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A New Bacterial Strain *Alcaligenes aquatilis* MAG 1 Associated with the Mediterranean Sea *Lithophaga* (Bivalvia) Precipitating in Nutrient Free Medium Shell-Like Calcium Carbonate Polymorphs

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ABSTRACT

Mediterranean Sea *Lithophaga* bivalve associated bacteria and their capability in calcium carbonate polymorphs formation have been investigated in the present study. Isolated bacteria were a single strain of Gram-negative, rod-shaped, and flagellated motile cells. Phylogenetic analysis based on 16S rDNA sequences consigned the isolated bacterium to *Alcaligenes aquatilis* species as a distinct strain assigned as *Alcaligenes aquatilis* strain MAG 1. Phenotypic and biochemical characteristics of the strain recorded no acids produced from different carbohydrates under aerobic conditions except for D-mannose, amygdalin, glucose, L-rhamnose, L- arabinose and D- melibiose. Isolates showed negative results for nitrate reduction, urea degradation, sulphate reduction (H_2S production) and tryptophan deaminase (TDA). *Alcaligenes aquatilis* strain MAG 1 precipitated in nutrient-free medium different polymorphs of anhydrous $CaCO_3$, initially, as a mixture of aragonite and calcite, and well-organized rhombohedral calcite crystals dominated by the fourth day. Conclusively, calcium carbonate biomineralization away from any added nutrients by this new strain magnifies its role as nucleation sites and retracts the effect of bacterial metabolic pathways. Besides, various $CaCO_3$ crystal polymorphs formed by the isolated strain advocate a potential complementary role in *Lithophaga* shell assembly.

INTRODUCTION

Calcium carbonate precipitation is a natural process occurred in terrestrial and aquatic habitats, for instance, sands, coral reefs and shells (Barabesi **et al.,** 2007; Kim *et al.*, 2016). Bivalves shell ornamentation is mainly carried out by animal mantle with a potential role in shell assembly could be considered for some associated beta proteobacteria (Peharda *et al.*, 2015). Some species members of the family Alcaligenaceae, belonging to the beta proteobacteria, are defined to induce calcium carbonate biomineralization (Daskalakis *et al.*, 2013).

The genus *Alcaligenes* (Castellani and Chalmers 1919) representing the genus type of family Alcaligenaceae has two type species *Alcaligenes faecalis* (subdivided into subspecies, namely, *faecalis*, *parafaecalis*, and *phenolicus*) and *Alcaligenes aquatilis* (Austin 2014; Basharat *et al.,* 2018; Sun *et al.,* 2015; Van Trappen *et al.,* 2005). *Alcaligenes* as a genus is chemoorganotrophic, obligate aerobe, motile via 1–9 peritrichous flagella and Gram-negative rods/coccobacilli of 0.5–1.2 x 1.0–3.0 µm in size bacteria that occur mostly singly and have non pigmented colonies on nutrient agar. The G+C ratio of the DNA is 56–60 mol%. The species *Alcaligenes aquatilis* produces non- and yellow-pigmented, smooth, low-convex and circular colonies and bacterial cells are Gram-negative rods of 0.7–1.1 x 1.0–2.5 µm in size and motile by peritrichous flagella. *Alcaligenes aquatilis* type strain is LMG 22996^T = CCUG 50924^T, which has a G+C ratio of the DNA of 56 mol% (Austin 2014; Van Trappen *et al.*, 2005).

Biomineralization of calcium carbonate various crystal polymorphs including calcite, the most stable polymorph; aragonite, the metastable polymorph; and vaterite have been reported to be produced by bacteria (Pedone and Folk 2010; Rodriguez-Navarro *et al.,* 2012). Bacterial calcium carbonate mineralization, family Alcaligenaceae included, is mostly produced through biologically induced mineralization mechanism (Dapurkar and Telang 2017; Daskalakis *et al.,* 2013), where bacteria have the highest surface area to volume ratio among microorganisms besides the prevalence of chemical groups that gives bacteria charged cell surfaces making their mineral-nucleation ability (Kawaguchi and Decho 2002).

Associated chemosynthetic bacteria have been described in seven Mollusca families: Mytilidae, Vesicomyidae, Solemyidae, Lucinidae, Nucinellidae and Montacutidae (Duperron *et al.,* 2013). The Mediterranean Sea has many mollusk bivalves of which *Lithophaga* spp. (Phylum: Mollusca, Class: Bivalvia, Order: Mytiloida, Family: Mytilidae) represent common bivalves in the Mediterranean Sea. Bivalve shell is composed mainly of calcium carbonate formed essentially by animal soft parts. Some associated bacteria, such as proteobacteria, could have a role in the formation of shell calcium carbonate polymorphs and others may participate in shell damage (Guidetti and Boero 2004; Mokady *et al.,* 1994; Ozsuer and Sunlu 2013; Peharda *et al.,* 2015). In shell mineralogy, mollusks use mainly two polymorphs of calcium carbonate, calcite and aragonite. Aragonite tends to transform into calcite, under the influence of diagenetic processes. Also, mollusks use transiently amorphous calcium carbonate and in exceptional circumstances (shell deformation) few of them use vaterite (Marin *et al.,* 2012).

