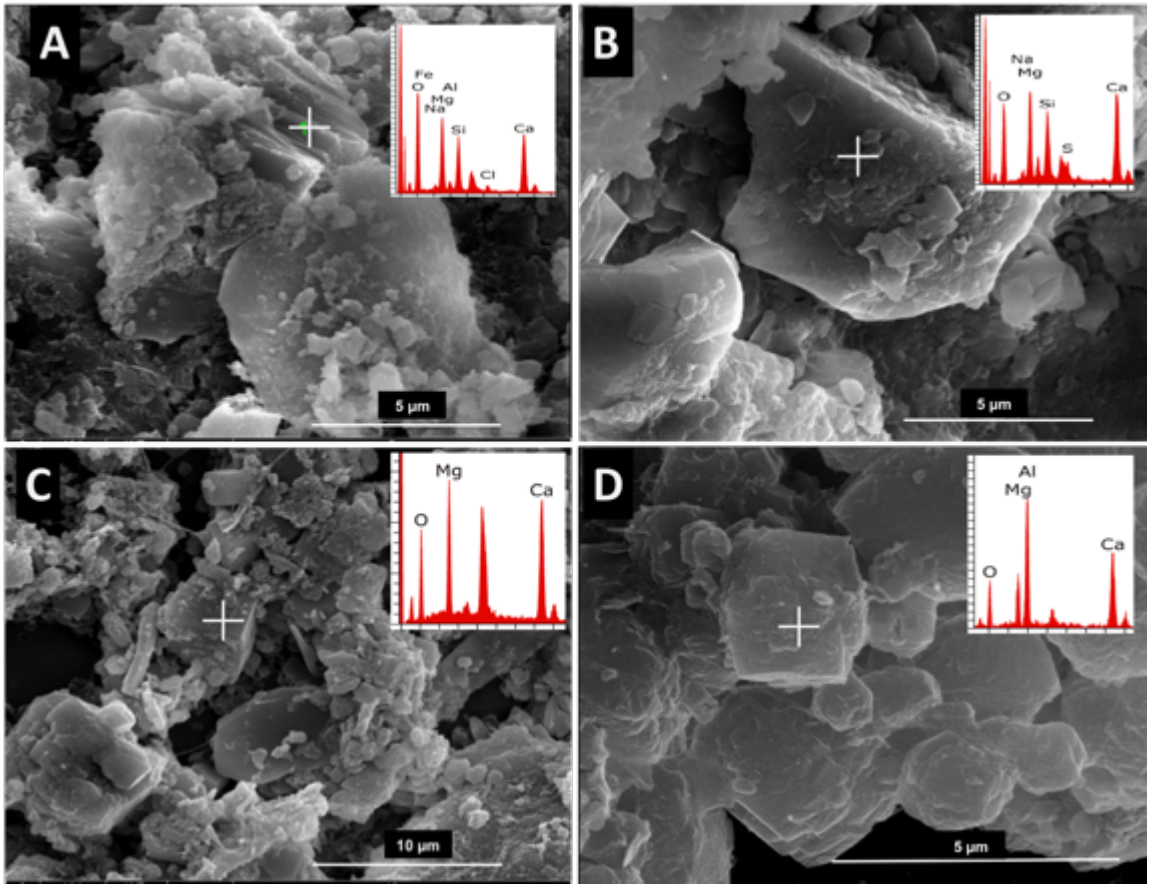

Figure 4.1. Representative SEM/EDS images of sediment core 1 and Core 2.
Core 1 at depth of - 2.5 cm (A) and) core 2 at -12.5 cm(B) -22.5 cm (C) and -37.5 cm(D).

The results of XRD analysis of sediments confirm the occurrence of dolomite, halite, gypsum and a mixture of carbonate minerals containing varying proportions of calcite and Mg-rich calcite (Figure 4.2). The dolomite main d104 peaks are broad with slight shifts to the right compared to the reference dolomite peak, suggesting the presence of a Mg-rich, non-stoichiometric composition.

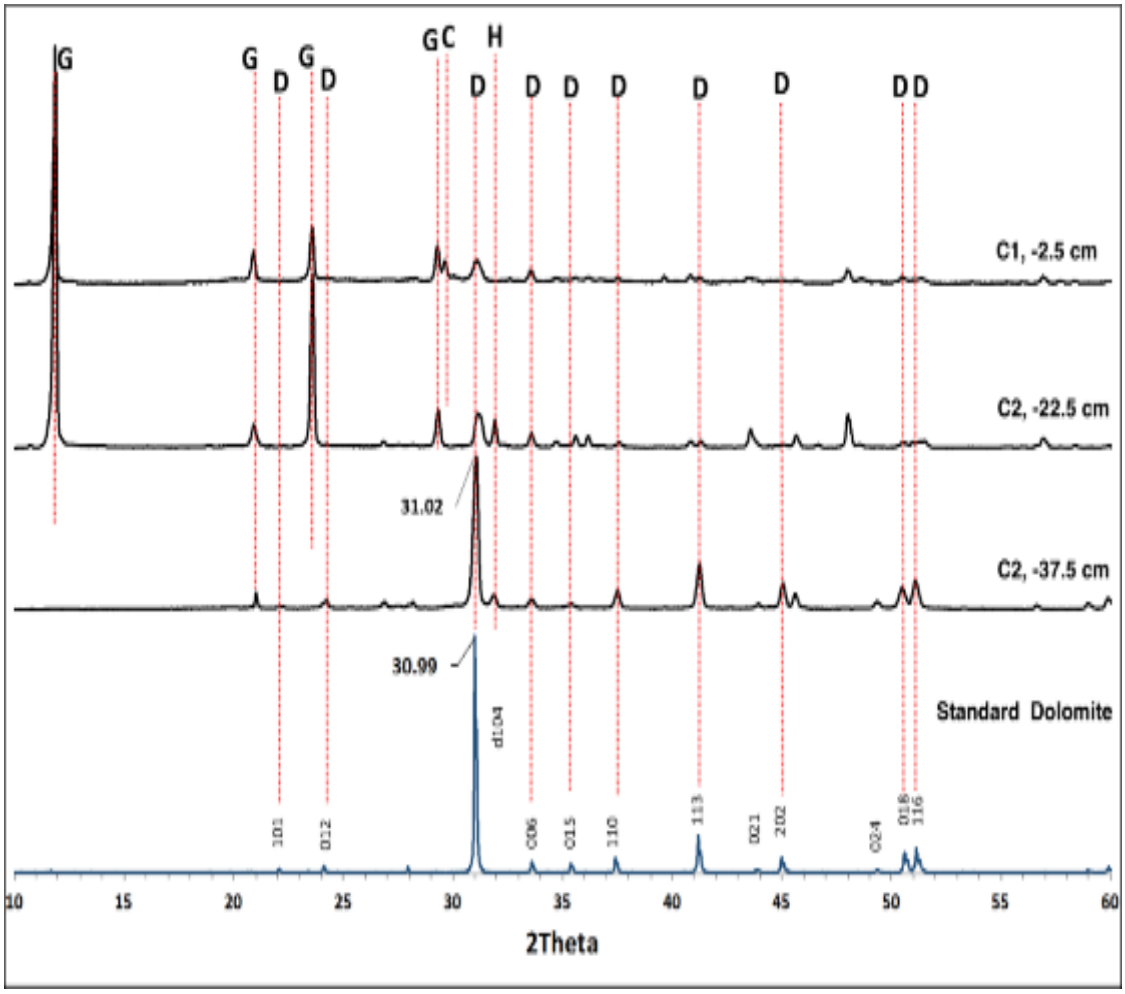

Figure 4.2: X-ray diffraction patterns of bulk sediments sampled from core 1 (C1) and core 2 (C2), at three different depths below the surface showing peaks of Dolomite (D), gypsum (G), calcite (C) and halite (H).

4.4 Isolation and identification of halophilic, heterotrophic, aerobic bacteria from Dohat Faishakh sabkha

4.4.1 Selection of the isolation media

An extensive literature review was performed on different types of media used for isolation of aerobic halophilic, heterotrophic bacteria as summarized in (Table 4-2).

Table 4-2: Media comparison

Component g/l	**MH** (Ventosa et al, 1989)	**M4** (Arahal et al., 1999)	**B4** (Rusznyák et al., 2012).	**D1** (Sánchez-Román, et al., 2008)	**MEC2** (Sanchez-Roman et al., 2007)
Yeast extract	10		4	10	10
Peptone		0.5			
Protease peptone	5			5	5
Glucose	1		10	1	1
NaCl	100	103		35	75
$Ca(C_2H_3O_2)_2$			2.5	1.9	1
$Mg(C_2H_3O_2)_2$				18	8
$MgCl_2\ 6H_2O$	7				
$MgSO_4\ 7H_2O$	9.6	18			
$CaCl_2\ 2H_2O$	0.36	0.25			
KCl	2	0.19			
$NaHCO_3$	0.06				
NaBr	0.026				
Agar			15	20	20
pH	7.2	7.2	8.0	7.2 (1M KOH)	7.2 (1M KOH)

Moreover, we checked Arabia Gulf water and sabkha water compositions, mainly for major ions (i.e., Mg^{+2}, Ca^{+2}, Na^{+}, Cl^{-}), as shown in (Table 4-3).

Table 4-3: Arabian Gulf & sabkha water compositions Compared with D1 and MEC2 culture media

Component	(Walker, 2012)		(Sánchez-Román, et al., 2008)	(Sanchez-Roman et al., 2007)
	Arabia Gulf mM /l	**Sabkha mM/l**	**D1 Medium mM/l**	**MEC2 Medium mM/l**
Ca^{+2}	11	31	11	6
Mg^{+2}	65	167	86	37
Na^{+}	1,215	1,304	875	1,750
K^{+}	17	33	-	-
SO_4^{-2}	34	103	-	-
Cl^{-}	1,000	1,600	875	1,750
$HCO3^{-}$	2.8	2.5	-	-
Mg^{2+}: Ca^{2}	5.9	5.4	7.8	6

Based on the available literature as demonstrated in (Tables 4-2 and 4-3), taking into consideration Mg^{2+}: Ca^{2} ratios and salinity values, we selected MEC2 medium to isolate aerobic, heterotrophic, halophilic bacteria.

4.4.2 Isolation of halophilic, heterotrophic, aerobic bacteria

An isolation program was employed to isolate halophilic, heterotrophic, aerobic bacteria from the sampled cores described in section (3.2). Core 1 was subdivided into seven layers of 4 cm each. Core 2 was subdivided into18 layers of 2.5 cm each. Core 3 was subdivided into five layers 4 cm each and core four was subdivided into 11 layers of 5 cm each. Layers were sampled and 1 g of homogenous sediments from each layer was used to isolate the corresponding bacterial strains in a selected medium. The isolated strains were coded by sampling site, corresponding core and respected layer. Total of 44 isolates was obtained with nine isolates from core 1, twenty from core 2, six from core 3 and nine from core 4 as shown in (Table 4-4 to Table 4-7).

Table 4-4: List of bacterial strains isolated from core 1

Serial No.	Isolate ID	Identity	Accession No.	Similarity score	Mediate mineral formation in solid MEC2?	GenBank Number of submitted sequences
1.	DF111	*Bacillus tequilensis*	KX129852.1	99%	No	KY367266
2.	DF112	*Virgibacillus marismortui strain GSP17*	AY505533.1	100%	Yes	KY361738
3.	DF124	*Staphylococcus sp.*	KX013438.1	99%	No	KY367396
4.	DF135	*Bacillus subtilis*	KX098334.1	100%	No	KY368133
5.	DF137	*Staphylococcus epidermidis*	KX646255.1	99%	No	KY361742
6.	DF141	*Bacillus licheniformis*	KY202705.1	100%	No	KY363571
7.	DF151	*Bacillus tequilensis*	KT758573.1	99%	No	KY367258
8.	DF153	*Salinivibrio costicola*	KF976348.1	99%	Yes	KY364636
9.	DF161	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY368143

Table 4-5: List of bacterial strains isolated from core 2

Serial No.	Isolate	Identity	Accession Number	Similarity	Mediate mineral formation in solid MEC2?	GenBank Number of submitted sequences
1.	DF211	*Bacillus licheniformis*	KX268487.1	100%	No	KY363572
2.	DF221	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY368130
3.	DF231	*Virgibacillus marismortui strain GSP17*	AY505533.1	100%	Yes	KY363850
4.	DF241	*Virgibacillus sp.*	KP795881.1	100%	Yes	KY363849
5.	DF251	*Virgibacillus sp.*	KP795875.1	100%	Yes	KY365009
6.	DF281	*Virgibacillus salarius*	KT008296.1	100%	Yes	KY364814
7.	DF282	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY364885
8.	DF291	*Virgibacillus sp. F2*	KC884680.1	100%	Yes	KY359388
9.	DF2101	*Bacillus licheniformis*	KX036560.1	100%	No	KY368576
10.	DF2102	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY368642
11.	DF2111	*Staphylococcus hominis*	KU184517.1	100%	No	KY359407
12.	DF2121	*Virgibacillus sp. KJ1-5-912*	KC989938.1	100%	Yes	KY373219
13.	DF2131	*Virgibacillus sp. H2–53*	KM979174.1	99%	Yes	KY360241
14.	DF2141	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY360309
15.	DF2151	*Virgibacillus sp. H2–53*	KM979174.1	100%	Yes	KY373210
16.	DF2152	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY373230
17.	DF2161	*Virgibacillus sp.*	KP795881.1	100%	Yes	KY366222
18.	DF2171	*Virgibacillus sp.*	KM979174.1	100%	Yes	KY360308
19.	DF2172	*Virgibacillus sp.*	KP795875.1	100%	Yes	KY369952
20.	DF2181	*Virgibacillus sp.*	KP795869.1	100%	Yes	KY369301

Table 4-6: List of bacterial strains isolated from core 3

Serial No.	Isolate	Identity	Accession Number	Similarity	Able to mediate mineral formation
1.	DF321	*Virgibacillus sp.*	KY359388.1	99%	Yes
2.	DF322	*Virgibacillus marismortui*	LC260007.1	99%	Yes
3.	DF331	*Virgibacillus salarius*	LC259998.1	99%	Yes
4.	DF332	*Virgibacillus marismortui*	LC260007.1	99%	Yes
5.	DF341	*Virgibacillus marismortui*	LC260007.1	99%	Yes
6.	DF351	*Virgibacillus salarius*	LC260006.1	99%	Yes

Table 4-7: List of bacterial strains isolated from core 4

Sample No.	Isolate	Identity	Accession Number	Similarity	Able to mediate mineral formation
	DF411	*Virgibacillus marismortui*	LC260007.1	99%	Yes
	DF431	*Virgibacillus sp.*	KY373230.1	99%	Yes
	DF441	*Virgibacillus marismortui*	LT714167.1	100%	Yes
	DF451	*Virgibacillus sp.*	KY364885.1	99%	Yes
	DF461	*Virgibacillus salarius*	LC259983.1	99%	Yes
	DF471	*Virgibacillus marismortui*	LC260007.1	99%	Yes
	DF472	*Virgibacillus marismortui*	LT714167.1	100%	Yes
	DF491	*Virgibacillus marismortui*	LC260007.1	99%	Yes
	DF4101	*Virgibacillus marismortui*	LT714167.1	100%	Yes

4.4.3 Identification of isolated strains

The sequences of 16S rRNA gene fragments were used to determine the most closely related species available in the database of GenBank using the NCBI Blast server. Lists of isolated strains are shown in (Table 4-4 to Table 4-7)

From core 1 layers, four different genera were identified: three *Bacillus* species *(B. licheniformis, B. subtilis* and *B. tequilensis),* two *Virgibacillus* species with one identified as *V. Marismortui,* two *Staphylococcus* species *(S. epidermidis* and *S.* sp.) and one *Salinivibrio costicola.* While from core 2, three genera were identified with majority 20 *Virgibacillus* sp. Strains with two species identified as *(V. salarius and V. marismortui),* two *Bacillus licheniformis* isolates and one *Staphylococcus* sp. Form core 3 six *Virgibacillus* sp., with two species identified *(V. salarius and Vs. marismortui)*. From core 4, nine *Virgibacillus* sp. two species were identified as *(V. salarius and Vs. marismortui)*. It is possible that some isolates may belong to the same species, although colonies were picked based on the morphological difference of the corresponding cells. These identified genera represent a preliminary assessment and a starting step for the investigation on mineral forming aerobic bacteria in the studied sabkha, obtained independently of their individual capability to mediate mineral formation.

