Microbial nucleation of Mg-rich dolomite in exopolymeric substances (EPS) under anoxic modern seawater salinity: New insight into an old enigma

Data Repository

Microorganism

Desulfobulbus mediterraneus strain 86FS1 is a gram-negative, chemoorganotrophic and strictly anaerobic bacterium (Sass et al., 2002). Growth temperature ranges from 10 to 30°C with an optimum at 25°C. The strain was isolated from deep-sea sediment (1268 m water depth, bottom water temperature 15°C) off the NE Spanish coast (Sass et al., 2002).

Culture medium

The experiments were conducted using an anoxic synthetic seawater medium (Medium 196, German Collection of Microorganisms and Cell Cultures) using Na-lactate as organic substrate. The concentration of magnesium and calcium were adjusted to 15.2 and 3 mmol l^{-1}, representing a modern seawater Mg/Ca molar ratio of 5. Medium headspace was gassed with 90% N_2, 10% CO_2 to remove oxygen. 200 µl resazurin solution was added as an indicator for oxygen contamination. After sterilizing under 90% N_2, 10% CO_2 atmosphere at 121°C for 20 minutes, the pH was adjusted to 7.4 with 0.1 M NaOH. pH was measured using a Schott Lab 850 pH meter, which was calibrated using NIST standards for pH 4.01, 6.87 and 9.18. The medium was transferred anaerobically into 50 ml serum vials with 90% N_2, 10% CO_2 headspace. The medium was kept in the dark until further use. Light microscopy observations did not show precipitation of carbonate material after several weeks.

Medium pH, alkalinity and sulfide concentration

During the 14 day incubation period pH changed from 7.4 to 7.8. Corresponding total alkalinity was measured by sample aliquot titration with 0.01 molar HCl and methyl

red/methylene blue indicator under N_2 ventilation in a vessel after Pavlova. IAPSO standard seawater was used for calibration. During 14 day incubations total alkalinity changed from 59 to 80 mEq. Total sulfide concentration was determined photometrically at a wavelength of 480 nm (Cord-Ruwisch, 1985). Sulfide concentrations increased from 1.2 to 8 mmol l^{-1} during 14 day incubations. The initial and final carbonate alkalinity was calculated with the following formula (1):

$$[CA] = [TA] - [HS^-] - [OH^-] + [H^+] \quad (1)$$

Calculations were performed according to Zeebe (2007).

Growing biofilms on glass slides

3 ml of active *D. mediterraneus* culture was inoculated into a vial containing 50 ml of sterile, anoxic medium 196. The vial was transferred into a glove box and opened under 90% N_2, 10% CO_2 atmosphere. A sterilized microscopy glass slide was placed on the opening of the vial and fixed with tape. Subsequently, the vial was turned upside and stored in the dark. According to the resazurin indicator samples remained anoxic during the entire experiment. After 3 and 14 days glass slides were carefully removed. The apparent biofilm was rinsed carefully in 1x phosphate buffered saline (PBS), and excess liquid was removed. Control experiments with sterile medium did not show precipitates.

Confocal laser scanning microscopy (CLSM)

D. mediterraneus biofilms growth on glass slides were investigated with CLSM the same day the anoxic culture experiments were stopped. Lectin, a constituent of extracellular polymeric substances (EPS) was stained with wheat germ agglutinin (WGA) conjugated with ALEXA FUOR 488. To prepare a 1.0 mg/mL wheat germ agglutinin (WGA) conjugate stock solution 5.0 mg of lyophilized WGA conjugate were dissolved in 5.0 mL of PBS. The stock solution was diluted to 1.0 mg/mL by adding WGA conjugate working solution into Hank's

balanced salt solution (HBSS). 100 µl of labeling solution was applied to each cover slip covering adhering cells. Samples were stained for 20 minutes at 20°C in the dark. Subsequently, excess solution was removed and biofilms were carefully washed twice with PBS. Excess liquid was removed, and cells were counterstained with freshly thawed 4′,6-Diamidin-2-phenylindol (DAPI) working solution (1 µg ml^{-1}) for 15 minutes in the dark. Investigation of stained samples and control materials was carried out using a Leica TCS SP5. Wheat germ agglutinin (WGA) ALEXA FLUOR 488 was excitated by the 495 nm laser line and emission was detected at 519 nm. DAPI was excited at 358 and emission recorded at 461 nm. Both fluorescence signals were recorded using a sequential mode. The 488 nm laser line was also used for recording the reflected light channel (469 - 498 nm). Image analyses were carried out using Imaris software version 6.