The present work aims to isolate the *Lithophaga* sp. associated bacteria and to investigate their efficiency in calcium carbonate crystals formation in absence of any added nutrients avoiding metabolic pathways and to clue a prospective role in *Lithophaga* shell assembly.

MATERIALS AND METHODS

Isolation of Mediterranean Sea *Lithophaga* Associated Bacteria:

Samples of Mediterranean Sea *Lithophaga* bivalves were brought from Alexandria, Egypt. Freshly collected samples were soaked in sterile 0.85% NaCl overnight at 4°C to get rid of most superficially attached marine water bacteria, then, they had been shaken in a fresh sterile 0.85% NaCl under aseptic conditions to collect the closely attached animal bacteria. Then, the saline solution shake was streaked on nutrient agar plates and incubated at 30°C for 48 hours. After incubation, all colonies were unpigmented and had the same colonial characteristics. Two single colonies were streaked for purification on nutrient agar plates and incubated as previously mentioned. Pure cultures were kept as

glycerol stocks at -80°C freezer and also on Nutrient agar slants at 4°C for future use.

Growth and Motility:

Bacteria were plated on brain heart agar, MacConkey agar and semisolid nutrient agar (0.2% agar) supplemented with tetrazolium chloride (5 mL of a 1% filter sterile stock solution/L) to monitor bacterial motility. The ability of bacteria to grow on high salt concentration was tested on nutrient agar that is supplemented with 3% and 7.5% NaCl, also its growth on the broth of M9 mineral medium supplemented with 1% glucose and soluble starch was investigated.

Flagella Staining:

Cultures (18-24 h) of well-isolated colony grown on nutrient agar slants were used for flagella stain. 1.5 mL of sterile distilled water was added to the agar slant and left at 30 °C for 1h to allow bacteria to swim to water forming a suspension. Air-dried smears from that suspension were prepared and stained with the Ryu stain solution (Kodaka *et al.,* 1982). Slides were examined by a light microscope (1000x magnification).

Transmission and Scanning Electron Microscopy:

Drops of diluted bacterial suspensions negatively stained with 4 % phosphotungstic acid pH 7.0 on electron microscope grids, which are previously covered with carbon membranes following up standard procedures for TEM bacterial sample preparation. Samples were examined by transmission electron microscope (JEM 100CX11-EM). For SEM, young culture of bacteria was grown on nutrient broth as previously described; and collected by centrifugation at 3000 rpm for 3 minutes. Bacteria were, then, subjected to SEM analysis using JEOL (JSM 5400 Lv) scanning electron microscopy.

Genomic DNA Extraction and Purification:

Total genomic DNA was extracted from overnight cultures of a single colony for each isolate that grown aerobically as previously described. 1.0 mL of the overnight culture for each isolate was centrifuged at 5000 rpm for 1 min., after the removal of the culture medium; the bacterial pellets were washed by resuspending each of them in 1 mL TE buffer (100mM Tris, 10 mM EDTA) and repelleted as above. DNA minipreps were prepared using Promega Wizard® genomic DNA purification kit (New York, USA) according to the manufacturer's instructions. The purified DNA was kept at -20 °C for future use.

PCR Amplification 16S rRNA gene:

The 16S rRNA gene was amplified from the genomic DNA using the 16S rRNA gene cloning universal primers synthesized by Clontech (Heidelberg, Germany). The nucleotide sequence of the forward primer (27F) is 5`-AGAGTTTGATCMTGGCTCAG-3` and the reverse primer (1492R) is 5`-TACGGYTACCTTGTTACGACTT-3` (Heuer *et al.,* 1997). Amplification was performed in 50 µL reaction volume containing 25 µL of KAPA2G fast ready mix (2X) master mix (Boston, Massachusetts, USA), 10 pMol of each primer, 1 µL of the total genomic DNA (1 ng/µL) and 21 µL of nuclease-free water. Amplification was carried out in thermocycler (ThermoHybaid-SPRT001), after an initial denaturation for 5 min at 95°C, 35 cycles were completed, each consisting of 45 sec at 94°C, 45 sec at 61°C annealing temperature, and extension for 120 sec at 72°C. A final extension step of 10 min at 72°C was applied. PCR product was stored at 4°C. The PCR product for each isolate was purified using GeneMark kit (GM Biolabs Co., Ltd., Taichung, Taiwan), eluted at 25 µL of Elution buffer. 1 µL was analyzed using 1% agarose submarine gel electrophoresis.

DNA Fragments Sequencing and Data Analysis:

PCR product of 16S rRNA gene fragments was sequenced by an automated ABI-3730xl model sequencer (Applied Biosystems, CA, USA) according to instructions of the manufacturer. Sequencing data were assembled using Serial Cloner (Version 2.6) program and compared with 16S rRNA gene sequences for bacteria

currently available in the GenBank database by BLAST homology search (www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments with 16S rRNA gene homologous sequences retrieved from GenBank were performed using ClustalW with default parameters (Higgins et al. 1994); genetic distances were estimated depending on Kimura 2-parameter model (Kimura 1980), and a phylogenetic tree was constructed by Neighbor-Joining method (Saitou and Nei 1987) using MEGA 6.0 software (Tamura *et al.,* 2013).