4.5 Investigation on the role of media components on biological activity (mineral formation)

4.5.1 Potential urease activity of the isolated bacteria

One of the reported mechanisms of biomineralization is the microbially induced calcite precipitation (MICP) mediated by the microbial enzyme, urease, which hydrolyzes urea and thus causes an increase in the pH. To check if the isolated strains from Dohat

Faishakh sabkha exhibit such enzyme activity, the solid urea medium in which urea serves as sole carbon and nitrogen source was used. The urease-positive strains are able to turn the pH indicator into red, phenolphthalein, due to ammonia released from urea hydrolysis. As expected, all *Bacillus* strains exhibited urease activity. In contrast, the *Virgibacillus* strains were shown urease-negative strains at the experimental conditions. Indeed, the negative strains were not growing in the medium. However, this finding is confirmed in the used urea-medium which may not be appropriate for *Virgibacillus* bacteria to express the urease gene(s). Supplemental of NaCl to the urea medium up to 3.5% did not promote the growth of the negative strains. This result indicates that most probably the *Virgibacillus* bacterium is not employing urease system in biomineralization process.

4.5.2 Role of Mg^{+2} and Ca^{+2} concentrations and ratios in biological activity

The aerobic bacterial strains were examined for their ability to precipitate minerals using media containing different Mg^{+2} and Ca^{+2} concentrations and ratios, at 30° C and incubation during 14 days. The plates were examined by optical microscope to detect the occurrence of solid phases corresponding to minerals.

The results obtained at these conditions are reported in (Table 4-8).

Table 4-8: Examination of isolates' ability to precipitate minerals at different Mg^{+2}: Ca^{+2} concentrations and ratios.

ID	M1	MEC2		M2		M3		M4		MR1	MR2		MR3		MR4	
	W 1&2	W1	W2	W1	W2	W1	W2	W1	W2	W 1&2	W1	W2	W1	W2	W1	W2
DF111	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF112	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF124	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF135	-	-	-	-	-	-	-	N	N	-	-	-	+	+	+	+
DF137	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF151	-	-	-	-	-	-	-	N	N	-	-	-	-	-	-	-
DF153	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+
DF161	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF171	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
DF211	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF221	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF231	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF241	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF251	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF252	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF281	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+
DF282	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF291	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF2102	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2111	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF2121	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+
DF2131	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2132	-	+	+	-	-	+	+	+	+	-	-	-	+	+	+	+
DF2141	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
DF2142	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2151	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
DF2152	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
DF2161	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+
DF2162	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
DF2171	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2172	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DF2181	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+

1w: 1week, 2w: 2 weeks, N: no growth

The results clearly show variability regarding the isolates' ability to precipitate minerals at different Mg^{+2}: Ca^{+2} concentrations and ratios within one week and two weeks of incubation. Mg^{2+}: Ca^{2+} concentrations and ratios are known to be important factors in terms of causing supersaturation with respect to dolomite (Morrow, 1982).

Media MEC2 and M2 may be considered as providing the most appropriate Mg^{+2} and Ca^{+2} concentrations and ratios and thus closest inorganic optimal conditions for all of the mineral precipitating isolates. Although strains produced crystals in the M2 medium in shorter incubation time, we considered that the medium MEC2 would allow a better differentiation between strains in further studies of their biomineralization potential. Moreover, MEC2 was selected, because it contains suitable composition for bacterial growth and crystal formation for all isolates and it has the closest salt concentration to Arabian Gulf water.

4.6 Study of the potential of isolated bacteria to mediate mineral formation

The ability of the bacterial isolates to mediate the formation of minerals was investigated using the solid MEC2 media. Each pure isolate was sub-cultured by streaking on to the surface of the solid medium. The plates were then incubated for 30 days at 30°C. The occurrence of crystals -solid phase mineral- was examined using optical microscopy on a daily basis. (Figure 4.3) illustrates an example of results, obtained from isolates DF2101 (a non-mineral forming bacterium) and DF251 (a mineral forming bacterium): (Figure 4.3 A) shows the growth of the non-mineral forming isolate DF2101, where no precipitates are associated with the bacterial growth. (Figure 4.3 B) Shows the border side of the growth and the association between the crystal formation and bacterial growth.

(Figure 4.3 C) Shows agglomeration of crystals with different sizes. (Figure 4.3 D) Illustrates the formation of three distinct colors and morphological types: the spherical, large size, dark brown (Figure 4.3 D1), the spherical, medium size, light brown (Figure 4.3 D2) and the very small, dumbbell, white shape (Figure 4.3 D3). All of the mineral forming isolates were able to form crystals within seven days of incubation (Table 4-4). For the most of the isolates, the initial mineral formation was noted after three days of incubation. Interestingly, most of our isolates were able to synthesize more than one morphological types of crystals.

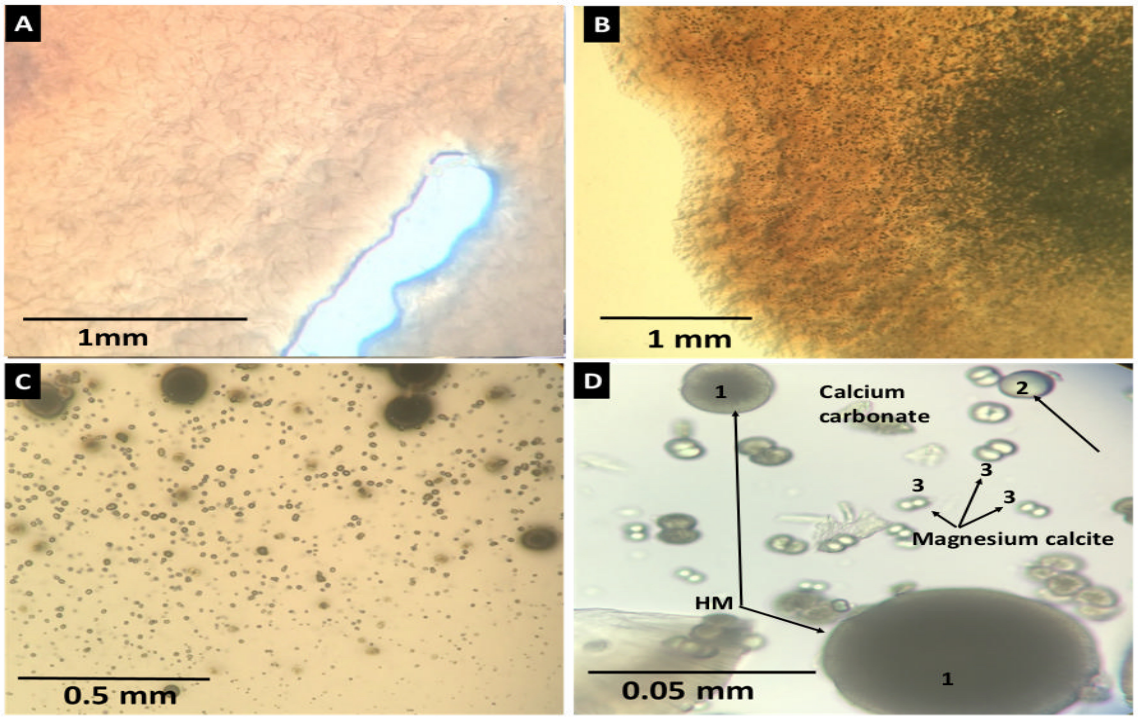

Figure 4.3: Microscopic observations of precipitates. (A) A plate of the non-mineral forming isolate DF2101, (B) Minerals observed in culture plates using optical microscopy at (40x). (C) of the minerals observed in the culture plates at (100x) and (D) Microscopic image at (1000x) of crystal obtain form DF251pure cultures showing mineral with different sizes, shapes and color.

Four controls were performed in parallel to confirm a further association between the production of crystals and bacterial growth and activity:

- Control 1: DF141 and DF2101 were able to grow on MEC2, a significant increase in pH (from 7.0 to ≈ 8.5) was detected, however, no mineral formation was detected at the studied conditions. (Figure 4.4) Shows extensive growth of DF2101, but without mineral formation.
- Control 2: No mineral formation was observed in the un-inoculated MEC2 solid medium.
- Control 3: All studied strains were able to grow on the M1 medium supplemented with low concentrations of acetate salts (3 mM Ca^{+2} and 18 mM Mg^{+2}), the pH increased to 8.5, but no mineral formation was observed.
- Control 4: Similarly, no mineral formation was observed upon artificially increasing the pH to 9 by adding Na_2CO_3.

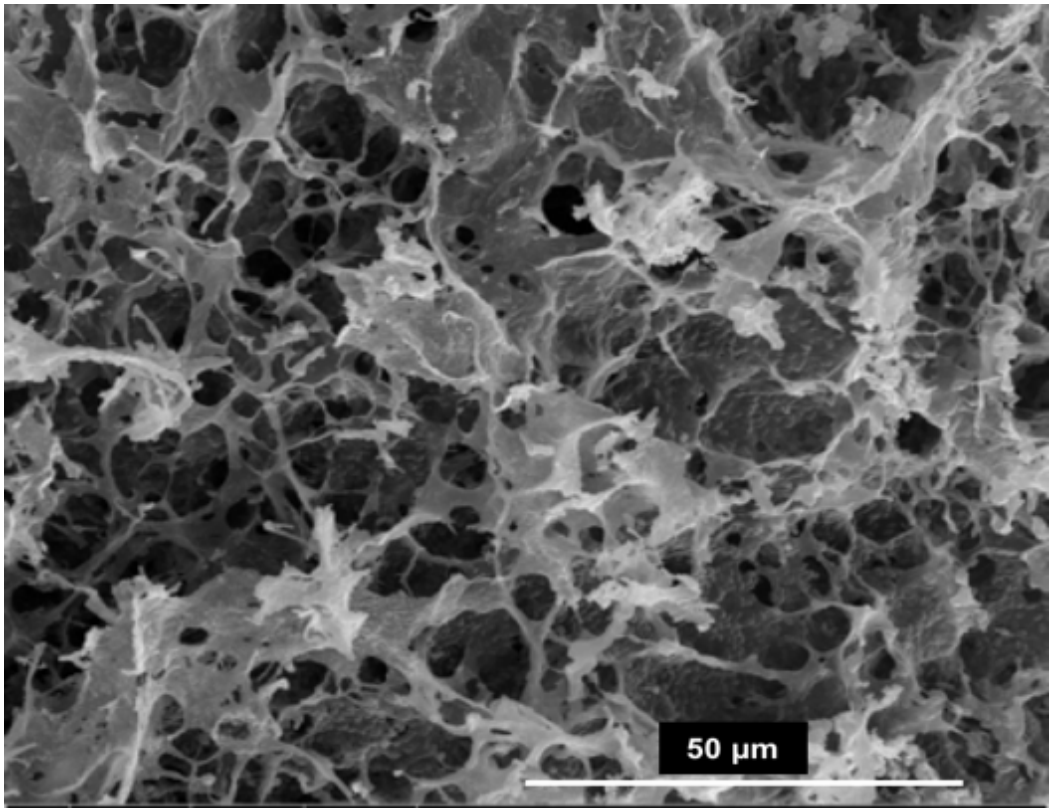

Figure 4.4: SEM images from a biofilm obtained from DF2101 pure cultures.

4.7 Involvement of aerobic bacteria in HMC formation

To determine the association between the growth of the isolated bacteria and the composition of formed minerals in solid media, the minerals recovered from MEC2 agar plates were analyzed by SEM/EDS. Four bacterial isolates: DF112 (*Virgibacillus marismortui* strain) and DF153 of *Salinivibrio costicola* and from core 1, DF2141, DF291 and DF251 (all *Virgibacillus sp*.) from core 2, were considered for their three types of crystals. The *Virgibacillus* and *Salinivibrio* strains have shown an ability to increase the pH from 7.0 to about 8.5 as well as induce mineral formation. The detected crystals exhibited different diameters and Mg^{+2}: Ca^{+2} ratios, although similar spherical

morphology (Figure 4.5). The largest spheres were the hydrated magnesium carbonate (hydromagnesite), having a diameter of approximately 100 μm. On the other hand, calcium carbonate made smaller spheres, with a diameter of approximately 50 μm, while the very high magnesium calcite (HMC) had the smallest dumbbell shaped crystals (approximately 10 μm).

This finding could explain the variability of mineral crystals occurring in the samples of Sabkha cores in our previous work reported in section 4.3.

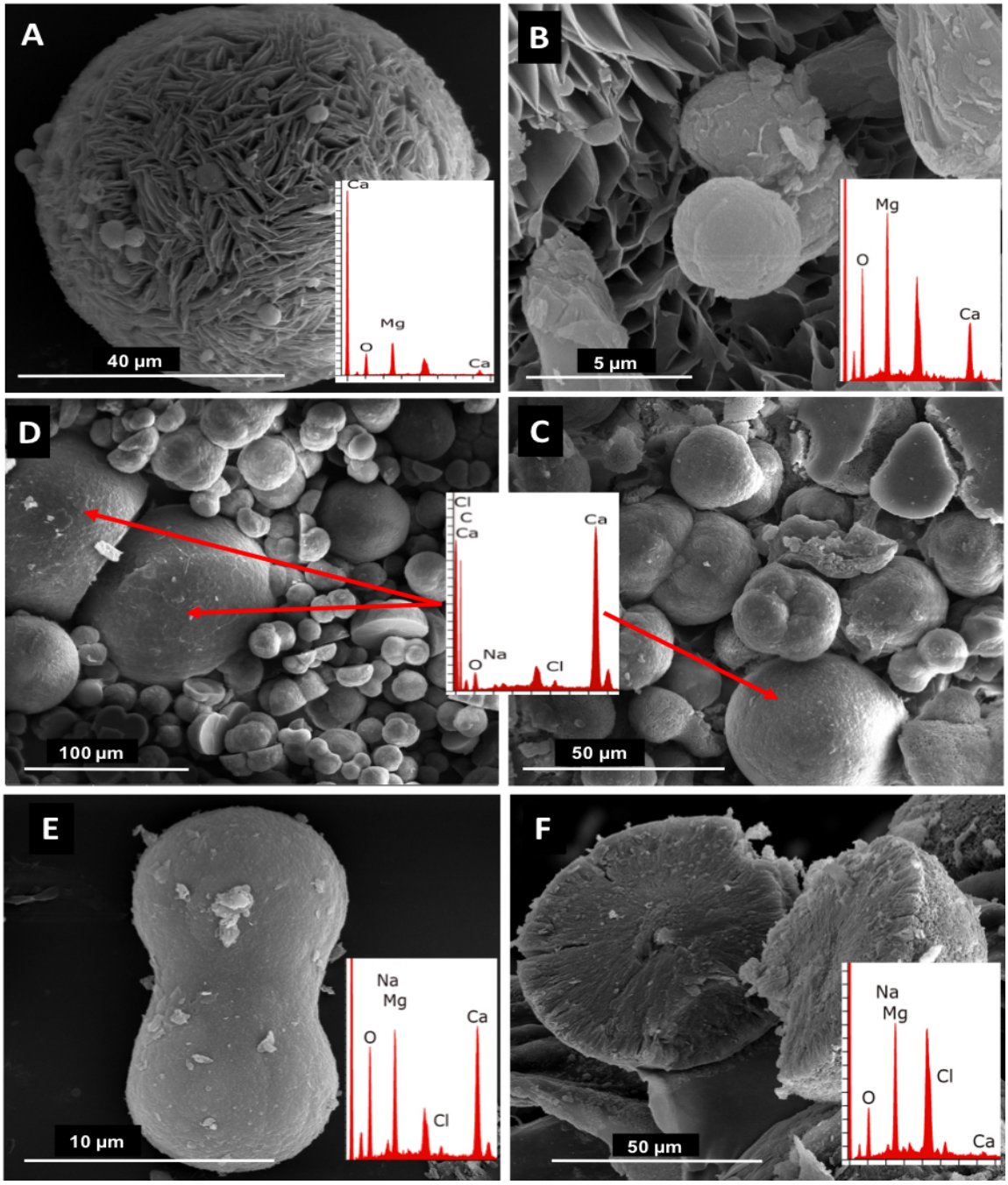

Figure 4.5: SEM/EDS of Crystal recovered from pure cultures.

(A) Huntite crystal obtained from DF112 cultures; (B) close up of small HMC crystals embedded into the big huntite crystal; (C) and (D) bulk mixture of crystals obtained from DF2141 and DF291 cultures, respectively, showing large crystals of calcium carbonate (EDS shown in overlaid panel), (E) a close up of representative small, dumbbell-shaped crystal of (HMC) recovered from DF112, DF291 and DF2141;(F) a representative image of broken hydromagnesite crystal was commonly recovered from all the studied cultures.