Scanning electron microscopy (SEM)

SEM imaging of *D. mediterraneus* biofilms were carried out using a Zeiss supra 50 VP equipped with an energy dispersive X-ray spectrometer (EDAX). Sample preparation started with careful initial washing with PBS for 5 minutes. For fixation samples were immersed in 2.5% paraformaldehyde for 15 minutes. After washing in PBS, 2% osmium tetroxide in PBS was applied for 10 minutes. Samples were then carefully washed 3 times in purified water. Glass slides were then broken into smaller pieces and dried in a series of ethanol solutions. Subsequently, critical point drying removed remaining water. Biofilm bearing glass pieces were mounted on aluminum stubs using conductive carbon cement. Samples were stored in a desiccator overnight. Before imaging, samples were sputter coated with an 8 nm gold layer. Images were obtained with secondary electron detector applying an accelerating voltage of 4 kV with a working distance of 8.2 mm. For EDX analysis the acceleration voltage was increased to 5.6 kV (Figure DR1).

Scanning Electron Microscopy with Cryogenic Preparation System (Cryo-SEM)

Cryo-SEM observations if *D. mediterraneus* biofilms were carried out with a Hitachi 4800s scanning electron microscope, equipped with a GARTAN GB cryo-unit. Biofilms on glass slides were cut and mounted on aluminum stubs with double sided sticking tape. Cryo-fixation of the samples was carried out by immersing the stub carefully into liquid nitrogen. Samples were then introduced into the cooled SEM. Images were obtained using a secondary electron detector, operated at acceleration voltage of 5 kV and 13.7 mm working distance.

Electron microprobe analysis (EMP)

For EMP analyses, *D. mediterraneus* biofilms were grown on sterile glass slides for 10 days, as described above. Elemental analysis of the crystals in the biofilms was performed using a Jeol JXA-8200 WD/ED combined with a microanalyzer Superprobe. Prior to the analysis, samples were carefully washed 3 times in purified water and dried overnight. Before analyzing, samples were coated with a carbon layer of 10 nm thick. The Mg/Ca ratios of the crystal precipitates, listed in Table 2, represent the average value of 42 measurements of individual crystals within the biofilm.

X-ray diffraction analysis (XRD)

For mineralogy analysis of the crystal precipitates, 3 x 30 ml of active *D. mediterraneus* culture was inoculated into 3 x l sterile medium. Biofilm growth, pH, total alkalinity and sulfide concentration were monitored weekly. After 23 days, sulfide concentration exceeded 11 mmol l^{-1} and biofilm growth was stopped. The clear medium was carefully removed. The biofilm was resuspended with the last remaining 10 ml medium and transferred into a centrifuge tube. The suspension was centrifuged with 4500x g for 10 minutes at 4°C. The clear supernatant was discarded. The remaining pellet was resuspended in 50 ml of purified water with a pH of 8-9 to avoid dissolution of carbonate material. After resuspension, the

105 sample was centrifuged again. The clear supernatant was discarded and replaced by 50 ml of
106 1% NaClO to bleach organic material. The sample was left to react overnight followed by
107 centrifuging and replacement of NaClO. In total, bleaching was repeated three times. The
108 remaining material was washed 3 times in purified water and dried at 37°C. For XRD
109 analyses, the material was powdered and placed on a silicon disc. Analyses were run from 0°
110 to 60° 2-theta angle (Figure DR2) on a Philips X-ray diffractometer PW 1710 with
111 monochromatic CuKα. The spectra showed additional peaks to those identified for dolomite.
112 As great care was taken during the purification to avoid crystal dissolution due to extensive
113 washing, additional peaks might originate from remnant medium components.

114

115 **Methods for element concentration and Ca isotope ratio measurements**

116 30 ml of active *D. mediterraneus* culture were inoculated into a Duran glass bottle
117 with 1 l of sterile 196 medium with adjusted Mg/Ca ratio (see above). After 23 days of
118 incubation, biofilm growth was stopped. The remaining medium was transferred step wise
119 into acid-washed 50 ml centrifuge vials and centrifuged at 4500x g for 10 minutes. The clear
120 supernatant was transferred into acid washed 50 ml centrifuge vials. The remaining pellet,
121 containing floating organic material was washed four times in purified water with a pH of 8-9
122 (adjusted with NH_4^+). The supernants of the washing steps were transferred separately into 50
123 ml acid washed centrifuge vials. The biofilm at the bottom was resuspended in 10 ml
124 centrifuged medium and transferred into a 50 ml acid washed centrifuge vial. The sample was
125 centrifuged at 4000 RPM for 10 minutes and transferred into a 15 ml Teflon beaker. 8 ml of
126 ultra pure NaClO (1%) bleaching solution was added and left to react for 12 hours.
127 Subsequently, the sample was centrifuged at 4000 RPM for 10 minutes and NaClO was
128 exchanged. This procedure was repeated 3 times. After bleaching, the sample was washed 4
129 times in purified water with adjusted pH (see above) and the supernatants of individual

washing steps were transferred separately into 50 ml centrifuge vials. Finally, the remaining pellet was transferred into a 6 ml teflon beaker.