Phenotypic and Biochemical Characterization:

Acid formation from carbohydrates under aerobic conditions was determined using API 50 CH system, which is a standardized system, consisting of 50 biochemical tests for the study of microbial carbohydrate metabolism according to the manufacturer's instructions (Biomerieux, Marcy l' Etoile, France). A suspension of pure bacterial culture with a turbidity equivalent to "2" McFarland in API 50 CHB/E medium was used for API50 CH strips inoculation. To investigate other biochemical features, API 20 E strips (Biomerieux, Marcy l' Etoile, France) were used. Suspension for inoculation of the API 20 E strips was prepared in API NaCl 0.85 % with turbidity equivalent to "2" McFarland. Strips were moistened and inoculated according to the manufacturer's instructions. API 20 E inoculated strips were incubated at 30 °C for 24 hours. In case of API 50 CH, inoculated strips were incubated for 24 and 48 hours. For API 50 CH, a positive test corresponds to acidification was revealed by the turnover phenol red indicator contained in the medium from red to yellow. For the esculin test (tube no. 25), a color change from red to BLACK is recorded as positive. Nitrate reduction test was performed separately in nitrate broth medium incubated at 30 °C for 48h. Other enzymes such as cytochrome oxidase and catalase were also tested.

Calcium Carbonate Polymorphs Precipitation:

To explore the quality of the isolated *Lithophaga* associated bacteria in the biological precipitation of calcium carbonate into crystals, a pure bacterial culture was used to inoculate a 20 mL nutrient broth medium in a 100 mL conical flask. The flask was incubated at 30 °C and a rotation rate of 150 rpm for 24 h. 0.5 mL from an overnight culture was centrifuged at 7000 rpm for 3 min to precipitate the bacterial cells. The pellet was washed by resuspending in 1mL of sterile distilled water and repelletted as described above. The cell pellet was resuspended in 0.5 mL of 1M solution of calcium chloride dihydrate ($CaCl_2.2H_2O$) and left at room temperature for 5 minutes to give the chance for all negatively charged groups on the bacterial cell surface to bind calcium ions. The bacterial suspension was added to a 19.5 mL sterile solution of 25 mM $NaHCO_3$ (in 100 mL conical flask). The final concentration of both $CaCl_2.2H_2O$ and $NaHCO_3$ was adjusted to have an equimolar of 25 mM. The conical flask was shaken at 150 rpm for proper mix and kept still at room temperature (15 - 25 °C). Separate sets of samples were collected at different sampling periods for microscopic examination (400x magnification).

Biocalcite SEM and X-ray Diffraction (XRD) Analysis:

To remove sodium chloride crystals, if formed, crystals were collected by centrifugation at 5000 rpm for 3 minutes; and resuspended in distilled water. Crystals were washed by strong vortexing and crystals were repelleted as above. The previous step was repeated once more before filtering the crystal suspension through a 0.45µm sterile filter. The membrane was dried at 60 °C for overnight. For X-ray diffraction measurements, crystals were mixed with an equal amount of finely crushed clean glass to increase the sample volume. Then, crystals were subjected to SEM analysis using JEOL (JSM 5400 Lv) to determine their morphology.

Dry crystals were examined using JEOL X-ray diffractometer model JSX-60PA of the central laboratory of Minia University,

Minia, Egypt. The analysis was run with CuKα radiation, Ni filtered (λ= 1.54184A°) The supply voltage of the X-ray tube was set at 35 kV and 15mA, under a normal scanning speed of 0.5 minutes, within the range of 2θ= 4-100. The crystalline phases were identified using the International Centre for Diffraction Data (ICDD) database (Joint Committee on Powder Diffraction Standards, JCPDS).

Nucleotide Sequence Accession Number:

The GenBank accession number of the 16S ribosomal RNA gene sequence of *Alcaligenes aquatilis* MAG 1 is KX 181570 and deposited in the Egypt Microbial Culture Collection (EMCC) under deposit no. EMCC 2288.

RESULTS

Morphological Characteristics of Isolated Bacteria:

All colonies, recovered after isolation, were identical in colonial characteristics denoting the similarity of their colony-forming units. Cells were singles, Gram-negative rods of about 0.7 - 0.9W x 1.2 -1.5L μm, and motile via flagella as shown by light microscopy, TEM and SEM micrographs (Fig 1). Bacteria from non-pigmented slightly raised and smooth colonies on nutrient agar plates, more profuse growth of raised colonies on brain heart agar and pale white (lactose non-fermentative) semitransparent colonies on MacConkey agar plates.