X-Ray Diffraction analysis was performed for mineral recovered from the cultures of the four studied strains, after 30 days of incubation (Figure 4.6). Interestingly, the XRD analysis confirms the conclusions made following the optical observations and the SEM/EDS studies. Indeed, XRD patterns indicate the presence of very high magnesium calcite peaks, located in the region between the calcite and dolomite peaks. The Mg content of the samples was calculated using the 104 peak shift in XRD patterns (Goldsmith et al., 1955). The mol% $MgCO_3$ ranged from 30% to 40%. *Salinivibrio* from core 1 and three *Virgibacillus* isolates from different layers of core 2 displayed similar XRD patterns. (DF112) The *Virgibacillus* isolated from the top layer of core 1 displayed a different XRD pattern, with HMC peak showing higher intensity than that of the other four strains. Altogether, these results demonstrate the ability of the *Salinivibrio* and *Virgibacillus* strains isolated from Dohat Faishakh sabkha to mediate the formation of HMC.

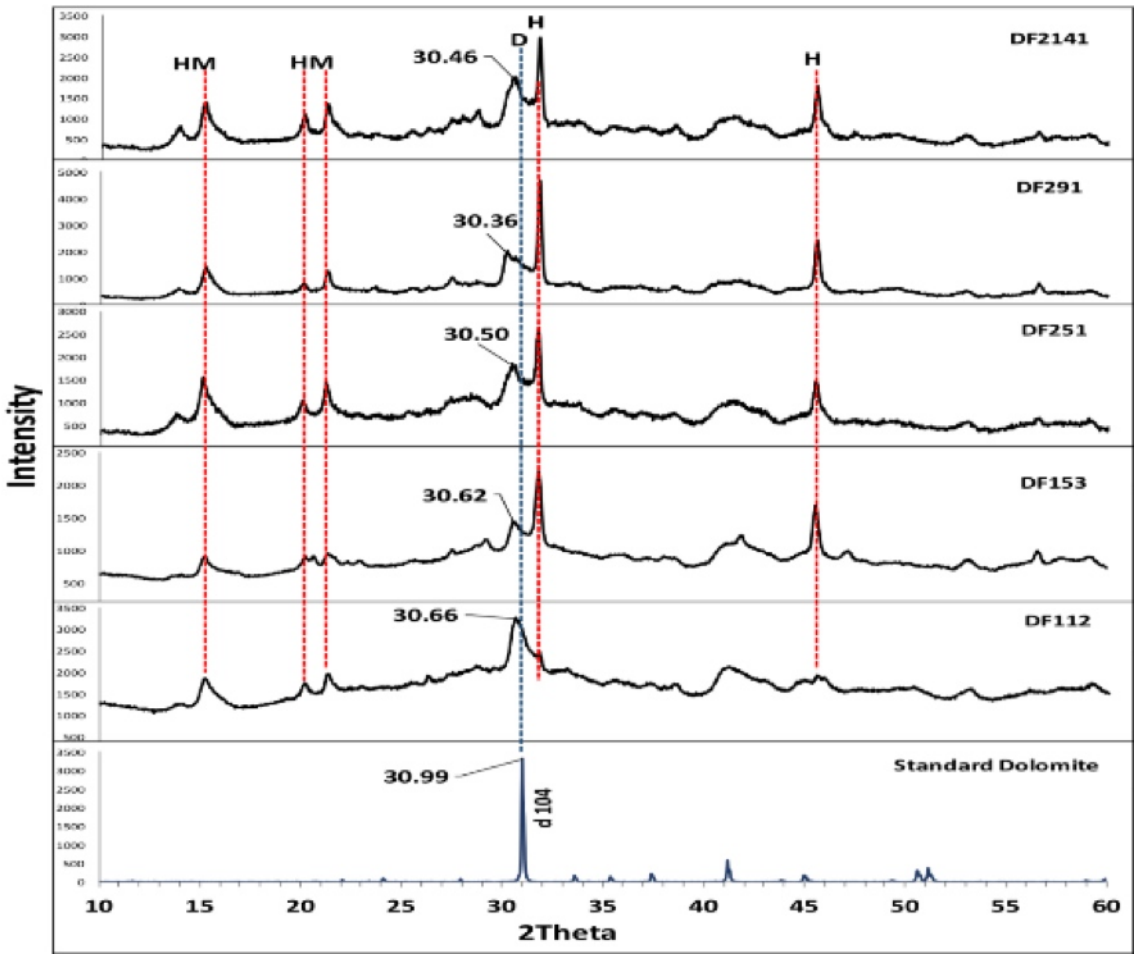

Figure 4.6: X-ray diffraction pattern of minerals recovered from pure cultures. DF112, DF153, DF251, DF291 and DF2141) cultures, compared with a standard dolomite XRD pattern.D: Dolomite, HM: Hydromagnesite, H: Halite

4.8 Discussion

Evidencing the occurrence of magnesium calcite in Dohat Faishakh sabkha emphasizes the importance of selecting the appropriate sampling sites to perform a study on biominerals in a specific environment. Our findings, confirms that Qatari evaporitic environments provide an efficient model for studying the magnesium calcite formation at ambient conditions. More interestingly, the precursors of dolomite were observed in tide

sediments, which are normally fed with oxygen at least momently. In such a tide sediments, strictly anaerobic bacteria would not be highly active due to the increase of the redox potential by movement of seawater. This represents an opportunity to study the role of aerobic and/or aerotolerant bacteria in the process of biomineralization. The recent study conducted by Vogt et al. (2017), on the hypersaline cyanobacterial mats in the intertidal flats in Sultanate of Oman, showed that bacteria represent a typical hypersaline community, most of the identified taxa are halophiles tolerating or requiring high temperatures. The occurrence of phototrophs, aerobic and anaerobic chemotrophs, in addition to sulfate reducers indicates the coexistence of aerobic and anaerobic microniches combined with active metabolic and element cycles within the microbial mat.

A first collection of aerobic bacteria was constructed from Dohat Faishakh sabkha in Qatar. It is well-known that the standard classical culture methods used for the isolation of target microorganisms are highly selective, thus, may not represent the absolute microbial community (Davis, 2014). Therefore, the bacteria isolated may represent specific populations of the microbial communities' present in the sabkha (Vaz-Moreira et al., 2011). We think that any laboratory study with isolated bacteria should consider that an eventual interaction between the populations may exit and that an eventual role of the non-isolated microorganisms may be of importance in the overall biomineralization occurring in the Sabkha. However, demonstrating that any of the isolated aerobic/aerotolerant bacterium is able to mediate miner formation in a Petri dish at the laboratory conditions, would have a merit to fill a gap in our knowledge. We think that biomineralization studies are devoted to the advancement of our knowledge on the role of

each separate communities to be further related to the interactions occurring in the environment. In our four core samples, the majority of cultivable isolates were from four main phyla; *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria*, which can be easily cultured at laboratory controlled conditions (Rastogi and Sani, 2011; Ajit Varma, 2017). *Virgibacillus* was the highest abundant genus, followed by *Bacillus*. Indeed, the bacterial genera and species found in Dohat Faishakh are described for the first time in Qatar; though, usually reported in other hypersaline areas. Moreno et al., (2013), isolated bacteria belonging to the genera *Bacillus*, *Staphylococcus* and *Salinivibrio* from several saline settings across the world, with evidence for their production of extracellular enzymes. The survivability and long-term preservation of the spore-forming bacteria *Virgibacillus* sp., was demonstrated by the isolation and growth of this bacteria from primary salt crystals aged 250 million years (Satterfield et al., 2005).

It was essential to select a culture medium which promotes bacterial growth and biomineralization if exhibited by the strain as well. Through a comparative study in many reported media, we have selected a medium named MEC2, in which most of the isolates were able to mediate mineral formation. This step was the basis of our research work as for all microbiology research. This MEC2 medium allows detecting minor differences between strains belonging to the same species. Indeed, combined analyses by SEM/EDS and XRD of minerals formed in our selected medium MEC2 showed the difficulties to mediate crystals formation in liquid cultures although extensive EPS production. In a solid MEC2 medium, different types of crystals were observed Interestingly, our cultural conditions allowed to detect at least four *Virgibacillus* strains producing three different

types of crystals. In the MEC2 solid medium, a *Salinivibrio* isolate and all the studied *Virgibacillus* isolates were able to mediate carbonate mineral formation.

The spherical and dumbbell morphologies were recognized as microbially induced carbonates in previous studies (Sánchez-Román, 2006; Mckenzie and Vasconcelos, 2009; Mettraux et al., 2015; Bahniuk et al., 2015). Moreover, the co-precipitation of dolomite and hydromagnesite was frequently reported in natural environments, suggesting that the hydrated-calcium magnesium carbonates may be precursors of dolomite formation (Lippmann, 1973; Baker & Kastner, 1981; Kelleher and Redfern, 2002 Martín-Pérez, Kosir, & Otonicar, 2015). Here, we confirmed that different aerobic *Virgibacillus* strains could mediate the formation of carbonate minerals with distinct mol% of $MgCO_3$. Interestingly, individual isolates can also mediate the formation of carbonate minerals with various Mg^{+2} content at the same experimental conditions. Moreover, we observed that all isolates were able to raise the pH in the culture media, although not all were able to mediate mineral formation at the studied conditions. Which suggests that the role of aerobic bacteria in carbonate mineral formation is not limited to the increase of pH and alkalinity by their biological activities. Rather, it is likely that the chemistry of their cell walls and/or their secreted extracellular polymeric substances (EPS) play a crucial role in the mineralization process (Dupraz et al., 2004; Bontognali et al. 2008; Bontognali et al., 2010; Roberts et al., 2013; Bontognali et al., 2014). The mechanism for magnesium is incorporation into the crystal lattice remains a topic of dispute in the literature. The involvement of microbial cell walls and/or the organic molecules (EPS) produced by microorganisms were recently discussed and reported (Kenward et al., 2013; Qiu, Yao, Wang, & Duan, 2107). EPS extensive production associated with these identified aerobic

bacterial species may play a key role in furnishing the templates or the nucleation sites that facilitate mineral formation and the incorporation of Mg into the mineral lattice (Bontognali et al., 2014).

4.9 Conclusion

The results of this work demonstrate that pure cultures of aerobic bacteria isolated from the Dohat Faishakh sabkha environment can mediate very high Mg-calcite VHMC formation, which has been proposed to constitute precursors for ordered dolomite. Four main bacterial genera were shown to exist in four studied cores sampled in different seasonal periods. All *Virgibacillus* and one *Salinivibrio* strain were able to mediate the formation of Mg-rich calcites in the laboratory under aerobic conditions. This study reveals that the Dohat Faishakh sabkha environment in Qatar –a unique place where dolomite from under the predominant weather conditions- is an appropriate site for investigating the role of halophilic, heterotrophic, aerobic bacteria in the formation of carbonate minerals.

The questions to which we tend to answer are:

- why a single strain mediates formation of more than one form and composition of crystals in highly-controlled experimental conditions: defined medium and incubation?

- What is the role of abiotic factors in the biological activities?

- What is the role of the cell structure and secretions (EPS) in mediating crystals or not and in types of mediated crystals.?

CHAPTER 5 : THE INFLUENCE OF TEMPERATURE, SALINITY AND Mg^{2+}: Ca^{2+} RATIO ON MICROBIAL MEDIATION OF Mg-RICH CARBONATES BY VIRGIBACILLUS STRAINS ISOLATED FROM A SABKHA ENVIRONMENT

5.1 Introduction

Studies conducted during the last 20 years have shown that microorganisms promote the formation of Mg-rich carbonate minerals. These studies are relevant to the long-lasting debate regarding the origin of sedimentary dolomite, which is often referred to as the "Dolomite Problem" (McKenzie, 1991; Vasconcelos & McKenzie, 1997). Despite its abundance in ancient sedimentary rocks, dolomite rarely is formed in natural environemnets and it is difficult (or virtually impossible in the absence of microorgansims) to precipitate in laboratory experiments that simulate the surface conditions on Earth (Petrash et al., 2017). For these reasons, the key factors that control its formation and distribution in ancient rocks remain unclear and are under debate (Arvidson & Mackenzie, 1999). Because sedimentary sequences rich in dolomite are often studied for paleoclimatic and paleonevironmental reconstructions and because dolomite comprises many economically important gas and oil reservoirs (Adam et al., 2014), much effort has been, and is still being invested in attempting to solve the Dolomite problem.

Microorganisms that have been shown to catalyze the incorporation of Mg into carbonate minerals at low temperatures include the sulfate reducers (Vasconcelos & McKenzie, 1997; Wright, 1999; Wright & Wacey, 2005; Warthmann et al., 2000; Bontognali et al., 2012), the methanogens (Roberts et al., 2004; Kenward et al., 2009), and the aerobic

heterotrophs (Sanchez-Roman et al., 2008; Al Disi et al., 2017). The exact mechanims by which these microrganims mediate the formation of Mg-rich carbonate minerals has not been fully resolved. Metabolism by mineral-mediating microorganisms leads to an increase of alkalinity and pH that favor the supersaturation and precipitation of carbonate minerals (Gallagher et al., 2012; Petrash et al, 2017). However, conditions of supersaturation with respect to dolomite are insufficent to explain its precipitation at low temeperature, which is likely inhibited by kinetic factors. An increasing number of studies suggest that microbially-produced organic molecules may play a key role in overcoming such kinetic barriers. A recurring and plausible hypothesis posits that some functional groups (e.g., carboxyl groups) present in extracellular polymeric substances (EPS) or in the cell wall adsorb ions (e.g., Mg^{2+}), creating a supersaturated local environment and also reducing the activation energy necessary for the initiation of crystal growth (e.g., by dehydrating Mg^{2+}) (Roberts et al., 2013; Bontognali et al., 2014; Cao et al., 2016).

Most microbial "protagonists" in the studies mentioned above were isolated from hypersaline environments characterized by high temperature, salinity and a high Mg^{+2}: Ca^{+2} ratio. From a purely physicochemical rather than a geobiological perspective, all of these factors are known to be important –even essential– for the formation of carbonate minerals (Buczynski & Chafetz, 1991; Silva-Castro et al., 2015; Balci & Demirel, 2016). Temperature is known to promote the incorporation of Mg into carbonates. Salinity and the consequently increased saturation of Mg^{2+} (and the Mg^{2+}: Ca^{2+} ratio) can also cause the supersaturation of dolomite (Morrow, 1982). It is therefore likely that a combination

of abiotic parameters determines the optimum conditions for the formation of microbially mediated Mg-rich carbonates (Nina et al., 2012). For instance, the sulfate reducing bacteria are virtually ubiquitous in the marine realm (Matsui, Ringelberg, & Lovell, 2004), but their ability to mediate the formation of dolomite may be expressed only in a hypersaline environment in waters that have a high Mg^{2+}: Ca^{2+} ratio (Vasconcelos & McKenzie, 1997; Van Lith et al., 2003; Warthmann et al., 2005; Bontognali et al., 2012). On the other hand, evaporitic environments where the positive effect of a high Mg^{2+}: Ca^{2+} ratio is thwarted by a salinity that is so high that it inhibits microbial growth may exist (Yan et al., 2015; Han et al., 2017). Knowledge of the optimal conditions for the microbially mediated formation of very high Mg calcite—often considered as a potential dolomite precursor—would be very helpful for interpreting the paleoenvironment associated with dolomite in ancient sedimentary sequences. Because microbial species react differently to changes in physiochemical parameters (Rachid et al., 2012), the identification of optimal conditions for the microbially mediated formation of Mg-rich carbonates is very challenging and caution is required before a generalized conclusion can be made. There are very limited studies evaluating the combined effect of several environmental factors on microbial (eco)physiology. With that in mind, our study is meant to be a first modest step in this direction.

We tested the combined effect of temperature, salinity and the Mg^{2+}: Ca^{2+} ratio the on microbially mediated formation of Mg-rich carbonates by two *Virgibacillus* strains isolated from the Dohat Faishakh sabkha (Qatar) that are known to mediate the formation of Mg-rich carbonates (Al Disi et al., 2017). Temperatures from 20ºC to 40ºC, salinities

from seawater salinity (i.e., 3.5%), to 10% (~3-times seawater salinity), and Mg^2; Ca^{2+} ratios of 1, 6, and 12 were tested. This range of values was meant to simulate conditions observed in the marine environment as well as those characterizing the intertidal and supratidal zone of the sabkha from which the microbial strains were originally isolated.

5.2 Selection of culture media

In chapter 4, we used the medium MEC2 to isolate bacterial strains from cores 1- 4 and to evidence their involvement in biomineralization, because it allowed differentiation between the strains. In order to study the effect of abiotic factors on biomineralization, we consider that the M2 medium it is more appropriate for all strains, in terms of the mineral formation kinetics. As previously shown in (Table 4-8) all the strains were able to form minerals within one week of incubation. M2 Modified media (from now on MD) were prepared, modifications were applied to study the impact of each separate factors (Salinity and Mg^{+2}: Ca^{+2} ratios) on mineral formation.