All samples were dried at 95°C for at least 12 hrs. Organic bearing samples and blanks were additionally resuspended in 1 ml purified water and 1 ml HNO_3 8N and dried at 95°C, followed by resuspension in 1 ml HNO_3 and 20 µl $HCLO_4$ and left to react for 3 hours at 95°C. All applied HCl and HNO_3 based acids refer to 2-step Teflon cascade still (pico trace) purification of initially per analysis quality. After drying at 180°C 0.75 ml 8N HNO_3 and 0.25 ml H_2O_2 were added and left to react for at least 12 hrs, and subsequently dried at 80°C.

In order to prepare source solutions for identical aliquots for different analytical methods and reproducibility tests the samples were re-dissolved in 4ml of 2.25 HNO_3. Simultaneous determination of element concentration (Mg, Ca, Sr) and ratios by ICP-OES (VARIAN 720-ES) was carried out on 0.5 ml of the source solution. Aliquots of up to 3000 ng Ca equivalent were taken for Ca-isotope preparation and mixed with a $^{43}Ca/^{48}Ca$ double spike. Spiked samples were dried at 95°C, resuspended in 2.2 N HNO_3, and Ca was separated using 600 µl columns (biorad) with MCI-Gel 75-150 µm. Whole procedure blanks were spiked at the beginning and revealed maximum amounts of 197 ng for multi-step bleach runs (dolomite crystals) and 30 to 60 ng for routine column chemistry runs (single step total dissolution).

The calcium isotope ratios of dolomite crystals, aqueous Ca of initial and remaining medium, and Ca remaining in the biological component were measured with a Finnigan Triton TI (Thermal Ionization Mass Spectrometer) following the method described in Heuser et al., (2002) and Böhm et al. (2006). Prior to filament loading samples were dried down and transferred into chloride form by evaporation in 2.2 N HCl. After uptake with loading solution, aliquots of about 300 ng were loaded with TaCl5 activator in a sandwich technique on a zone-refined Re filament.

155 Measurements were made on single filaments at temperatures around 1470 °C and a
156 typical ^{40}Ca signal intensity of 9-10 V. Data acquisition was performed in dynamic mode. The
157 double-spike correction was carried out with the algorithm used by Heuser et al. (2002).The
158 isotope values of Ca are reported as $ä^{44/40}Ca$ (‰) values relative to the NIST standard
159 SRM915a, where $ä^{44/40}Ca = [(^{44}Ca/^{40}Ca)_{sample}/(^{44}Ca/^{40}Ca)_{SRM915\ a} - 1] \times 1000$. For each sample
160 at least 3 independent filaments were measured in one session and individually normalized to
161 the average $^{44}Ca/^{40}Ca$ of four SRM915a analyses, distributed throughout the same turret. The
162 precision is expressed as two times the standard error of the average (2SEM = 2r/n0.5). Some
163 samples were repeated in different sessions during the study. A representative external
164 uncertainty for the method of at least 0.11‰ is reflected in the 2 SE for NIST-SRM-915a
165 measurements throughout the study. The results are summarized in Table DR1.
166
167 Notes: (1) Some samples were reproduced better within their single session run than
168 the standard used for normalization regarded throughout the whole study.
169 (2) The last two washing supernatant liquids for the organic component had an
170 isotopic fractionation similar to the corresponding total dissolved sample. This result shows
171 that the intermediate fractionation of the organic material is not due to artifacts during sample
172 preparation, but represents the original Ca isotopic fractionation of this component.
173 (3) The isotope signature of the whole procedure blank was heavier than all
174 samples. Therefore, its only potential influence was a shift towards apparent heavier
175 signatures, which would be mostly expressed in the smallest and multistep bleached samples,
176 the dolomite crystals. Consequently, after blank correction original crystal isotope signature
177 pointed to even lighter values. The fractionation step between the divalent cation reservoir,
178 attached to the organic phase, and the dolomite precipitate must be assumed to be even larger
179 than reflected in these initial measurements.