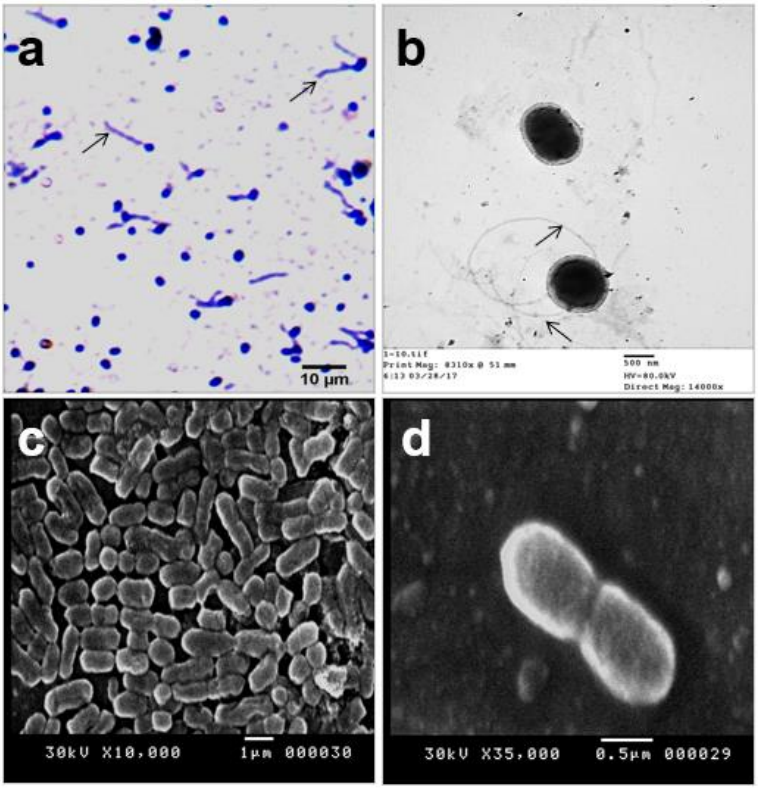

Fig. 1 Photomicrographs of *Alcaligenes aquatilis* MAG 1 grown on nutrient agar. a, bacterial flagella stained with Ryu stain, arrows, (Light microscopy, 1000x). b, TEM micrograph indicating rod shaped bacteria with their flagella, arrows; and c and d, SEM micrographs showing bacteria of about 0.7W x 1.2L μm, in dimensions (Flagella were sheared off because of spin down).

16S rRNA Gene-based Identification of Isolated Bacteria:

Fragments of 1449 bp were cloned from bacterial isolates taken from *Lithophaga* sp. using 27F and 1492R 16S rDNA universal primers. Identical sequences of amplified fragments were recorded except at position 305, where most clones recorded "C" nucleotide at position 305 and some others had "T" at that position. However,

there were no colonial characteristics or physiological differences noticed for both types of isolates. Nucleotides composition of cloned 16S rRNA gene sequence recorded "T" of 20.2%, "C" of 22.8%, "A" of 26%, "G" of 31% and calculated "G~C" of 53.8%. Isolates cloned 16S rRNA gene sequencing data were reviewed by BLAST homology search for similarity with other prokaryotic taxa, where no complete identity with GenBank records was confirmed. 16S rRNA gene sequence of isolates showed close similarities with records of other 6 proteobacteria strains with values of E=0.0 and 97-98% sequences covered. The strain showed the highest similarity of 99 % with *Alcaligenes aquatilis* strain LMG 22996 and the lowest similarity of 95% with *Paenalcaligenes suwonensis* strain ABC02-12 (Table 1).

Multiple sequence alignments of the 16S rRNA gene nucleotide sequence of isolated bacteria with the nucleotide sequences of the other closely related proteobacteria strains reference homologs provided many identical nucleotide sequences located between 28 and 1427 base positions of the isolated strain 16S rDNA. Moreover, there are distinct conserved sequences among isolated strain 16S rRNA gene and those of *Alcaligenes aquatilis* strain LMG 22996^T, located between 1429 and 1449 base positions of isolate sequencing, giving an evidence, in addition to the isolate host and the host habitat, that the present isolate is a member of *Alcaligenes aquatilis* strains, but not the same strain LMG 22996^T.

Table 1: Isolated strain 16S rDNA nucleotide sequences data and the close related reference homologs (E-value = 0.0).

Strain	16S rDNA GenBank Accession Numbers	Sequence length (bp)	Sequence covered (%)	Identity scores (%)
Alcaligenes aquatilis strain MAG 1	gb| KX181570.1	1449	Itself	Itself
Alcaligenes aquatilis strain LMG 22996	ref| NR_104977.1	1491	98	99
Alcaligenes faecalis strain NBRC 13111	ref| NR_113606.1	1462	98	98
Alcaligenes faecalis strain IAM 12369	ref| NR_043445.1	1470	98	98
Alcaligenes faecalis parafaecalis strain G	ref| NR_025357.1	1414	97	99
Alcaligenes faecalis phenolicus strain J	ref| NR_042830.1	1503	97	96
Paenalcaligenes suwonensis strain ABC02-12	ref| NR_133804.1	1439	97	95

Phylogenetic tree (Fig 2) constructed between the pairwise of isolated strain sequences of 16S rDNA and the closely similar homologs depending on the number of base substitutions per site (1378 in total) showed varied genetic distances among them. The isolated strain closest strain was found to be *Alcaligenes aquatilis* strain LMG 22996^T with the least distance in between. The cluster of *Alcaligenes faecalis* strain NBRC 13111, *Alcaligenes faecalis* strain IAM 12369 and *Alcaligenes faecalis phenolicus* strain J showed distances of 0.007, 0.011 and 0.020, respectively. The most distant (0.041) species from the isolated strain was *Paenalcaligenes suwonensis* strain ABC02-12, which is considered as an out-group.

