

Isolation and identification of halophilic archaea with polysaccharide-converting enzyme potential from a Vietnamese solar saltern

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Abstract: Solar salterns are the most accessible hypersaline habitats for the recovery of extremely halophilic archaea (haloarchaea), yet the culturable fraction of Vietnamese saltern communities has never been assigned taxonomically. Here the culturable haloarchaeal component of the Hon Khoi solar saltern (Khanh Hoa, Vietnam) was characterised and the previously reported polysaccharide-degrading isolate VS7M5.3 was placed phylogenetically. Four brine, microbial-mat and sediment samples spanning 165–340 g L⁻¹ total salts, pH 6.8–7.6, redox potential –50 to +243 mV and in-situ temperatures of 36.5–60.2 °C were plated on modified growth medium containing 23% (w/v) salts and incubated at 40 °C for up to 30 days. Twenty-seven isolates were purified. Colonies were scored for pigmentation: 13 (48%) were unpigmented and 14 (52%) were yellow (9), orange (2), pink (2) or red-brown (1), a distribution consistent with the coexistence of moderately halophilic bacteria and carotenogenic haloarchaea. No isolate grew after transfer to media containing less than 20% (w/v) salts, identifying the collection as obligately halophilic. Five isolates were susceptible to anisomycin, and genomic DNA of adequate purity (A_{260}/A_{280} 1.8–2.0) was recovered from four of them. Near-complete amplification of the 16S rRNA gene succeeded for VS7M5.3, whose 1,453-nucleotide sequence and neighbour-joining phylogeny placed it robustly within the genus *Halorubrum* but did not resolve it to species level, the closest relatives being *Halorubrum luteum* and *Halorubrum aethiopicum*. The strain is therefore designated *Halorubrum* sp. VS7M5.3. These results provide the first taxonomic anchor for a culturable, enzymatically active haloarchaeon from a Vietnamese saltern and delimit what single-gene data can establish in this recombination-prone genus.

Keywords: 16S rRNA gene; Bacterioruberin; *Halorubrum*; Obligate halophile; Polysaccharide-converting enzymes; Solar saltern; Vietnam

1. Introduction

Hypersaline environments in which the salt concentration approaches saturation are dominated by extremely halophilic archaea of the class *Halobacteria*, collectively termed haloarchaea. These organisms maintain osmotic balance by the energetically economical "salt-in" strategy, accumulating molar concentrations of intracellular potassium and evolving a proteome enriched in acidic surface residues, so that their proteins require multi-molar salt for correct folding and catalysis [1,2]. Most species are also strongly pigmented, accumulating the C₅₀ carotenoid bacterioruberin and its derivatives, which rigidify the ether-lipid membrane and quench reactive oxygen species generated under the intense insolation of shallow brines [3]. Because their enzymes remain active at salt concentrations, temperatures and pH values that inactivate mesophilic counterparts, haloarchaea are an attractive source of robust biocatalysts and of pigments, biopolymers and compatible solutes for industrial use [4,5].

The functional image of this group has changed substantially over the past decade. Long regarded as heterotrophs restricted to amino acid mixtures and simple sugars, haloarchaea are now known to mineralise recalcitrant polysaccharides. Cellulose-

and chitin-utilising representatives have been isolated from hypersaline lakes [6,7], selective enrichment on a broad polysaccharide spectrum has yielded entirely new metabolic and taxonomic groups [8], and comparative genomics has shown that cellulotrophic haloarchaea are significantly enriched in glycoside hydrolase families GH5, GH9 and GH12 and can be partitioned into substrate specialists and nutritional generalists [9]. A parallel survey across the class *Halobacteria* has mapped the prevalence and genomic organisation of chitin-utilisation loci [10], and glycosaminoglycan catabolism — previously unknown in archaea — has now been demonstrated in *Natronoarchaeum* and *Haloarcula* strains enriched on hyaluronic acid, including material from Vietnamese salt-production fields [11].