5.3 Brine chemistry variability in Dohat Faishakh Sabkha

Naturally, brine is the source of physical and chemical factors which affect the biological activities of microorganisms. Since, the cores, sampled in this study, showed the occurrence of many forms of minerals including those considered precursors of dolomite, the corresponding brine was analyzed to mimic the local water composition in term of main parameters that could be involved in biomineralization. The chemical characteristics of the major elements of the five core brine samples that were collected at different times from the Dohat Faishakh sabkha are illustrated in (Table 5-1). The data demonstrate large variations in temperature, concentrations of major elements, Mg^{2+}:Ca^{2+}

ratios and salinity. These results consolidate our hypothesis that fluctuations in physical and chemical factors could lead to many types of effects on microorganisms. From the microbiology point of view, the relationship existing between structure and function of the individual cell of each microorganism and the response of the corresponding metabolism to environmental factors (physical and chemical) orient the final behavior. Consequently, the internal synthesized molecules and the secreted ones may change accordingly, as balance, composition and structure. One should consider that the brine could exert stress effect on cells, with many consequences on the level of signaling processes and responses to an individual or combined stressors, including those related to gene expression and regulation and molecules charges and folding.

Table 5-1: Concentrations of major ions (mM) in the water samples collected during different seasons from the sabkha.

Sample	**SW1**	**SW2**	**SW3**	**SW4**	**SW5**
Collection date	Mar-2015	Oct-2015	Mar-2016	Oct-2016	Mar-2017
Temp. (°C)	25	40	23	39	23
Mg^{+2}	213.5	386	207	341.1	296.8
Ca^{+2}	25.1	11.8	12.3	25.7	29.9
Mg^{2+}: Ca^{2+} ratio	8.5	34.4	16.8	13.3	9.9
$SO4^{-2}$	98.1	76.8	96.2	90.7	89.7
K^{+}	35.8	72	36.1	62.1	53.9
Br^{-}	2.2	1.4	0.5	4.4	3.7
Na^{+}	2716	3238	1827	3269	2789
Cl^{-}	2327	3999	1728	3244	3850
Salinity (%)	19.6	27	14	33	18

SW: Sabkha water

5.4 Impact of salinity, temperature and Mg^{2+}: Ca^{2+} ratios on the growth of *Virgibacillus* and crystal formation

In order to investigate the impact of the three abiotic factors, two strains previously isolated, identified and characterized as HMC forming strains (Al Disi et al., 2017), were compared to a non-crystal producing bacterium (Table 5-2). Interestingly, combined effects of these parameters were shown. Indeed, the three strains grew faster when incubated at high temperatures combined with low salinities and low Mg^{2+}: Ca^{2+} ratios. The earliest growth began after 24 h incubation at 40°C, while the precipitation often started 1–3 days after extensive growth was observed. Extensive growth followed by precipitation started the earliest in cultures containing the lowest Mg^{2+}: Ca^{2+} ratio of 1. However, by the end of incubation period, the total number of formed crystals was still relatively low in cultures containing a Mg^{2+}: Ca^{2+} ratio of 1 compared to cultures with Mg^{2+}: Ca^{2+} of 6 and 12. There were no significant differences observed in the frequency of formed crystals between the two *Virgibacillus* strains. No indications about the composition could be postulated at this stage. No mineral formation was observed in the cultures used as negative controls (non-crystal producing bacterium, autoclaved cells, non-inoculated media and modified MD medium with no acetate salts). Indeed, although the qualitative observations, the growth of both crystal-forming *Virgibacillus* strains and the non-forming strains is mostly affected by the three factors, which is expected from the microbiology point of view. However, interestingly, the kinetic of crystals formation is not necessarily proportional to the kinetics of growth. This means that the growth stage and the corresponding cell structure and secreted metabolites are also associated with

crystal formation. Further study should focus on the composition of the crystals formed with varying the effect of the three factors.

Table 5-2: Results of culture experiments at different temperatures and NaCl concentrations observed by optical microscope (40X)

Temperature (°C)	NaCl (%)	Mg^{2+}: Ca^{2+} Ratio	Initial growth (d)	Initial precipitation (d)	Extensive precipitation (d)	Amount of formed crystals*
20	3.5	1	3	5	8	++
20	7.5	1	3	5	10	++
20	10	1	3	5	10	+
20	3.5	6 & 12	3	5	10	+++
20	7.5	6 & 12	3	5	10-12	+++
20	10	6 & 12	3	7	10-12	+++
30	3.5	1	2	3	5	+++
30	7.5	1	2	3	5	++
30	10	1	2-3	5	7	++
30	3.5	6 & 12	2	3	5	+++
30	7.5	6 & 12	2	3	5	+++
30	10	6 & 12	2-3	5	7	+++
40	3.5	1	1	2	3	++
40	7.5	1	1	2	5	++
40	10	1	1-2	3	5	++
40	3.5	6 & 12	1	2	3	++++
40	7.5	6 & 12	1	2	5	++++
40	10	6 & 12	1-2	3	5	++++

*Qualitative estimation, average number of crystals/mm^2, +: <5, ++: 10-20, +++: 20-30, ++++: >30

5.5 Impact of salinity, temperature and Mg^{2+}: Ca^{2+} ratios on morphology and composition of crystals

The SEM/EDS of the minerals formed in the cultures performed at various salinities, temperatures and Mg^{2+}: Ca^{2+} ratios indicated the presence of bioliths composed of various Ca^{2+} and Mg^{2+} carbonates.

(Figure 5.1A–D). The most dominant morphologies were spheres, which varied in size and surface roughness. Fewer dumbbell- and cap-shaped bioliths were observed. Consistently, many surfaces of spherulites showed embedded bacteria cells, bacterial molds and nanoparticles. The EDS spectra of the bioliths indicated the presence of C, O, P, Ca^{2+} and Mg^{2+} but at various ratios. The size and composition of the bioliths varied within each pure culture and between different abiotic conditions. Larger spheres and cap-shaped bioliths were observed when the cultures were incubated at lower temperatures (Figure 5.1 E and F).

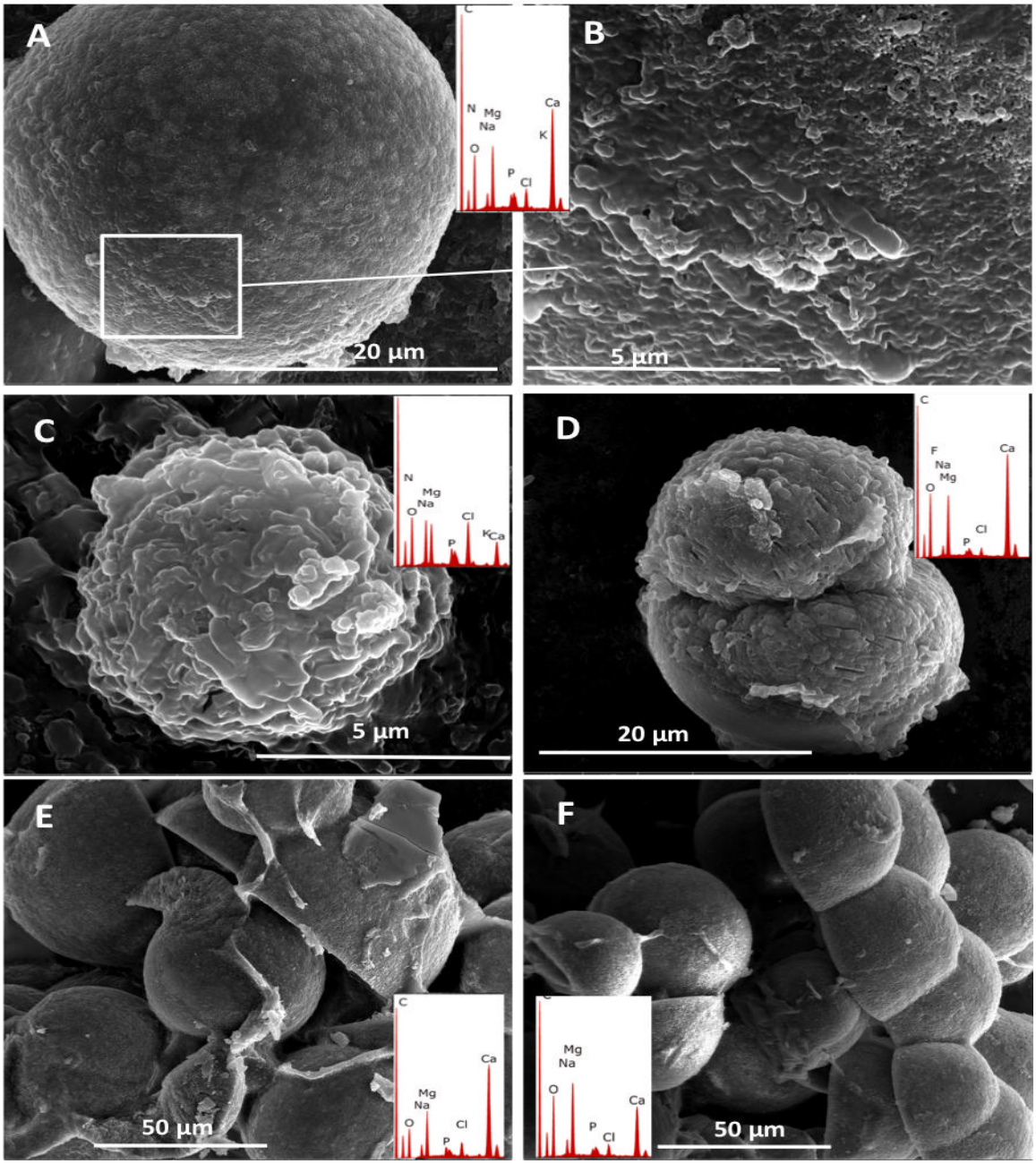

Figure 5.1: SEM/EDS exploration of minerals obtained from DF112 and DF2141 cultures.
(A) Calcium carbonate crystal formed in DF112 cultures at 20℃. (B) Close-up of (A) showing bacterial cells embedded in the calcium carbonate crystal. (C) Mineralized bacterial cells. (D) High-magnesium carbonate crystal retrieved from DF112 cultures at 30℃ showing molds of bacterial cells. A mixture of magnesium carbonate crystals formed in DF2141 cultures at (E) 30℃ and (F) 40℃, showing similar morphologies but with smaller crystals and more incorporation of Mg^2 at 40℃.

Moreover, it was possible to detect chains of bacterial cells covering the formed minerals (Figure 5.2) and a close-up image revealed an aggregation of nanoglobules on the outer bacterial cells (Figure 5.2B).

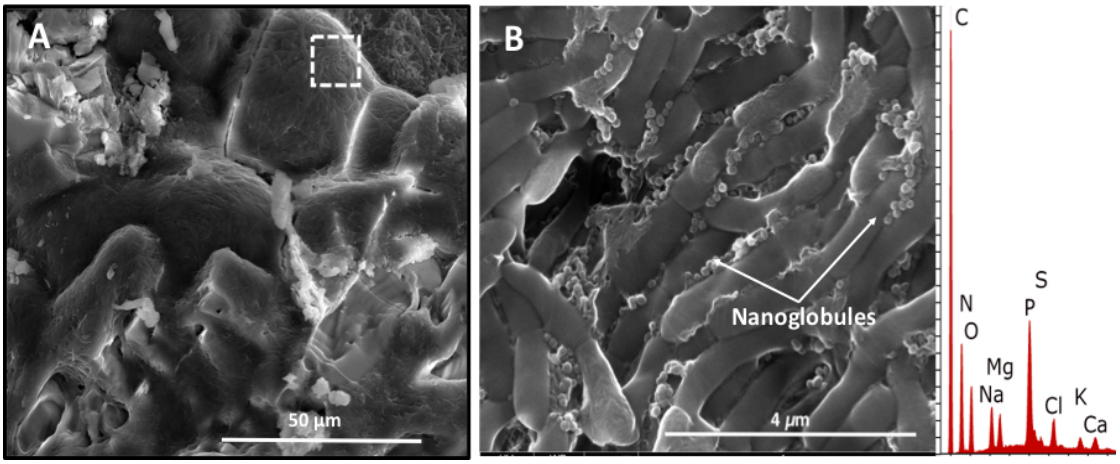

Figure 5.2: (A) Chains of Virgibacillus cells covering a formed mineral. (B) Close-up image is showing nanoglobules aggregates on the outer bacterial cells. (C) EDS results are indicating the bulk elemental composition of (B).

The XRD analysis of the recovered minerals revealed the presence of very high-magnesium calcite (VHMC) with a variable mol% of Mg in cultures performed with initial Mg^{2+}:Ca^{2+} ratios of 6 and 12 (Figure 5.3). However, HMC peaks were not detected in the minerals recovered at a Mg^{2+}:Ca^{2+} ratio of 1; these cultures only showed calcite and halite peaks (Figure 5.4). At Mg^{2+}:Ca^{2+} ratio of 6 and 12, the incorporation of Mg^{2+}

into the carbonate crystals increased as the salinity and/or the temperature increased (Table 5-3, Figures 5.5 and 5.6)

The Mg mol% of the carbonate minerals formed in the experiments was calculated using the formula of Goldsmith et al., (1961), which is based on the position of the (d104) peak in the XRD pattern.

An ANOVA analysis revealed significant differences in the incorporation of Mg^{2+} among the different conditions for each *Virgibacillus* strain. However, the differences between the two *Virgibacillus* strains were not statistically significant. The multilinear regression revealed that the main effect for each of the three studied parameters was statistically significant on the incorporation of Mg into carbonate minerals. Indeed, the adjusted R-squared value of 0.584 indicated that 58% of variation in the data is explained by the model, with temperature producing the largest R-squared increase. The standardized coefficients (Beta) values were (0.545, 0.379, 0.285) for temperature, salinity and Mg^{+2}: Ca^{+2} ratio respectively, indicating that temperature had the highest impact on the change of % mole Mg, while the Mg^{+2}: Ca^{+2} ratio had the lowest impact. Among the three parameters, the only significant interaction was between temperature and Mg^{+2}: Ca^{+2} ratio is significant, while all the other combinations were not statistically significant since their corresponding *P* value is > 0.05.

No mineral formation was detected in the negative control cultures (i.e., non-producing bacteria, autoclaved cells, uninoculated medium, and medium with no acetate salts).

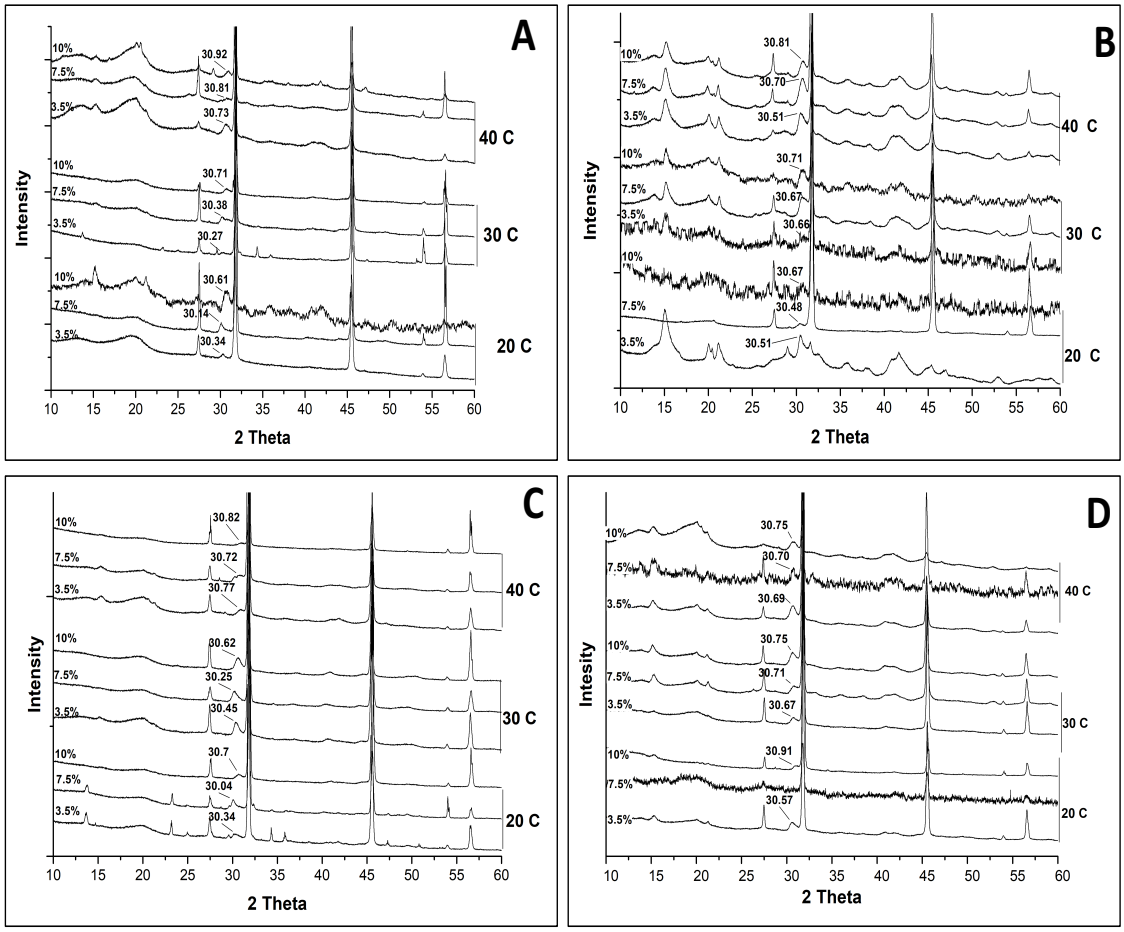

Figure 5.3: X-ray diffraction patterns of minerals retrieved from DF112 and 2141 cultures using different media, salinity levels and temperatures.
DF112 culture with an Mg^{2+}: Ca^{2+} ratio of (A) 6 and (B) 12. DF2141 culture with an Mg^{2+}: Ca^{2+} ratio of (C) 6 and (D) 12.