Accordingly, in addition to the morphological and biochemical characteristics, the isolated strain was given the name *Alcaligenes aquatilis* strain MAG 1 and 16S rDNA sequence had been submitted to the GenBank databases as *Alcaligenes aquatilis* MAG 1 16S rRNA gene, partial sequence, under accession number gb|KX181570.1.

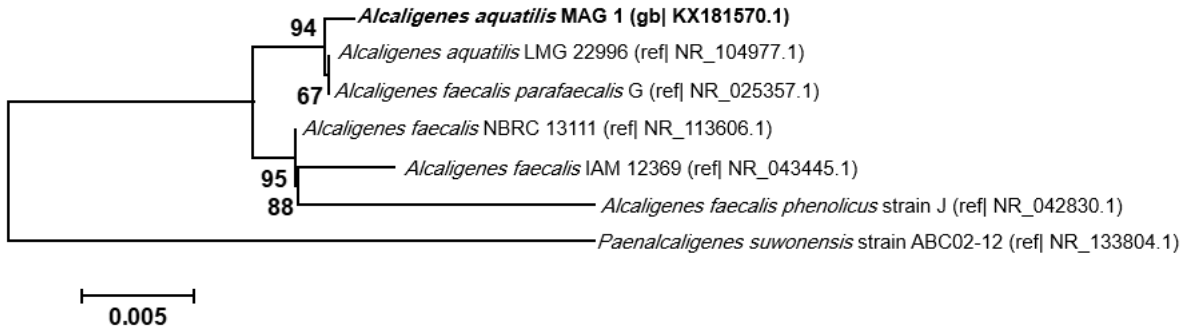

Fig. 2 Neighbor-Joining phylogenetic tree constructed for *Alcaligenes aquatilis* strain MAG 1 and the close related 6 homologs bacterial strains. Distances used were computed using the Kimura 2-parameter model. All positions containing gaps and missing data were eliminated. There were a total of 1378 positions in the final dataset. Numbers at nodes refer to bootstrap values for 1000 replicates and scale bar denotes 0.05% nucleotide divergence.

Phenotypic and Biochemical Profile of the Isolated Bacterial Strain:

The ability of the isolated *Alcaligenes aquatilis* strain MAG 1 to form acid from different carbohydrates under aerobic conditions, as tested using the API 50 CH strips (a whole list of the carbohydrates used is shown in Table (2), revealed that no acids were formed except for D-mannose (sugar no. 13) and amygdalin (no. 23). D-mannose exhibited weak acidification in the first 24 h then turned to be stronger after 48 h; and amygdalin, which is a poisonous cyanogenic glycoside, exhibited weak fermentation after 48 h. Isolated *Alcaligenes aquatilis* strain MAG 1 showed negative growth in M9 broth supplemented with 1% starch as the sole carbon source.

Results of reactions tested in API 20 E system are indicated in Table 3. While it is recommended by the manufacturer to record only the first 12 tests in API 20 E, as the rest of the tests represent carbohydrates that were already tested in API 50 CH, the obtained data on filling API 20 E whole strips revealed additional characteristics, where sugars such as glucose, L-rhamnose and L-arabinose indicated acid formation. Also, acid formation from D- melibiose was formed. Positive bacterial growth in M9 broth supplemented with 1% glucose was observed which supports positive glucose acidification obtained from API 20 E test strip. Cytochrome oxidase and catalase tests were positive. Moreover, *Alcaligenes aquatilis* strain MAG 1 recorded negative results for nitrate reduction, urea degradation, sulphate reduction (H_2S production) and tryptophan deaminase (TDA) (Table 3).

Carbonic anhydrase activity was abolished by avoiding the addition of Zn^{2+} to the used media or crystallization solution, moreover, calcium chloride was used as the sole calcium source.

Table 2: Carbohydrates forming acid tested in *Alcaligenes aquatilis* strain MAG 1 by API 50 CH test strips

Strip 1		Strip 2		Strip 3		Strip 4		Strip 5	
0	Control	10	Galactose	20	ά-methyl-D-mannoside	30	Melibiose	40	D-turanose
1	Glycerol	11	D-glucose	21	ά-methyl-D- glucoside	31	Sucrose	41	D-lyxose
2	Erythritol	12	D-fructose	22	N-acetyl-glucosamine	32	Trehalose	42	D- tagatose
3	D-arabinose	13	D-mannose[a]	23	Amygdalin[a]	33	Inulin	43	D-fucose
4	L- arabinose	14	L-sorbose	24	Arbutin	34	Melezitose	44	L-fucose
5	Ribose	15	Rhamnose	25	Esculin	35	D-raffinose	45	D-arabitol
6	D- xylose	16	Dulsitol	26	Salicin	36	Starch	46	L- arabitol
7	L- xylose	17	Inositol	27	Cellobiose	37	Glycogen	47	Gluconate
8	Adonitol	18	Mannitol	28	Maltose	38	Xylitol	48	2-keto-gluconate
9	β-methyl-xyloside	19	Sorbitol	29	Lactose	39	β-gentiobiose	49	5-keto-gluconate

[a] denotes acid formation.