Despite this progress, the culturable fraction of haloarchaeal diversity remains small. Growth requires stable multi-molar salinity, generation times are long, colonies commonly need three to four weeks to become countable, and cell lysis on dilution is a persistent obstacle, so isolation success is low relative to the diversity revealed by sequencing [12]. Cultures nonetheless remain indispensable: physiological limits, enzyme activities and formal taxonomic descriptions cannot be obtained from metagenomes alone.

Vietnam offers favourable but almost unexploited material for such work. Artisanal solar salterns extend along more than 3,000 km of coastline, and the crystalliser ponds at Hon Khoi (Ninh Hoa, Khanh Hoa Province) reach brine salinities above 300 g L⁻¹ under mid-day temperatures well above 40 °C. Metagenomic analysis of Vietnamese saltworks has documented a distinctive community in which *Halorubrum*, *Halobacterium* and *Haloarcula* dominate the haloarchaeal fraction and Nanohaloarchaeota can be locally abundant [13]. By contrast, the strains actually held in culture from these ponds have not been identified. In a previous report we described the isolation of 27 strains from Hon Khoi, the selection of five anisomycin-susceptible candidates, and their plate-based screening for cellulase/xylanase, chitinase and hyaluronidase activity, which identified VS7M5.3 as active on all three substrates [14]. That study assigned no taxonomy, characterised no colony architecture and included no molecular data; the present work does not re-examine the enzyme phenotypes, which are cited only as the rationale for strain selection.

The aims of the present study were therefore (i) to describe the physicochemical setting and colony architecture of the culturable community recovered from Hon Khoi and to quantify its pigmentation, (ii) to establish whether the isolates are obligately or facultatively halophilic, and (iii) to determine the phylogenetic position of the multi-substrate isolate VS7M5.3 by 16S rRNA gene sequencing, while defining explicitly the limits of a single-gene assignment within *Halorubrum*.

2. Materials and methods

2.1. Study site and sampling

Samples were collected in June 2023 from the Hon Khoi solar saltern at Ninh Diem, Ninh Hoa, Khanh Hoa Province, Vietnam, one of the largest salt-production areas in central Vietnam. Material was taken from residual brine remaining after salt crystallisation, in two cases together with underlying sediment or microbial mat. Four sampling points were used, designated VS1 (12°32'07.7" N), VS7 (12°32'16.7" N), VS8 (12°32'20.9" N) and VS9sed (12°32'23.5" N). Samples were collected into sterile polypropylene bottles, held at 4 °C and transported to the laboratory. Temperature, salinity, pH and redox potential were measured on site with a thermometer, a hand-held refractometer and a portable pH/ORP meter, respectively. Sample material was provided by the Nhatrang Institute of Technology Research and Application within the framework of the HALOPHARM project.

2.2. Medium and isolation procedure

Modified growth medium (MGM) contained 1% (w/v) soy peptone, 0.5% (w/v) yeast extract and 1.5% (w/v) agar in a salt mixture at a total concentration of 23% (w/v), comprising 3.1 M NaCl, 0.1 M MgCl₂·6H₂O, 0.1 M MgSO₄·7H₂O, 0.07 M KCl and 5 × 10⁻³ M CaCl₂·2H₂O; the CaCl₂ solution was autoclaved separately and added after sterilisation. The pH was adjusted to 7.2–7.4 with HCl or NaOH and the medium was autoclaved at

121 °C for 15 min before being poured into sterile Petri dishes in a biological safety cabinet (Telstar AV-100).

Aliquots of 100 µL of each sample were spread onto MGM plates and incubated at 40 °C for 15–30 days in a Memmert incubator, with periodic inspection for colony development. Discrete colonies were transferred repeatedly onto fresh MGM until pure. Purified isolates were maintained at 4 °C for short-term use and cryopreserved at –80 °C for long-term storage.

2.3. Colony morphology and pigmentation scoring

Colonies on the third purification passage were photographed and scored for outline, elevation, margin, surface texture, opacity and pigment. Pigmentation was recorded in five categories — white (unpigmented), yellow, orange, pink and red-brown — and expressed as counts and percentages of the total collection. Scoring was performed on plates of equivalent age (30 days) to avoid the drift in colour intensity that accompanies continued incubation.