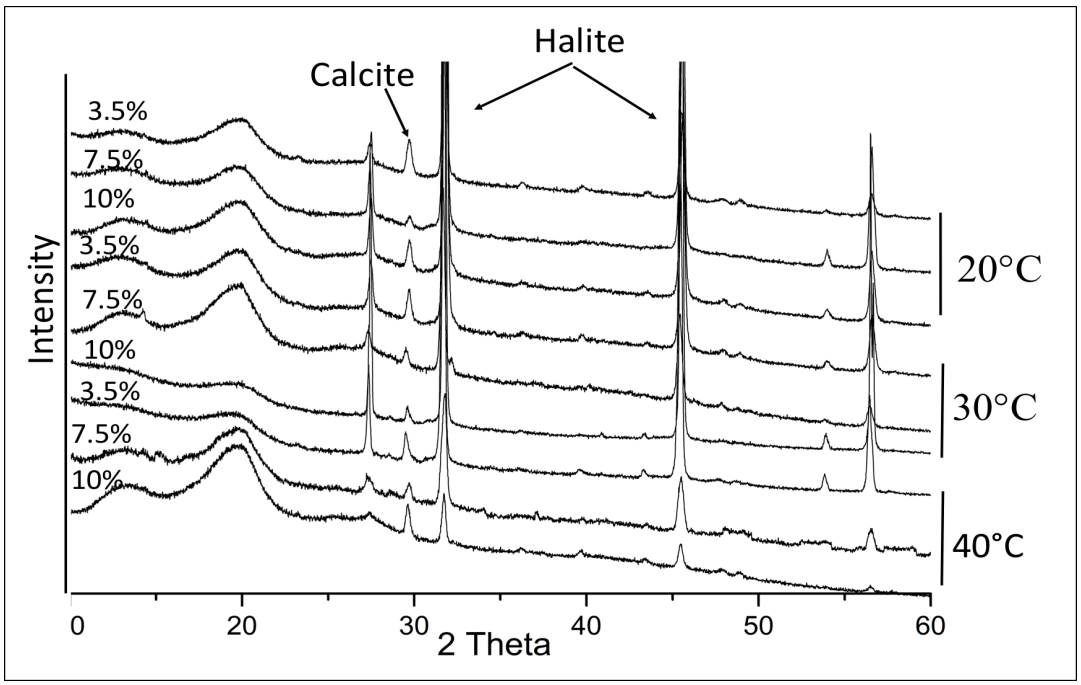

Figure 5.4: Representative X-ray diffraction patterns of minerals retrieved from DF112 and 2141 cultures at Mg^{2+}: Ca^{2+} ratio of 1 combined with different salinity levels and temperatures.

Table 5-3: % Mole Mg for the crystals recovered from different cultures.

Strain	Medium	Mg^{2+}: Ca^{2+}	NaCl (%w/v)	Temperature (°C)	% Mole Mg
DF112	MD4	6	3.5	20	30.02 ± 2.6
	MD5	6	7.5	20	26.59 ± 1.65
	MD6	6	10	20	38.45 ± 1.35
	MD4	6	3.5	30	27.7 ± 2.0
	MD5	6	7.5	30	31.63 ± 0.62
	MD6	6	10	30	41.52 ± 1.71
	MD4	6	3.5	40	42.17 ± 2.02
	MD5	6	7.5	40	44.43 ± 0.97
	MD6	6	10	40	46.82 ± 1.14
	MD7	12	3.5	20	35.32 ± 0.99
	MD8	12	7.5	20	34.44 ± 2.24
	MD9	12	10	20	40.21 ± 2.65
	MD7	12	3.5	30	38.21 ± 1.96
	MD8	12	7.5	30	40.23 ± 2.03
	MD9	12	10	30	41.52 ± 1.8
	MD7	12	3.5	40	40 ± 1.91
	MD8	12	7.5	40	41.41 ± 1.31
	MD9	12	10	40	44.64 ± 1.52
DF2141	MD4	6	3.5	20	29.93 ± 1.38
	MD5	6	7.5	20	20.87 ± 1.25
	MD6	6	10	20	41.6 ± 1.06
	MD4	6	3.5	30	33.34 ± 2.38
	MD5	6	7.5	30	27.15 ± 1.51
	MD6	6	10	30	38.49 ± 0.82
	MD4	6	3.5	40	43.25 ± 1.52
	MD5	6	7.5	40	41.95 ± 1.46
	MD6	6	10	40	44.85 ± 1.29
	MD7	12	3.5	20	36.49 ± 1.36
	MD8	12	7.5	20	34.12 ± 2.21
	MD9	12	10	20	47.37 ± 2.71
	MD7	12	3.5	30	37.61 ± 2.29
	MD8	12	7.5	30	41.63 ± 1.04
	MD9	12	10	30	42.92 ± 0.67
	MD7	12	3.5	40	40.98 ± 1.04
	MD8	12	7.5	40	42.05 ± 1.52
	MD9	12	10	40	43.25 ± 1.3

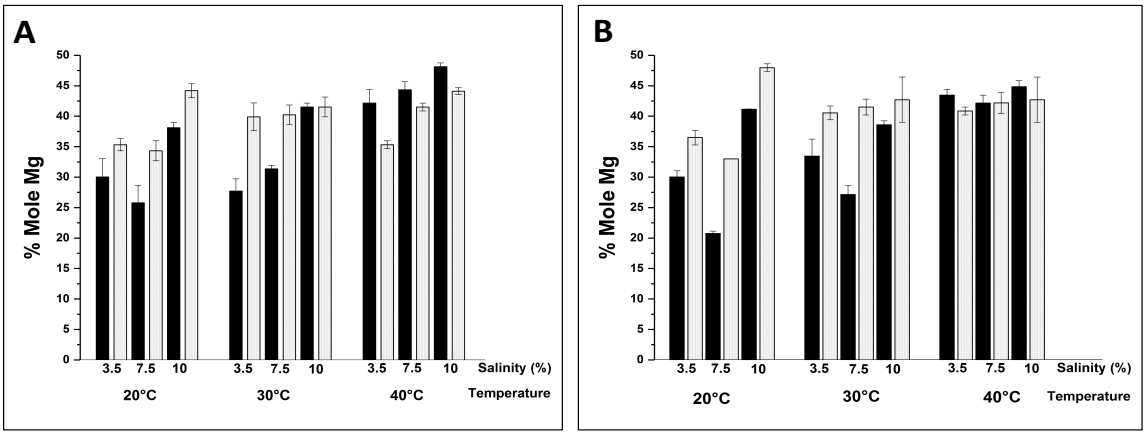

Figure 5.5: Effect of temperature, salinity and Mg2+: Ca2+ ratio on incorporation of Mg (% Mole Mg) into the carbonate minerals recovered from pure cultures of: DF112 (A) and DF 2141 (B). Mg^{2+}: Ca^{2+} 6 ■, 12 □

5.6 Combinatorial Impact of Salinity, temperature and Mg^{2+}: Ca^{2+} ratios on the incorporation of Mg into the formed crystals

The 3D illustration of the combinatorial impact of the studied factors in cultures with Mg^{2+}: Ca^{2+} ratios 6 and 12 (Figure 5.6) shows that the incorporation of magnesium increased with the increase of salinity and/or temperature and Mg^{2+}: Ca^{2+} ratios. The results of Mg^{2+}: Ca^{2+} ratio of 1 are not shown since no HMC peaks were detected.

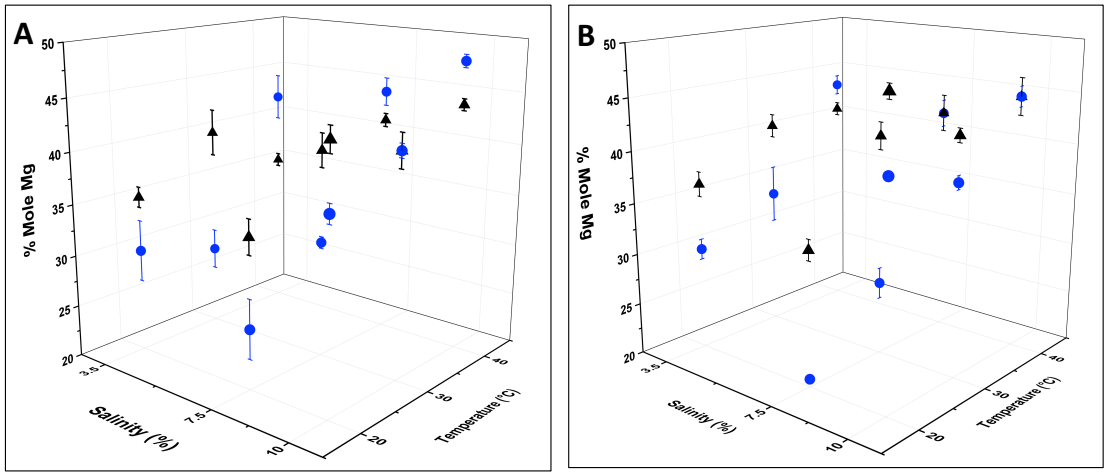

Figure 5.6: 3D Illustration of the combined effect of temperature, salinity and Mg^{2+}: Ca^{2+} ratios on the values of % Mg, for minerals recovered from (A) DF112 and (B) DF2141, at Mg^{2+}: Ca^{2+} ratios 6 ● and 12 ▲.

5.7 Discussion

Temperature is known to favor the precipitation of carbonate minerals by increasing the saturation index (Arvidson & Mackenzie, 1999). Temperature is also known to help overcome kinetic barriers that otherwise prevent the incorporation of Mg into a crystal. Indeed, Dolomite is not difficult to form in laboratory experiments performed at high temperature (i.e. Kaczmarek & Duncan, 2011; Rodriguez-Blanco, Shaw, & Benning, 2015). Previous studies using microbial culture experiments showed a positive correlation between temperature and the amount of Mg incorporated into carbonate minerals (Sanchez-Roman et al., 2011). With respect to microbially mediated formation of Mg-rich carbonates, the role of temperature might be twofold: it promotes mineral formation for thermodynamic and kinetic reasons, but also because many

microorganisms have an optimum growth temperature or may tolerate relatively high temperatures. However, the optimal growth temperature for *Virgibacillus* bacteria genetically close to that used in the present study was reported to be approximately 37°C (Sanchez-Porro et al., 2014). All these considerations are consistent and confirmed by the results of our experiments carried ot at 20, 30, and 40°C, which showed a positive correlation between temperature and the Mg mol% content of a carbonate mineral.

In natural evaporitic environments, all dissolved elements are simultaneously concentrated through progressive evaporation of the seawater (Warren, 2016). Therefore, salinity is generally positively correlated (although not in a linear manner) with the saturation index of the carbonate minerals. However, in our experiments, the Na^+ and Cl^- concentrations increased independently of the concentrations of Ca^{2+} and Mg^{2+}, allowing the separate evaluation of the effect of salinity and the Mg^{2+}:Ca^{2+} ratio. The effect of salinity and ionic strength on the incorporation of Mg into the carbonate minerals are not fully resolved, and existing studies show contrasting results. Stephenson et al., (2011) reported an inverse relationship between the ionic strength and the Mg content in Mg-rich calcite obtained abiotically. In addition, a theoretical geochemical model for dolomite formation predicted a higher saturation index (SI) at higher salinity (Kaczmarski et al., 2016). Differently, various microbial mediation experiments conducted at terrestrial surface temperatures suggested that high salinity (if not sufficiently high to inhibit microbial growth) generally promoted the formation of Mg-rich carbonates (Rivadeneyra et al., 2004; Rivadeneyra et al., 2016; Han et al., 2017). This correlation was also clearly observed in our study. For example, the Mg mol% content increased from 27.7 ± 2.0 to

31.63 ± 0.62 to 41.52 ± 1.71 when the salinity increased from 3.5% to 7.5% to 10% respectively, while the temperature was maintained at 30°C and the Mg^{2+}:Ca^{2+} ratio at 6 (Table 5-3).

A similar positive correlation was observed between the mol% of Mg and Mg^{2+}:Ca^{2+} ratio. A ratio of 1:1 was not sufficient to form any high-Mg calcite; only Ca-carboantes and Mg-calcite with a low mol% of Mg^{+2} were detected. However, a ratio of 12:1 compared to 6:1 significantly affected the percentage of Mg^{+2} incorporated into the mineral. This result is consistent with previous studies, showing that high Mg^{2+}:Ca^{2+} ratio as a key factor for the formation of dolomite in a natural environment (Illing et al., 1965; Petrash et al., 2017). For instance, dolomite fromation in the Dohat Faishak sabkha –the site where the microbes used for this study were isolated– has been linked to the precipitation of gypsum, which removes Ca^{+2} from solution and abruptly increases the Mg^{2+}:Ca^{2+} ratio (i.e., from ≈1 to ≈12) (Illing et al., 1965; Illing & Taylor, 1993). Our findings using *Virgibcillus* support this hypothesis, but also show that temperature and salinity have the highest impact on the incorporation of Mg^{2+} into carbonate minerals. This result would further illuminate the link between the mol% of Mg into the carbonate mineral and high concentrations of dissolved Na^{+} and Cl^{-}, which is intuitively not so direct. An increase in ionic strength does not enhance incorporation of Mg into the carbonate under abiotic conditions, but has rather the opposite effect (Stephenson et al., 2011). We therefore propose that the correlation between salinity and Mg-rich carbonates should be investigated. Considering that many of the most recent studies on the microbially mediated formation of dolomite and Mg-rich carbonates suggest that EPS

have key importance in the mineralization process (Bontognali et al., 2010; Roberts et al., 2013; Bontognali et al., 2014; Petrash et al. 2017. Such EPS may in turn contain functional groups that, by affecting the kinetics of the nucleation process, promote the incorporation of Mg into carbonate minerals at low temperature, for example by promoting the dehydration of Mg (Roberts, et al., 2013). In future studies, it would be interesting to investigate how salinity influences the net production of EPS as well as its general composition.

The hypothesis that EPS played an important role in our experiments is also consistent with the morphology of the precipitates. Braissant et al., (2003) investigated the effects of exopolysaccharides and amino acids on the morphology of calcium carbonate crystals and concluded that minerals formed in the presence of these organic molecules tend to have spherical morphologies with a surface texture that is very similar to that of the minerals produced in our experiments (Figure 5.1).

Finally, the observation that different minerals (i.e. calcium carbonate, high magnesium calcite, hydromagnesite) were formed simultaneously in the same pure culture is consistent with mineralization under the influence of heterogenic functional groups present in various types of EPS and/or bacterial cells. Organic molecules with different cation-adsorption properties would indeed explain the co/occurrence of minerals with different morphologies and chemical compositions.

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cation-adsorption properties would indeed explain the co/occurrence of minerals with different morphologies and chemical compositions.

5.8 Conclusion

All of the three tested parameters—temperature, salinity, and Mg^{2+}:Ca^{2+} ratio—have an impact on the microbially mediated formation of high-Mg calcite. High temperature causes the stimulation of bacterial growth. The increase of salinity likely causes ecological stress. Bacterial growth and EPS production respectively stimulated by temperature and salinity were likely to cause an increase of magnesium incorporation into the minerals formed. The highest level of Mg^{2+} incorporation in the carbonate crystals formed was obtained at 10% rather than 3.5% or 7.5% salinity, while calcium carbonates were mostly detected at 3.5% salinity. At a Mg^{2+}:Ca^{2+} ratio of 1, the dominant mineral phase was calcium carbonate; no or rare magnesium calcium carbonates were observed. Increasing the Mg^{2+}:Ca^{2+} ratio from 6 to 12 resulted in an increase of Mg^{2+} incorporation into the formed carbonate crystals . Thus, a high Mg^{2+}:Ca^{2+} ratio seems to be crucial as proposed by most previous models of dolomite formation in a sabkha environment.