Figure caption:

Figure DR1: Scanning electron microscopy images of Mg-rich dolomite in *D. mediterraneus* biofilm. A: Spheroidal dolomite nanocrystals (~50-200 nm) embedded in EPS were visible after 3 days of biofilm growth. B: After 14 days, larger crystals were observed within the biofilm. EDX spot analysis indicates the relative abundance of Mg and Ca in the crystals (Crystal) with respect to the bacterium (Cell). The prominent peak for silicon (Si) is caused by the glass slide the biofilm was grown on. Note: Due to de-hydration during sample preparation the EPS was compressed and crystals might not be located in their original position.

Figure DR2: X-ray diffractograms of reference dolomite material and dolomite crystals formed in *D. mediterraneus* biofilms at 21˚C (sample SK0101, sample SK0120). Note: The presence of superstructure reflections (odd labeled hkl's) indicates that the rhombohedral carbonate is an ordered dolomite (Reeder, 1983).

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Figure DR1.

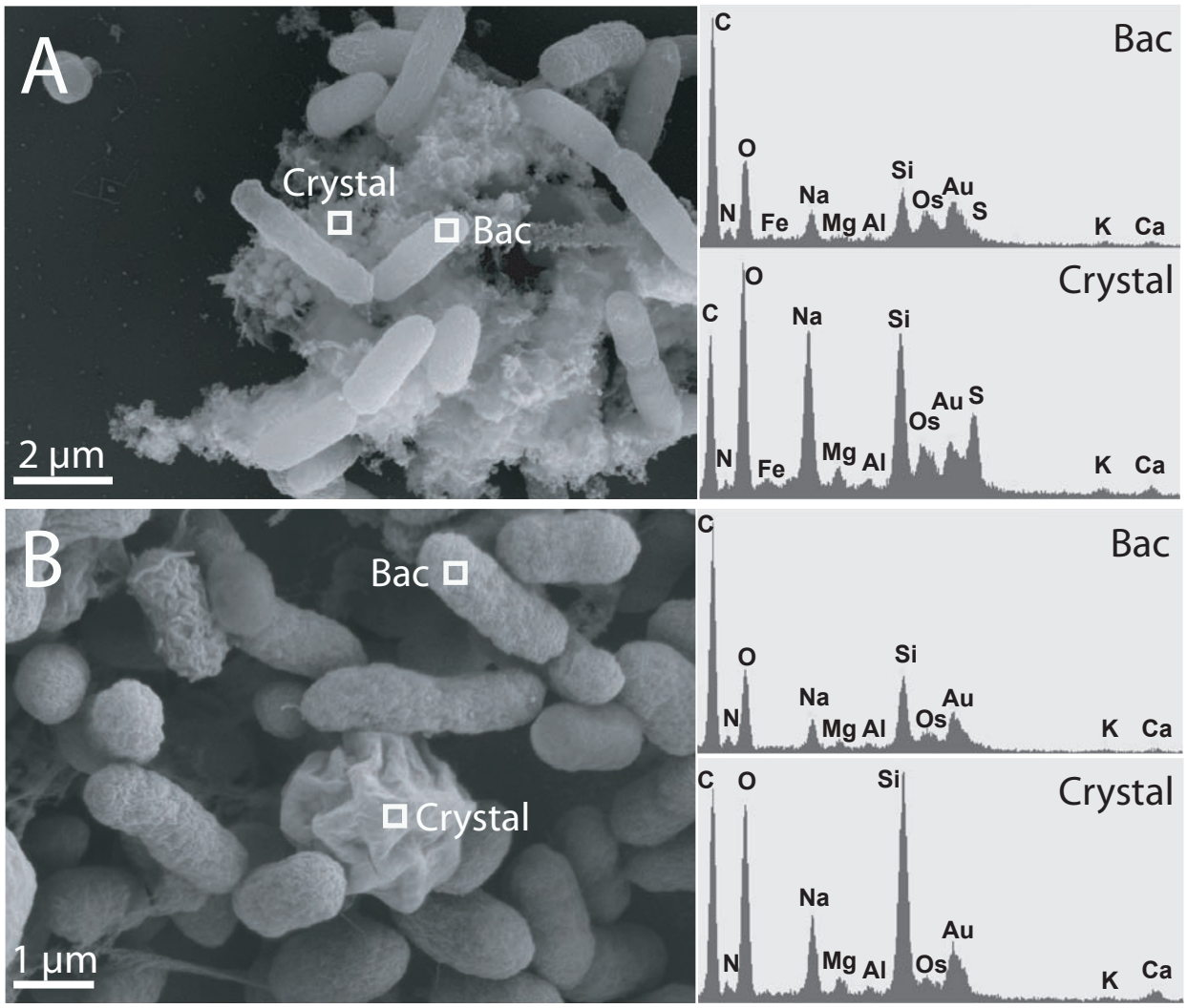
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Figure DR2.