Table 3: Biochemical characteristics of *Alcaligenes aquatilis* strain MAG 1 analyzed by API 20 E test strips.

	API 20 E tested reactions	Results
ONPG	Beta-galactosidase	-
ADH	Arginine dihydrolase	-
LDC	Lysine decarboxylase	-
ODC	Ornithine decarboxylase	-
CIT	Citrate utilization	-
H_2S	H_2S production	-
URE	Urea hydrolysis	-
TDA	Tryptophane deaminase	-
IND	Indole production	-
VP	Acetoin production (Voges Proskauer)	-
GEL	Gelatinase production	-
GLU	Acid production from D-glucose	+
Nit	Nitrate reduction	-
MAN	Acid production from D-mannitol	-
INO	Acid production from inositol	-
SOR	Acid production from D-sorbitol	-
RHA	Acid production from L-rhamnose	+
SAC	Acid production from D-sucrose	-
MEL	Acid production from D-melibiose	+
AMY	Acid production from amygdalin	+
ARA	Acid production from L-arabinose	+

Calcium Carbonate Crystals Formation by Isolated Bacterial Cells:

Isolated *Alcaligenes aquatilis* strain MAG 1 bacteria induced, in absence of any added nutrients, the formation of calcium carbonate crystal polymorphs. The yield of calcium carbonate precipitate was approximately 700 mg/20 mL of crystallization solution in a week (four replicates used for each experiment). Microscopically, monitoring of bacterially formed calcium carbonate crystals showed both multiple-needles/plate-shaped aragonite and rhombohedral calcite polymorphs with aragonite/calcite content ratio changed over time. Both aragonite and calcite were precipitated with predominant aragonite in the first three days. From the fourth day,

calcite dominated in a uniformly elapsed time manner to be the only documented well-designed rhombohedra of different sizes by the seventh day and afterward. Bacterial calcium carbonate different polymorphs precipitation were taken place in the presence of *Alcaligenes aquatilis* strain MAG 1bacteria compared with amorphous precipitation obtained in control sample where the addition of bacteria was absent (Fig. 3).

At the end of fifteen days, X-ray diffraction is used as a sensitive instrument to identify the crystalline phases of harvested calcium carbonate crystals precipitated by *Alcaligenes aquatilis* strain MAG 1 strain. XRD spectrum showed relative intense peaks at 2θ values of 29.40, 39.80, 43.50, 47.80, 48.80, and 57.20, respectively, with d(A)-values of 3.04, 2.27, 2.08, 1.90, 1.86, and 1.59, respectively. The highest intensity peak was at a 2θ angle of a value 29.40° corresponding to (hkl) index of 104. Therefore, attained XRD data (PDF No. 72-1652) confirmed the crystalline characteristics for calcite polymorph (Fig 4).