Although our experimental approach does not simulate the complexity of the natural environment, the results obtained with two different *Virgibacillus* strains suggest that temperature, salinity values higher than in average seawater favor the microbially mediated formation of Mg-rich carbonates, which are often considered as potential dolomite precursors. In ancient sedimentary sequences, intervals rich in dolomite (as opposed to limestone) may therefore reflect a paleoenvironment that was warm and/or characterized by high salinity.

Actually, we consider that our results are consistent with the hypothesis that each single bacterial strain or populations exhibit a separate behavior with regard to cell structure or metabolic pathways. In turn, this behavior is a response to each of the major physical and chemical environmental parameters or the combined factors. This response may mediate the formation of more than one form and composition of crystals in controlled conditions: defined medium and incubation.

Moreover, it is now obviously clear, based on the results that the role of abiotic factors on biomineralization is through the cell structure and/or their biological activities.

Actually, the interest is to be focused on what is the role of the cell structure and secreted (EPS) in mediating minerals or not and in types of mediated minerals?

CHAPTER 6 : THE EFFECT OF SALINITY AND TEMPERATURE ON THE COMPOSITION OF EPS PRODUCED BY DOLOMITE MEDIATING BACTERIA

6.1 Introduction

The role of specific macromolecules - Exopolymeric Substances (EPS) - secreted by bacteria in the mineralization process has been progressively highlighted (Braissant et al., 2008; Bontognali et al., 2014; Roberts et al., 2013; Tourney & Ngwenya, 2014; Petrash et al., 2017). The majority of EPS are mainly composed of polysaccharides (exopolysaccharides) and proteins, but may also include other macromolecules (i.e., DNA, lipids, humic substances, etc.) (Decho, 1993). EPS contain repeated chains of carboxylated compounds, methylated carboxyl-rich uronic acids, non-carbohydrate components, such as pyruvate and succinate, in addition to minor amounts of amines, thiosulfates and phosphates (Flemming & Wingender, 2001). The secreted EPS by microbial cells into their growth environment is influenced by the factors that control microbial metabolisms including many physiochemical and biological factors such as genotype, growth conditions, carbon to nitrogen ratios (C/N ratio), pH and temperature (More et al., 2014).

Despite numerous research on biofilms, many fundamental questions concerning the extracellular activity of the EPS enzymes within the biofilm matrix remain unresolved. EPS enzymes can render vast quantities of dissolved polymers that are readily bioavailable for further degradation. Thus, they contribute to the global carbon cycle (Flemming & Wingender, 2010).

Temperature is one of the most important parameters the influence the production of EPS (Wingender et al., 1999). Literature reports suggested that the EPS production could vary for different microorganisms at a similar growth temperature. Depending on the microbial strain, it is also possible that optimum temperatures for EPS production may vary from those optimum for the microbial growth (Yang, 2007). This difference between the optimum temperature for growth and EPS production could also result in the increase of enzymatic activity for the synthesis of the EPS precursors (Nichols et al., 2005). Salt stress can cause physiological changes to the microbial community, which in turn, may influence the consequent biofilm formation. For example, microorganisms enhance the production of EPS and stimulate several osmoadaptation reactions as a protective response to salinity stress, which results in accumulation of osmoprotectants (i.e., glycine betaine, proline and carnitine (Kim & Chong, 2017).

In the evaporitic environments such as sabkha, it is proposed that high temperature and subsequent increase in salinity and supersaturation caused by the substantial evaporation, may promote an ecological stress that stimulates the extensive production of EPS by microorganisms (i.e., Bontognali et al., 2010; Petrash et al., 2017). The EPS produced at these different stress conditions may have specific characteristics that may be, in turn, related to mineral formation.

In the previous chapters, we confirmed a positive relationship between temperature and salinity and the incorporation of Mg^{+2} into the carbonate crystals. Here, we Investigate if at the same conditions that favor the formation of high magnesium calcite, the secreted EPS has a particular composition that promotes the high incorporation of magnesium into

the carbonate crystals. And, if a relationship between the heterogeneity of EPS composition and structure and the various forms and compositions of crystals could be established.

In the present study, three bacterial strains previously isolated from Dohat Faishakh in Qatar, two *Virgibacillus* strains (DF112 and DF2141) and one *Bacillus licheniformis* (DF141) were used to study how temperature and salinity affect the composition of EPS which, in turn, affect the composition of the formed Mg-calcite minerals. *Virgibacillus* strains are mineral forming strains, while *Bacillus* is the non-mineral forming one. The EPS produced by the three bacterial strains were chemically characterized to understand their potential role in carbonate mineral formation was studied. The EPS produced at three different salinities (3.5%, 7.5% and 10%) and two incubation temperatures (30º C and 40º C), were used to evaluate the characteristics of EPS and their potential role in mineral formation.

6.1 Chemical characterization of EPS

6.1.1 Study of functional groups using FTIR

EPS from the selected strains were prepared and analyzed by FTIR (Figure 6.1). The FTIR spectra demonstrate general similarity between the EPS produced by DF112, DF2141 and DF141. They show the presence of several functional groups, mainly, carboxyl, amino, phosphorus and sulfate groups. The FTIR spectrum is characterized by a broad peak above 3000 cm^{-1} and strong absorptions in the range of 1660 to 1040 cm^{-1}. The absorption peaks around 3500–3200 cm^{-1} are attributed to the N–H stretching of the amino groups (Guo et al., 2016). The N–H stretching peak is located in the board

spectrum region attributed to hydroxyl groups (Dittrich & Sibler, 2010). The very weak peak at 2970 cm-^{1} is ascribed to the presence of saturated carbohydrates (Dittrich & Sibler, 2010). Regarding the protein related bands: amide I is present at 1660 cm^{-1}, the amide II peak is present at 1560 cm^{-1} region and the amide III band at 1240 cm^{-1} (Cavalu & Simon, 2007; Kong & Shaoning, 2007). The band at 1420 cm^{-1} is located in the region of CH_2 deformation modes (Dittrich & Sibler, 2010). According to Kazy et al., (2008) the observed peaks in the spectra at 1400 to 1450 cm^{-1} region are characteristic of the existence of carboxyl groups (Kazy et al. 2008). Bands at 1350 cm^{-1} are assigned to the C—O vibration of carboxylic acids. Vibrations due to the carbohydrate backbone were commonly observed in all spectra. Intense, complex absorptions centered at 1070 to 1080 cm^{-1} are attributed to complex vibrations of the carbohydrate skeleton ring structures (Omoike & Chorover, 2004). However, the presence of bands between 1070–1080 cm^{-1} usually corresponds to ring vibrations of C-O-C in polysaccharides and to the stretching vibrations of P=O bonds. Indeed, due to their overlapping, it is difficult to separate the phosphate bands and polysaccharide vibrations bands (Masaki et al., 2017). Polysaccharides and phosphoryl groups can be recognized as components of the EPS produced by the three studied strains.

The amide I and amide II correspond to the protein secondary structure (Kong & Shaoning, 2007; Chadefaux et al., 2009). EPS produced by the mineral forming strains DF112 and DF2141 in cultures incubated at 30ºC, showed a shift in the amide I band from 1654 cm^{-1} (at 3.5%) to 1644 cm^{-1} (10%), while no shift in amide I band was observed in the EPS produced by the non-mineral forming strain DF141 at increasing salinity. In addition, the amide II band was significantly attenuated in the EPS produced

by mineral forming strains DF112 and DF2141in cultures incubated at 40ºC at the three-different salinity levels. Indeed, differences in the amide II band in the EPS produced by DF141 at 30ºC and 40ºC are not major.

At 40ºC, the FTIR band at 1350 cm^{-1} attributed to the carboxyl group, was not detected in the EPS of mineral forming strains DF112 and DF2141 produced, but, it was just attenuated in the EPS of non-mineral forming strain DF141 produced at 40ºC.

It is then clearly observed that increasing salinity caused shifts in the amide I band in EPS produced by the mineral forming strains, while increased temperature caused attenuation of the amide II band.

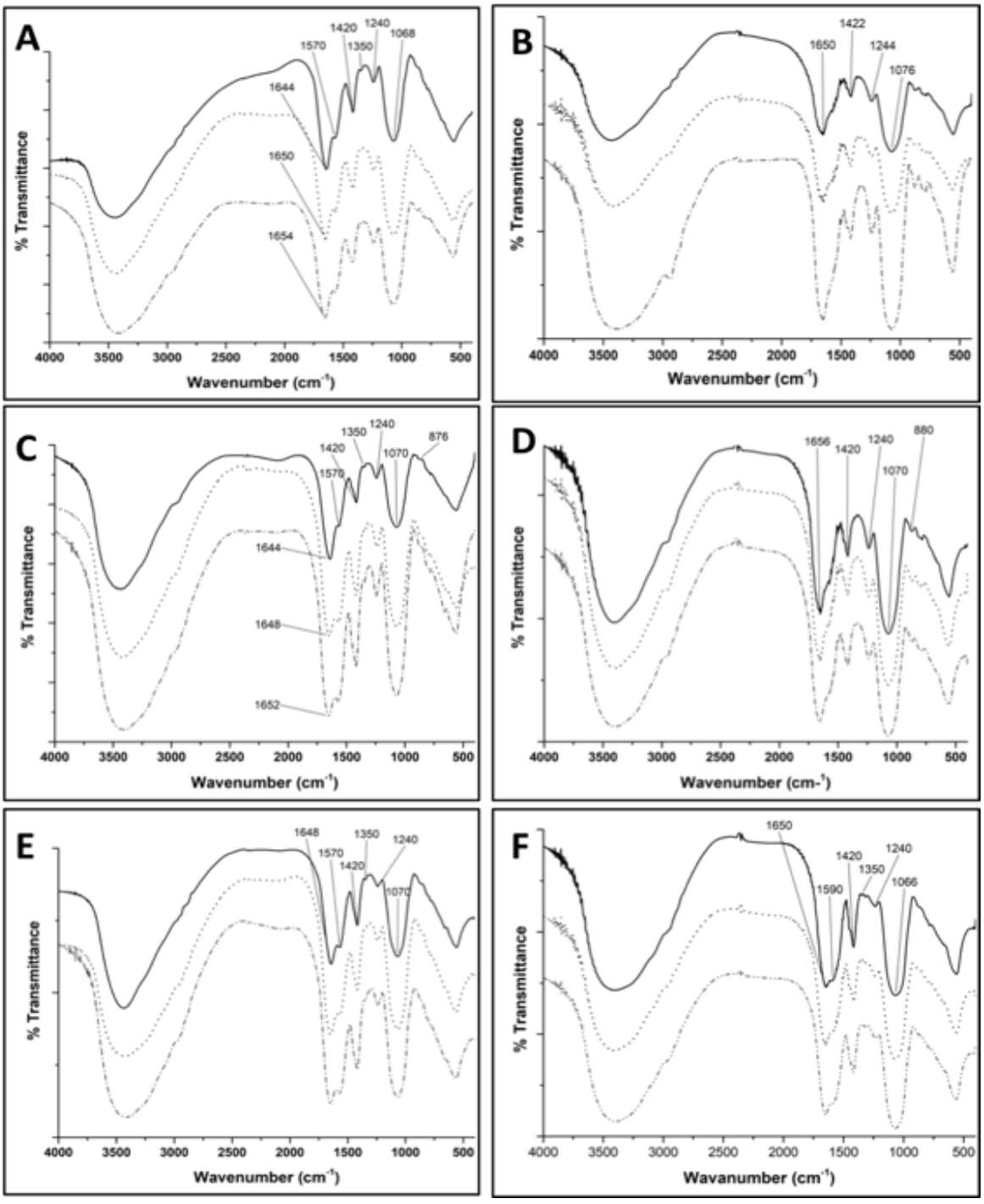


Figure 6.1: FTIR spectra of DF112: A, at 30ºC, B, at 40ºC, DF2141: C at 30ºC, D at 40ºC and DF141: E at 30ºC, F at 40ºC and salinity levels: 3.5% - - - , 7.5% ·····,10% ——

6.2 Estimation of Total Carbohydrate (TCHO) and Total Proteins (TP) in the soluble EPS

The importance of EPS is mainly attributed to carbohydrates and proteins which proportion may vary depending on the bacterial strains and the ecological conditions. As shown in (Table 6-1), the content of both TCHO and TP increased significantly (≈ 2 - 3 folds) in the EPS produced in the cultures incubated at 40ºC compared to cultures incubated at 30ºC. In order to understand their role, TCHO and TP contents were discussed separately and then their combinatory effect was studied.

Table 6-1: Content of Total Carbohydrate (TCHO) and Total protein (TP) in the soluble EPS produced in the cultures incubated at 30ºC and 40ºC at different salinities.

Temperature (ºC)	**30**		**40**	
Salinity (%)	**TCHO (mg/g(EPS)**	**TP (mg/g (EPS)**	**TCHO (mg/g(EPS)**	**TP (mg/g(EPS)**
DF112 (Mineral forming)				
3.5	412 ± 31	6.19 ± 0.74	927 ± 23	9.89 ± 0.74
7.5	440 ± 35	6.70 ± 0.95	893 ± 60	7.85 ± 0.51
10	313 ± 64	3.45 ± 0.6	978 ± 15	10.47± 1.34
DF2141 (Mineral forming)				
3.5	429 ± 44	7.18 ± 1.02	939 ± 63	10.19 ± 0.73
7.5	491 ± 19	5.09 ± 0.86	651 ±64	9.85 ± 1.07
10	363 ± 58	5.13 ± 0.35	822 ± 50	10.39± 0.85
DF141 (Non-mineral forming)				
3.5	454 ± 44	7.27 ± 0.64	743 ± 73	16.88± 1.11
7.5	362 ± 33	7.34 ± 0.72	718 ± 83	16.76± 1.07
10	296 ± 33	5.12 ± 1.09	407 ± 76	26.41 ± 0.64

6.2.1 Impact of temperature and salinity on TCHO

The TCHO content in the EPS is shown in (Figure 6.2). In cultures incubated at 30°C, the content of TCHO varied between 296±33 mg/g(EPS) and 491±19 mg/g(EPS) among the three studied EPS. Based on one way ANOVA analysis at 95% confidence level, there is a significant difference in the content of TCHO in the EPS produced by each strain at 3.5%, 7.5% and 10% salinity levels. However, the three strains exhibit similarity as no significant difference between the content of TCHO was observed.

In the cultures incubated at 40°C, the content of TCHO varied between 407±76 mg/g(EPS) and 978±15 mg/g(EPS) among the strains. In the EPS of one mineral forming strain DF112, the content of TCHO in the EPS did not change significantly with the increase in salinity. In contrast, the TCHO content in EPS of the other producing strain DF2141 and the non-producing strain significantly varied with salinity. However, there is no significant difference of TCHO content in EPS produced by the three studied strains at 3.5% and 7.5% salinities. The significant difference between the three strains was observed at 10% salinity.

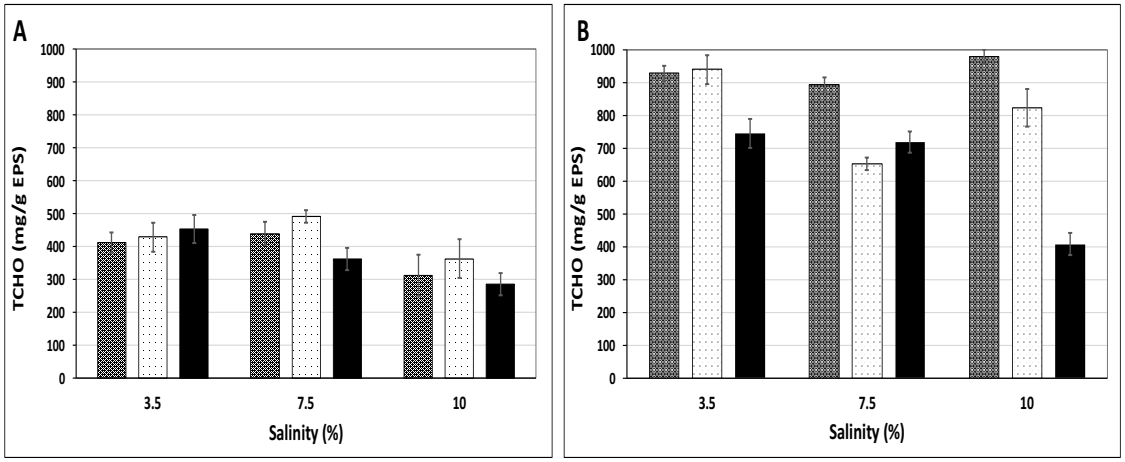


Figure 6.2: Total carbohydrate content in the EPS of DF112 , DF2141 and DF141 at different salinity levels; (A) at 30ºC and (B) 40ºC.