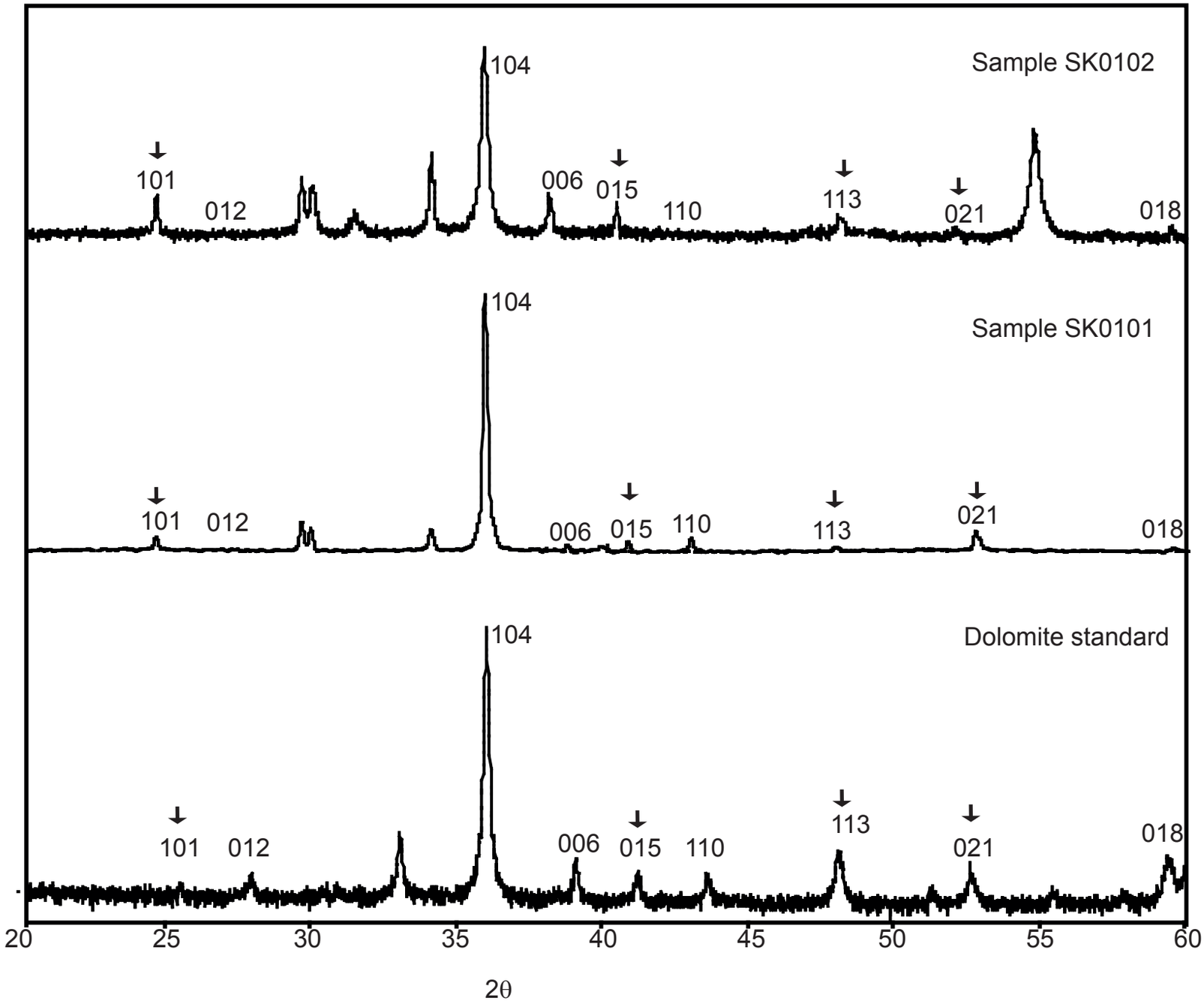
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TABLE DR1. Ca ISOTOPY OF MEDIUM, ORGANIC COMPONENT AND CRYSTALS

Sample	Average $\delta^{44/40}$Ca (‰)	2 SE	n
Standard NIST-SRM-915a	0.00	0.11	8
Whole procedure blank	1.16	0.38	2
Chemistry blank	1.24	0.03	2
Initial medium	0.95	0.05	3
Remaining medium	1.10	0.12	6
Organic (EPS+Bacteria)	0.48	0.07	6
3rd wash Organic	0.46	0.08	5
4th wash Organic	0.48	0.26	3
Dolomite crystals	0.05	0.24	3