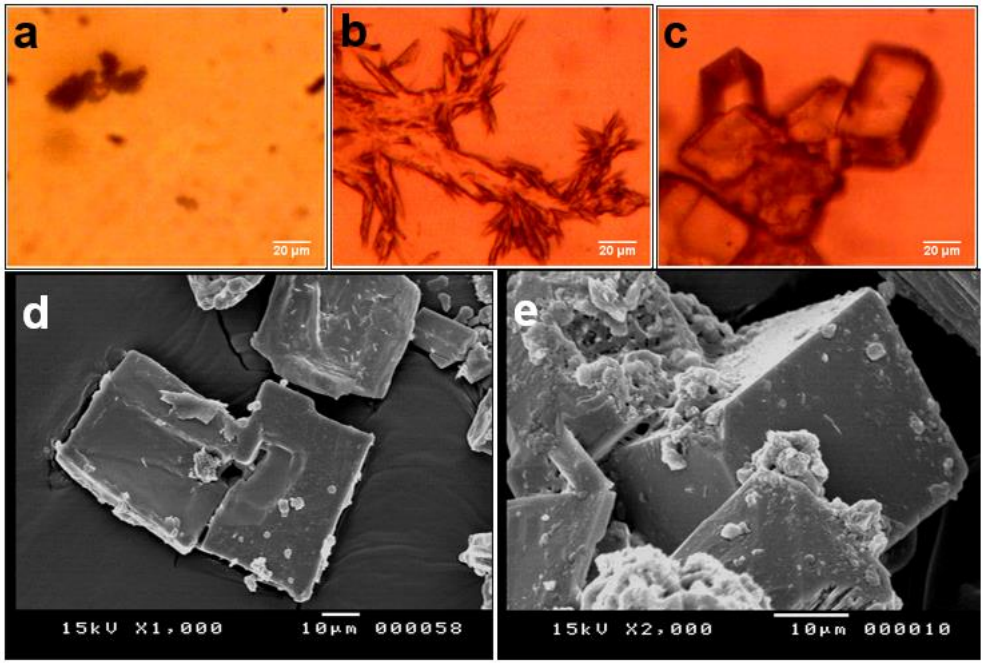

Fig. 3 Calcium carbonate crystal polymorphs produced by *Alcaligenes aquatilis* strain MAG 1. Calcium carbonate was precipitated at 25 mM $NaHCO_3$ and 25 mM $CaCl_2$. $2H_2O$. a, an amorphous precipitate in absence of bacteria (400x). In presence of bacteria, well organized crystals are formed: b, light microscope image of aragonite crystals (400x), c, light microscope image of calcite crystals (400x), d, SEM micrograph showing developing calcite crystals and associated bacterial imprints and e, SEM micrograph showing calcite crystals of variable sizes.

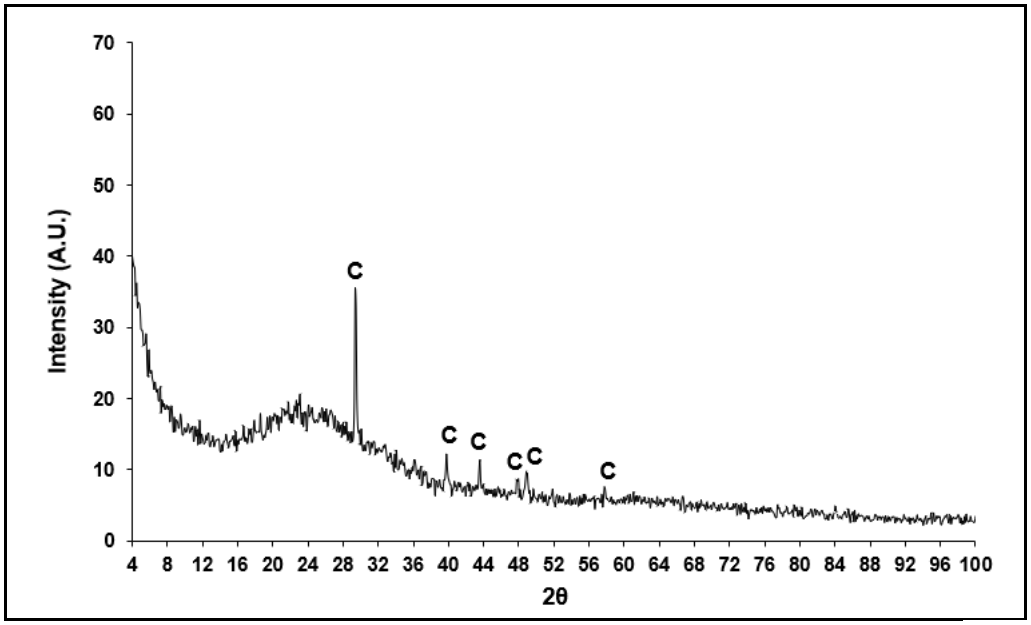

Fig. 4 XRD curve of calcite crystals (C= calcite, $CaCO_3$) precipitated by *Alcaligenes aquatilis* strain MAG 1 (PDF No. 72-1652). Crystals were collected by centrifugation at 5000 rpm for 3 minutes; washed and repelleted. Then, crystals suspension was filtered through a 0.45µm sterile filter, and crystals were dried at 60 °C for overnight.

DISCUSSION

Bacteria are, ecologically, important participants in the carbon cycle via calcium carbonate precipitation, as this process sequesters a large amount of carbon dioxide (Kim *et al.,* 2016; Vahabi *et al.,* 2015; Zhang *et al.,* 2018). Bacterial calcium carbonate biomineralization is influenced by four key factors: calcium concentration, pH, the concentration of dissolved inorganic carbon, and nucleation site availability (Muynck *et al.,* 2010). Bacteria can alter the first three factors, mostly by increase the alkalinity of the surrounding environment and the fourth by affording nucleation sites (Hammes and Verstraete 2002). Bacterially induced $CaCO_3$ precipitation was suggested for mechanisms involving degradation of urea, photosynthesis, ammonification, denitrification, and/or sulphate reduction (Castanier *et al.,* 2000; Wang *et al.,* 2018). Isolated *Alcaligenes aquatilis* strain MAG 1 recorded negative results for nitrate reduction, urea degradation, sulphate reduction (H_2S production) and tryptophan deaminase (TDA). Therefore, *Alcaligenes aquatilis* strain MAG 1 worked as nucleation sites for crystalline calcium carbonate precipitation following the theory of Urrutia Mera *et al.* (1992). This theory proposed: in general, the bacterial cell does not control the mineral precipitation on its surface; it happens because of the physico-chemistry of the bacterial cell surface and the chemistry of its surrounding environment. Furthermore, mineral formation on the cell surface of actively metabolizing bacteria is inhibited because its plasma membrane is highly energized and the cell wall is flooded with protons that compete with metal cations (Hammes and Verstraete 2002; Silva-Castro *et al.,* 2015; Urrutia Mera *et al.,* 1992).