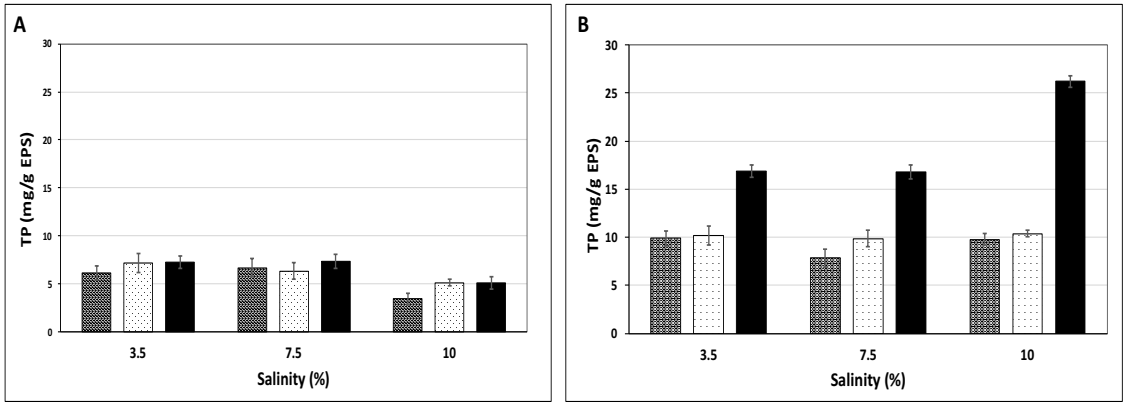


Figure 6.3: Total protein content in the EPS of DF112 , DF2141 and DF141 at different salinity levels; (A) at 30ºC and (B) 40ºC.

6.2.2 Impact of temperature and salinity on TP

The TP content in the EPS is shown in (Figure 6.3). In cultures incubated at 30°C, the content of TP varied between 3.45±0.6 mg/g(EPS) and 7.34±0.72 mg/g(EPS) among the three studied strains. There is a no significant difference between the TP content of EPS produced by the three studied strains. However, the content of TP in the EPS produced by mineral producing by DF2141 and DF141 did not change significantly by the increase of salinity from 3.5% to 10%. While the TP content in EPS produced by DF112 was significantly different at the three studied salinity levels.

In cultures incubated at 40°C, the content of TP varied significantly between 7.85±0.51 mg/g(EPS) and 26.41±0.64 mg/g(EPS) among the three studied EPS. However, The content of TP in the EPS produced by DF112 and DF2141 did not change significantly with the increase of salinity from 3.5% to 10%. However, the TP content was significantly different in both EPS produced by DF141 at the three studied salinity levels.

Moreover, the TCHO/TP ratios are not significantly varying at different salinity level in the EPS produced by all strains at 30°C, while at 40°C a significant difference in the TCHO/TP ratios between the EPS produced the forming and non-forming strains was detected as shown in (Figure 6.4)

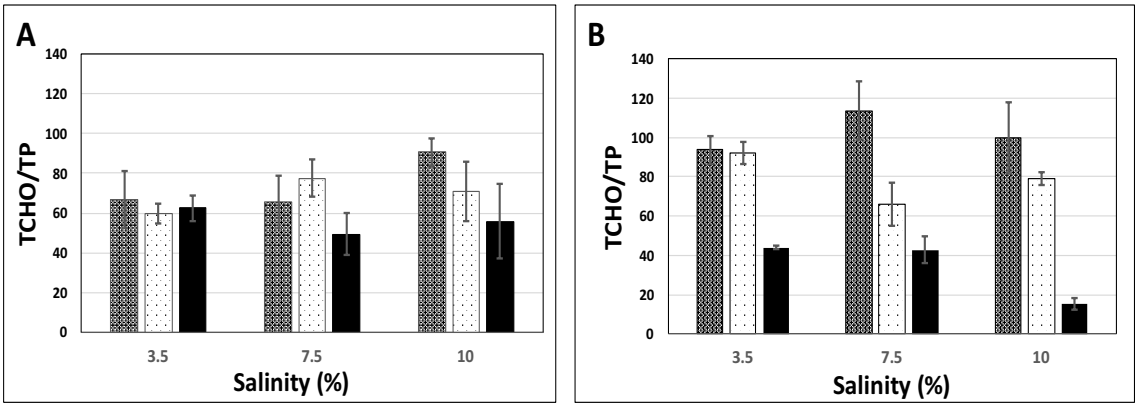


Figure 6.4: TCHO/TP Ratios for EPS produced by DF112 , DF2141 and DF141 at different salinity levels; (A) at 30ºC and (B) 40ºC.

6.3 Discussion and conclusion

By using FTIR analysis, it was shown that differences exist between the composition of EPS produced by mineral forming strains (DF112 and DF2141) and the non-mineral forming strain (DF114). When cultured at 30°C, the increase of salinity caused a shift in amid 1 band in the EPS produced by the two mineral forming strains, while no shift was observed with the non-forming strain. Moreover, the amide II band was significantly attenuated in the EPS produced of the mineral forming strains at 40°C independently of the salinity levels, which was not observed with the non-forming strain. It is then clear the temperature and salinity affect protein components in the EPS of mineral forming strains. The correlation between FTIR amide peaks and protein secondary structure (α-helix, β-sheet and β-turn structures) is established in the literature (Kong and Shaoning 2007, Chadefaux et al. 2009; Yang et al., 2015). So, we can hypothesize that the

disruption of amide I and II bands caused by increasing salinity and/or temperature could be due to the process occurring in the specific sites corresponding to these bands.

In terms in effect on eps composition, temperature increase caused a significant increase of TCHO in EPS of forming and non-forming strains at 3.5% and 7.5% salinity. Interestingly, the increase of TCHO was not observed with the non-forming strain with 10% salinities.

TP content was increased in the 3 EPS at all temperature and salinity condition, but, with an excessive increase in the EPS of the non-mineral forming strain, for which the overproduction of TP was even higher at 10% salinity, although TCHO did not increase.

Our results, clearly show that biomineralization activity may not be exclusively related to TCHO and TP content, but, mainly to the specific functional groups including composition carboxyl and amide.

CHAPTER 7 : SIMULATION OF THE NATURAL EPS TO INVESTIGATE THE ROLE OF CARBOXYL AND AMIDE GROUPS IN EPS

7.1 Introduction

The complete biochemical profiling of EPS remains extremely challenging, due to the difficulty of purifying the components of the EPS apart from other constituents such as cells and other associated macromolecules (Wingender et al., 1999).

Xanthan was used to mimic EPS function, as a carbohydrate and pure amino acids (Glutamine, Glutamic acid and Aspartic acid) as proteins' functional groups. This will help to study the role of specific functional groups in carbohydrates and amino acids in carbonate minerals forms and composition.

Xanthan Gum , an extracellular polysaccharide produced by Xanthomonas campestris, is composed of repeating units of glucose, mannose, glucuronic acid, acetyl and pyruvic acid (Figure 7.1). Xanthan forming homogeneous aqueous diffusions also exhibits high viscosity (Moorhouse et al., 1977).

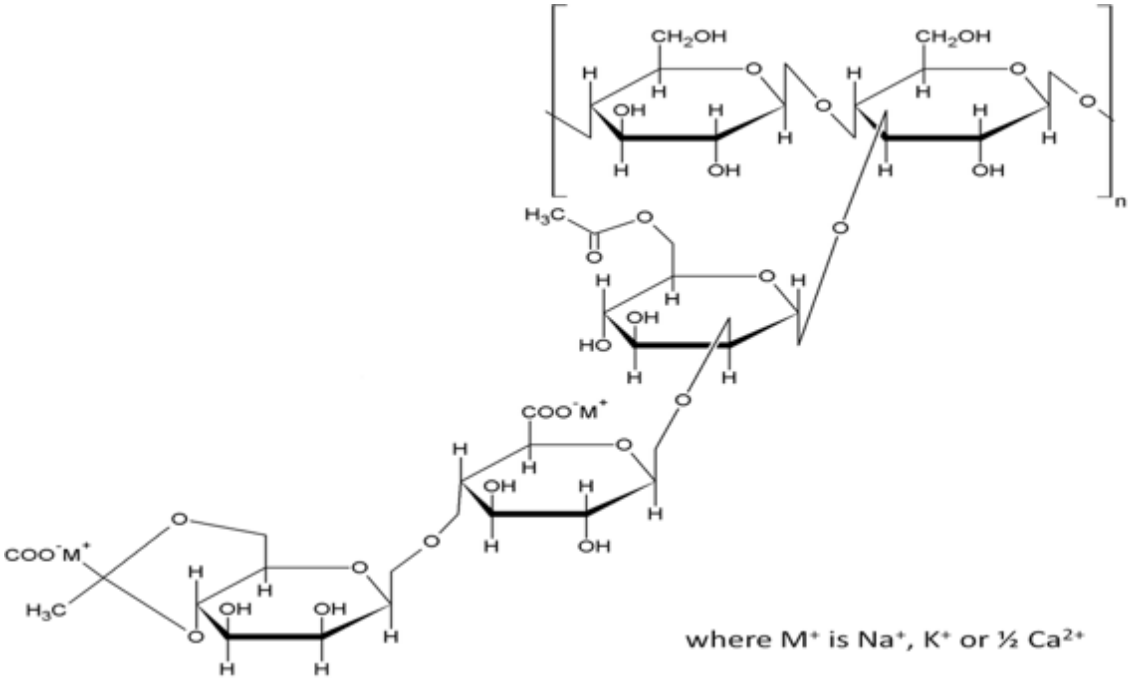
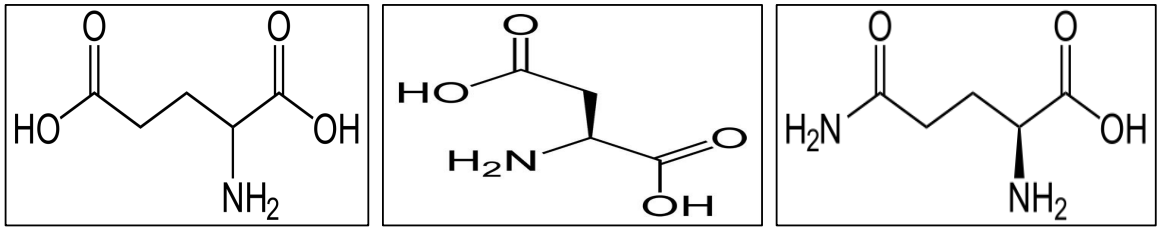


Figure 7.1: Structure of xanthan gum (Kulkarni, Butte, & Rathod, 2012)

Amino acids provide carboxyl and amide groups, L-Glutamic Acid ($C_5H_9NO_4$) and L-Aspartic Acid ($C_4H_7NO_4$) are acidic polar and negatively charged amino acids with two carboxyl groups. L-Glutamine ($C_5H_{10}N_2O_3$), is polar, neutral amino acid with two amide side chain (Figure 7.2). Moreover, these three amino acids were chosen for this study because they are normally found in the parietal structure of many bacterial species (i.e., *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Sporosarcina halophila*, *Synechococcus* sp.) (Braissant et al. 2003). For studying the role of xanthan (representing carbohydrates) and amino acids (representing proteins), it is necessary to consider also the Mg^{+2}: Ca^{+2}

ratios, which play an important role in the incorporation of Mg into the carbonate mineral.



Glutamic Acid **Aspartic Acid** **Glutamine**

Figure 7.2: chemical structures of Glutamic acid, aspartic acid and glutamine.

In our previous studies, we selected the Mg^{+2}: Ca^{+2} ratio of 6 as optimal to screen the isolates for their biomineralization potential. Meister et al., (2011) demonstrated that high Mg^{+2}: Ca^{+2} ratios above 5, led to the abiotic formation of spherical shape carbonate crystals. This is solid evidence that the spherical shape of crystals may not only be a result of biological mechanism.

Two Mg^{+2}: Ca^{+2} ratios of 1 and 14 were compared to study the role of xanthan and amino acids. The ratio of 1 which was shown to promote the formation of all crystals shapes (current research) and the ratio of 14 causing spherical crystals formation. These two conditions would clarify the role of the EPS components in varying the crystal shape and Mg incorporation.

7.2 Analysis of morphology and composition of crystal by SEM/EDS

(Figure 7.3) Shows that at Mg^{+2}: Ca^{+2} ratio of 1, using the NH_4CO_3 free-drift method, the reaction between Ca^{+2}, Mg^{+2} and CO_3^{-2}, in the absence of xanthan and/or amino acids produced rhombohedral carbonate crystals. This condition can be considered as a control to the conditions in which the biochemical precipitation was tested. As expected, incorporation of Mg into the formed rhombohedral carbonate crystals in control was very low. However, the synthesis of carbonate in the presence of 0.1% xanthan resulted in a change of crystal morphologies to spherical and dumbbell shapes; EDS analysis shows that higher Mg incorporation is associated with the spherical and dumbbell shapes. A potential role of the carbohydrates components of EPS in the morphology and composition of minerals could be concluded. The carbonate crystals formed in the presence of Glutamic acid, but without xanthan, were spherical with rough surface morphology and high incorporation of Mg. In solutions containing Glutamic acid combined to 0.1% xanthan, three different crystal morphologies (spherical, rhombohedral and rectangular prism) with smoother surfaces. However, the content of Mg in each of these crystal types was variable. In the presence of Glutamine or Aspartic acid, the prismatic crystals have a high content of magnesium. Prismatic, elongated nesquehonite crystals were formed only with glutamine. Nesquehonite ($Mg(HCO_3)(OH)•2(H_2O)$) is a hydrated metastable form of magnesium carbonate. When xanthan was added to these two amino acids, both rhombohedral and spherical crystals were formed. Here, contents of Mg incorporated into the carbonate crystals were variable, which means that with the

EDS analysis it is not possible to discriminate the influences of xanthan and each of the three amino acids on the incorporation of Mg^{+2} in the formed carbonates.

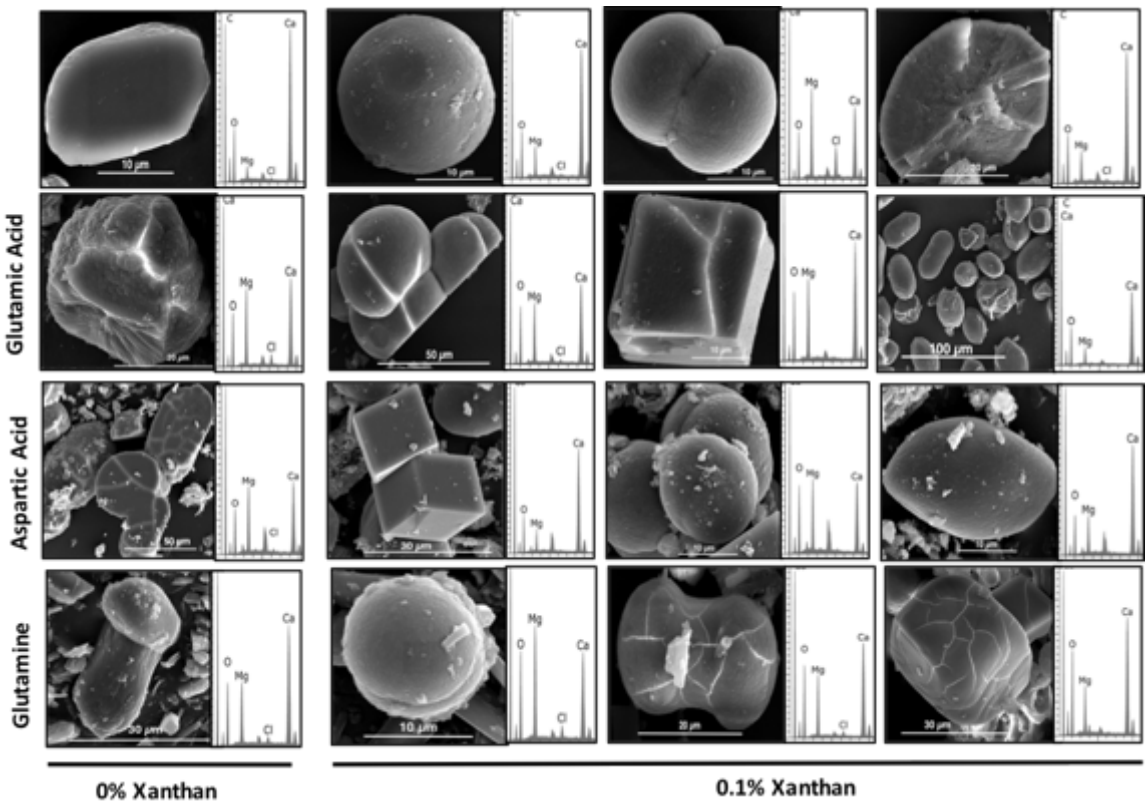


Figure 7.3: SEM/EDS exploration of crystal formation in the presence or absence of xanthan and/or amino acids at Mg^{+2}: Ca^{+2} of 1.

At Mg^{+2}: Ca^{+2} of 14, the majority of crystals obtained with amino acids and xanthan were nesquehonite crystals besides fewer carbonate crystal aggregates with spherical and dumbbell shapes. In the control performed in the absence of xanthan and/or amino acids

spherical, dumbbell-rough surface calcium carbonate and platy shaped magnesium carbonate crystals were formed. If 0.1% xanthan was supplemented, smooth-surface dumbbell and spherical Mg-calcite crystals were formed beside the platy shaped Mg-carbonate crystals. The crystals formed in the presence of any of the three tested amino acids were spherical with rough surface morphology. Large spheres with needle-like structures composed of calcium carbonate and small spheres with honeycomb like structures composed of magnesium carbonate were observed as well. In the presence of both xanthan and amino acids, dumbbell-shaped and spherical crystals with smooth and rough surfaces were observed. Based on the EDS analysis, the main types of crystals which were formed are; primarily, nesquehonite, calcium carbonate crystals with various Mg content and hydromagnesite.

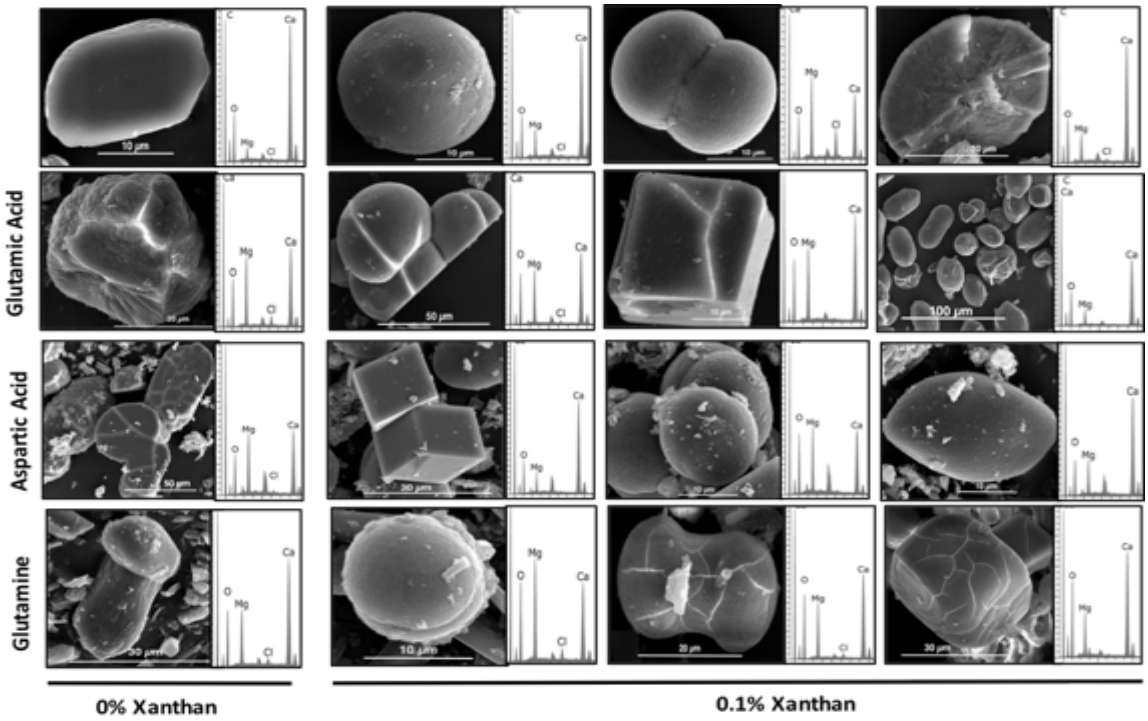


Figure 7.4 : SEM/EDS exploration of crystal formation in the presence or absence of xanthan and/or amino acids at Mg^{+2}: Ca^{+2} of 14.

7.3 Phase identification by XRD

In the experiments performed with Mg^{+2}: Ca^{+2} of 1, the XRD spectra of the minerals formed in the control sample, indicated the existence of calcite peaks (Figure 7.5). The XRD spectra of the minerals formed in the presence of xanthan and/or amino acids, exhibit the presence of high magnesium calcite (HMC) peaks. The HMC peaks are located in the region between the calcite and dolomite main peaks and have variable values corresponding to variable incorporation of Mg^{+2} into the formed Mg-calcite

crystals. Glutamine promoted the formation nesquehonite in the presence or absence of xanthan. This confirms our previous observation with SEM/EDS.

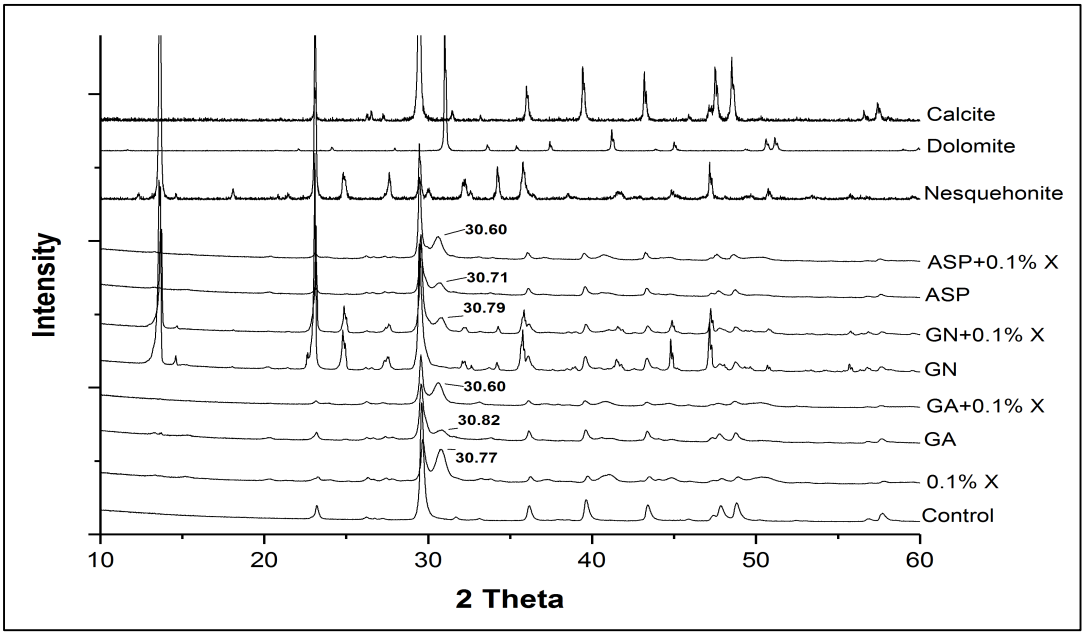


Figure 7.5: XRD Patterns of crystals formed in precipitation experiments at Mg^{+2}: Ca^{+2} of 1. X: Xanthan, GA: Glutamic acid, ASP: Aspartic acid.

The XRD analysis of the minerals formed in the control sample performed at Mg^{+2}: Ca^{+2} ratio of 14 (Figure 7.6) showed nesquehonite peaks and small peaks in the region corresponding to aragonite. In the XRD of minerals formed in the presence of 0.1% xanthan with or without the presence of amino acids, small peaks appear in the position the d104 main dolomite peak, in addition to HMC peaks with variable values.

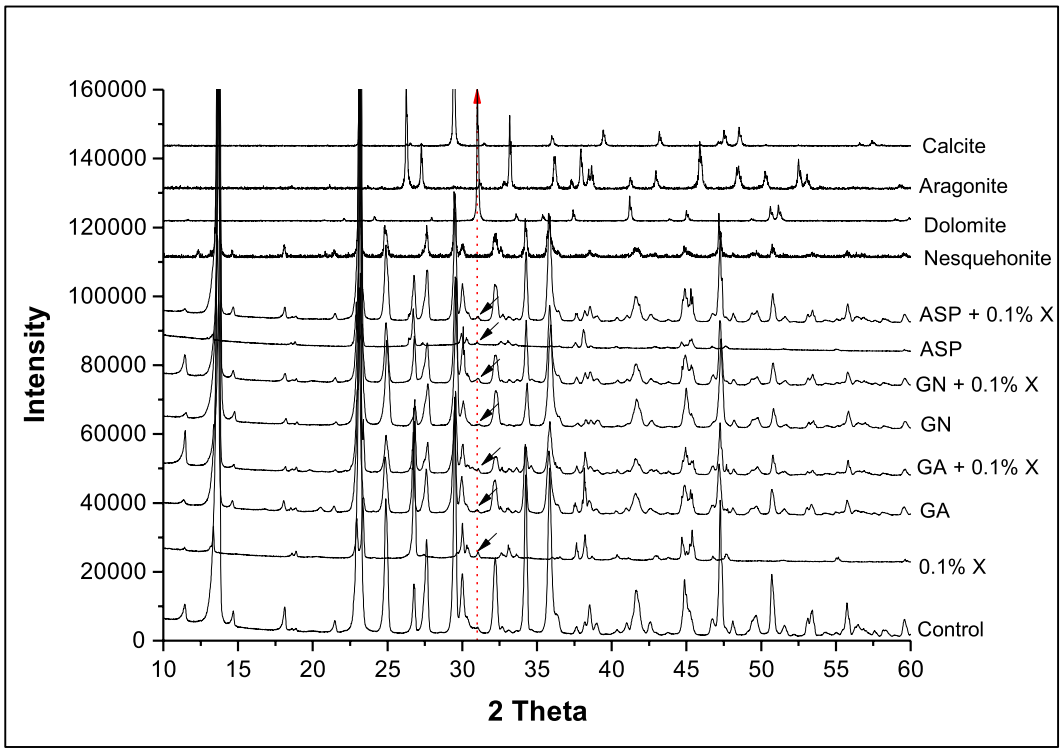


Figure 7.6: XRD Patterns of crystals formed in precipitation experiments at Mg^{+2}: Ca^{+2} of 14. Arrows point to the small peak representing disordered dolomite.

X: Xanthan, GA: Glutamic acid, ASP: Aspartic acid.

7.4 Discussion & conclusion

The results of biochemical precipitation using simulated EPS condition demonstrated a role of Mg^{+2}: Ca^{+2} ratios in the morphology and composition of the precipitated mineral phases. At Mg^{+2}: Ca^{+2} ratio of 1, calcite and HMC were formed with xanthan, glutamic and aspartic but not with glutamine. In turn, glutamine also promoted nesquehonite

crystals formation. In the presence of xanthan, glutamine promoted the formation of nesquehonite and HMC as well.

At Mg^{+2}: Ca^{+2} ratio of 14, only nesquehonite and aragonite was formed. When using any of the amino acids and xanthan separately or combined, disordered dolomite was observed.

The results of biochemical precipitation study with xanthan as representing polysaccharides and three amino acids representing proteins in EPS evidenced the role of polysaccharides and proteins functional groups in the mediation of disordered dolomite at room temperature.

Further research is required, to optimise best conditions for Mg-rich carbonate/dolomite formation.

CHAPTER 8 : CONCLUSION & PERSPECTIVES

8.1 Conclusion

Together, the results of this thesis showed that aerobic microorganisms isolated from the Dohat Faishakh sabkha are capable of mediating the formation of Mg-rich carbonates.

In previous works, dolomite formation in the sabkha was mostly linked to the sulfur cycle both through precipitation of gypsum that causes an increase in the Mg^{+2}: Ca^{+2} ratio, and through the metabolism of sulfate-reducing bacteria that remove sulfate (considered an inhibitor for dolomite formation) and increase the SI with respect to dolomite. Our results show that other microbial metabolism not linked to sulfur (as our aerobic isolates) may play a significant role in mediating the formation of dolomite.

Despite the fact that they all increased the alkalinity of the growth medium, not all the isolated strains showed the ability to mediate the formation of Mg-rich carbonates. This suggests that the studied mineralization process to be referred to as a microbially influenced rather than microbially induced process. For this reason, we focused our attention on cell wall composition and associated exopolymeric substances (EPS) rather than on respiration reactions.

The fact that more than a mineral phase is often formed in the same experiment also points to the existence of heterogeneous sub-environments within the same microbial culture. This may correspond to a heterogeneous EPS composition including different functional groups.

In the sabkha, at the site where the bacteria used for our study were isolated, environmental conditions are extreme and highly variable with respect to a more stable

conventional marine environment. Considering that the isolated bacteria are ubiquitous in marine environment, but they do not seem to precipitate dolomite there, we hypothesized that the extreme conditions of the sabkha (in particular in terms of salinity, Mg^{+2}: Ca^{+2} and temperature) concur with microbial activity creating the ideal condition for dolomite precipitation.

Our experiments conducted at variable conditions have indeed confirmed that, at high temperature and high salinity, the isolated microbes produce carbonate minerals that have a higher mol% of Mg. High temperatures seem to stimulate microbes to produce more extracellular polymeric substances.

Therefore, in line with the hypothesis that EPS play a key role for low-temperature dolomite formation, we made experiments to evaluate how the composition of EPS changes depending on the temperature and salinity under which to bacteria are cultivated. We found that, in experiments conducted at high temperatures, EPS have a higher content of carbohydrates (i.e., an increased carbohydrate to protein ratio), which is consistent with the results of other works indicating that carboxyl groups promote the dehydration of Mg^{+2}. Dehydration of Mg^{+2} at low temperature is considered to be one of the main factors inhibiting the formation of dolomite even from supersaturated solutions. Our results provided experimental data that support this view. At high temperature, the EPS produced by the studied bacteria indeed contain more carbohydrates, which correlates with the formation of carbonates rich in Mg.

To confirm this result, we have also performed precipitation experiments with xanthan and various types of amino acids (acidic and neutral). Formation of Mg-rich carbonates was clearly favored by xanthan - composed of repeating units of pentasaccharide with

glucuronic and pyruvic acid on their sidechains - and also by the acidic amino acids, while almost no Mg-carbonates were produced in the presence of the neutral amino acid.

Together, these results suggest that dolomite formation in sabkha environments may be the result of a microbially influenced process, whereby EPS rich in carboxyl groups play a key role in the mineralization process. The production of this type of EPS is stimulated by microbial growth at high temperature (that typically characterize sabkha environment vs. standard marine setting) and to some extent also by high salinity.

Dolomite formation in evaporitic settings may, therefore, be the result of an ecological stress that causes the production of an EPS with a chemical composition that is particularly favorable to promote precipitation of Mg-rich carbonates.

Our findings provide new insight for interpreting the uneven occurrence of dolomite in ancient sedimentary sequences: more dolomite was formed under conditions causing a sort of ecological stress on the microbial community inhabiting the sedimentary basin. Dolomite would, therefore, record environmental instability rather than a set of specific stable environmental conditions. Variable conditions (i.e., extreme fluctuations of temperature and salinity) are indeed a characteristic of the sabkha environment from where the studied bacteria were isolated.

8.2 Perspectives

To test the hypothesis proposed above, it would surely be of great interest to measuring the concentrations of carboxyl groups present in the sabkha sediments and see whether there is a correlation between dolomite abundance and carboxyl group contents.

Assuming the importance of carboxyl groups, it would be important to identify which component is the most efficient and under what ecological conditions it is produced and accumulated in seawater. Such organic molecules may "behave" differently in solutions with different chemistry (i.e., alkalinity, pH, cation concentrations). Systematic experiments may help to have a better understanding of the optimal organic and inorganic conditions promoting dolomite formation.

An intriguing aspect of our experiments relates to the morphology of the produced mineral. It is unclear, among the various abiotic and biotic factors, what ultimately control the shape of the crystal. Again, systematic experiments may help to associate specific morphologies to specific physicochemical and microbially-influenced conditions. In parallel, it would also be important to elucidate whether the role of such carboxyl groups is –as proposed- really that of promoting de-hydration of Mg. A computer-modeling approach may be beneficial to achieve this goal.

In many of our experiments – particularly those at high temperatures and with high Mg^{+2}: Ca^{+2}ratio -- we observed co-precipitation of Mg-rich calcite and hydromagnesite. The reasons for this co-precipitation remain unclear. A better investigation of this aspect of our experiment may provide critical insight for interpreting similar mineral assemblage that has been described from various natural environments.

Finally, the best EPS for dolomite formation may not accumulate in the environment as the result of the activity of a single microbial species. On the contrary, it may result from a synergy of microbial processes where some microbial communities degrade and transform EPS previously produced by other microbial communities. Working with pure

cultures may, therefore, be insufficient to capture and elucidate to the full complexity of the process that leads to dolomite formation in modern environments.

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