Moreover, bacteria precipitate calcium carbonate at a faster rate than chemical approaches, and an increase in bacterial cell numbers increases available nucleation sites for precipitation (Stocks-Fischer *et al.,* 1999). In addition, it was reported that supplementing bacterial growth media with peptone as a carbon source inhibits carbonate precipitation (Silva-Castro *et al.,* 2015), while Zn^{2+} addition stimulates carbonic anhydrase (a zinc-containing enzyme that catalyzes the reversible conversion of CO_2 to bicarbonate) dependent biological precipitation of carbonate (Achal

and Pan 2011). Present study results were consistent in the formation of amorphous calcium carbonate in absence of bacteria and well-organized crystal polymorphs were formed in presence of *Alcaligenes aquatilis* strain MAG 1bacterial cells. Unlike other Alcaligenaceae members pathways of calcium carbonate formation (Dapurkar and Telang 2017; Daskalakis *et al.,* 2013), *Alcaligenes aquatilis* strain MAG 1 precipitated calcium carbonate in absence of any added organic nutrients or acid-forming carbohydrates or any other nutrients that could support any kind of metabolic pathway. Furthermore, this study banned the use of nutrients and just kept the basic constituents of calcium carbonate precipitation, sodium bicarbonate ($NaHCO_3$) as a source of CO_3^{2-} ions, calcium chloride ($CaCl_2$. $2H_2O$) as a source of Ca^{2+} ions and the isolated bacterial strain cells as nucleation sites, as well, Zn^{2+} was dismissed from crystallization solution, which ensures its role as nucleation sites for calcium carbonate precipitation.

Biomineralization of calcium carbonate is a natural process occurred in terrestrial, marine and freshwater habitats especially sands, coral reefs and shells (Kim *et al.* 2016). Crystalline calcium carbonate is of three polymorphs: calcite, aragonite and vaterite, where bacteria could precipitate calcite as well as aragonite and rarely vaterite, of which induced calcite dominates other polymorphs (Wei *et al.,* 2015). Aragonite is a metastable polymorph with high mechanical strength and is considered to be suitable for varied tissue engineering applications (Hoque *et al.,* 2013). In shell formation, mollusks use mainly two polymorphs of calcium carbonate, aragonite and calcite (Marin *et al.,* 2012). Calcium carbonate formation in Mollusca is counted mainly for the carbonic anhydrase pathway, which is evidenced by a triggered carbonic anhydrase expression in the mantle tissues of oyster (Bivalvia) hypercapnia, which may denote changes in carbonate chemistry of oyster extrapallial fluid (Beniash *et al.,* 2010). In current results, *Alcaligenes aquatilis* strain MAG 1 associated with Mediterranean Sea *Lithophaga* sp. (Bivalvia) precipitated both aragonite and calcite calcium carbonate polymorphs away from the carbonic anhydrase pathway, which may proclaim for a possible complementary role in shell formation and ornamentation.

Calcite is a more stable polymorph compared to aragonite, thus, it could be used for valuable applications such as bioremediation of deteriorated stones, subsurface fractures and permeability in porous materials, where the advantage of microbially induced calcite over cement-based sealants was testified (Jimenez-Lopez et al. 2007; Phillips et al. 2016). Jimenez-Lopez *et al.* (2007) recommended investigations to focus on the retention of nutrients and metabolic products in treated stone owing to the chemical reactions between the stone minerals and some by-products originating from the metabolism of viable heterotrophic bacteria, and the formation of stained patches as a result of the growth of air-borne fungi related to the presence of organic nutrients (Tiano *et al.,* 1999). *Alcaligenes aquatilis* strain MAG 1 precipitated, in absence of any added organic nutrients or acid-forming carbohydrates, both aragonite and calcite calcium carbonate polymorphs, and exhibiting an outstanding tendency towards forming well organized rhombohedral calcite crystals. Therefore, the way of calcium carbonate precipitation by *Alcaligenes aquatilis* strain MAG 1 might be convenient for bioremediation of deteriorated stones and similar conservational purposes.

In conclusion, morphological, physicochemical, and 16S ribosomal RNA gene outcomes ensure that isolated strain associated with Mediterranean Sea *Lithophaga* sp. is a distinctive strain of *Alcaligenes aquatilis* species assigned as *Alcaligenes aquatilis* strain MAG 1. This strain outstanding ability to form aragonite and calcite polymorphs, in sterile water amended with just $CaCl_2$. $2H_2O$ and $NaHCO_3$, maximize their role as excellent nucleation sites. These findings create a

paradigm for future studies on a potential complementary role in bivalves shell assembly, a role affecting the ratio of soluble to insoluble calcium carbonate in a marine environment and being a way for CO_2 sequestering, moreover, *Alcaligenes aquatilis* strain MAG 1 could be suitable for bioremediation of deteriorated stones and similarly conservational purposes.

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