2.4. Determination of salt dependence

To distinguish obligate from facultative halophily, purified isolates were subcultured onto MGM in which the total salt concentration had been reduced below 20% (w/v), incubated in parallel with 23% (w/v) control plates at 40 °C, and examined for growth over 30 days.

2.5. Anisomycin susceptibility

Susceptibility to anisomycin, which inhibits the peptidyl transferase centre of archaeal and eukaryotic but not bacterial ribosomes, was used as a presumptive discriminator between haloarchaea and halophilic bacteria [15,16]. Isolates were grown in liquid MGM, harvested and suspended in sterile 0.85% (w/v) NaCl to a density equivalent to 0.5 McFarland (approximately 1.5 × 10⁸ CFU mL⁻¹). Suspensions were spread over the entire surface of MGM plates in three perpendicular directions and allowed to dry for 10–15 min under aseptic conditions. Disks containing 30 µg anisomycin (Santa Cruz Biotechnology, USA) were applied and gently pressed onto the agar. Plates were incubated at 37–42 °C for 7–20 days according to the growth rate of each isolate and inspected for zones of inhibition. Isolates producing a discernible zone were scored as susceptible.

2.6. Extraction of genomic DNA

Genomic DNA was extracted from the anisomycin-susceptible isolates with the GNOME DNA kit (MP Biomedicals). Cells were lysed in the manufacturer's lysis buffer containing lysozyme (37 °C, 30 min), followed by sodium dodecyl sulfate and proteinase K treatment at 55 °C for 1–2 h. Nucleic acids were extracted at least twice with phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v), precipitated with cold isopropanol at –20 °C for 1 h, washed with 70% ethanol, dissolved in TE buffer and stored at –20 °C. Concentration and purity were determined spectrophotometrically from absorbance at 260, 280 and 230 nm, and integrity was verified by electrophoresis on 0.8–1.0% (w/v) agarose gels stained with ethidium bromide.

2.7. Amplification and sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified in a 25 μ L reaction containing 12.5 μ L master mix (Taq DNA polymerase, buffer, $MgCl_2$ and dNTPs), 1 μ L each of the archaeal primers 4F (5'-TCCGGTTGATCCTGCRG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [17] at 10 μ M, 2 μ L template DNA (20–50 ng μ L⁻¹) and 8.5 μ L nuclease-free water. Cycling comprised initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s; and a final extension at 72 °C for 10 min. Products were resolved on 1.5% (w/v) agarose in TAE buffer at 100 V for 40 min, visualised under UV illumination and sized against a molecular-weight ladder, the expected amplicon being approximately 1.5 kb. Amplicons were purified and sequenced commercially (Macrogen Inc., Seoul, Republic of Korea), and the forward and reverse reads were assembled into a consensus sequence.

2.8. Sequence analysis and phylogenetic reconstruction

The assembled sequence was compared with the NCBI database using BLASTn, and identity, query coverage and E-value were recorded for the four closest type-strain entries. Sequences were aligned with reference 16S rRNA genes of Halorubrum type strains and with uncultured Nanohaloarchaeota clones recovered from polysaccharide enrichments of the same saltern system. A phylogenetic tree was inferred by the neighbour-joining method in MEGA 11, with branch support assessed by

1,000 bootstrap replicates, and rendered with the ChiPlot web server [18].

2.9. Data availability

The 16S rRNA gene sequence of strain VS7M5.3 has been deposited in GenBank.

3. Results

3.1. Physicochemical setting of the sampled ponds

The four sampling points spanned a wide physicochemical range within a single saltern system (Table 1). Total salts varied from 165 g L⁻¹ at VS7 to 340 g L⁻¹ at VS1 and VS9sed, the upper values approaching NaCl saturation. The brines were circumneutral, with pH between 6.8 and 7.6, and no alkaline pond was encountered. Redox potential differed markedly among points, from –50 mV at VS7, indicating reducing conditions associated with the mat and sediment layer, to +243 mV at VS9sed. In-situ temperatures recorded at midday ranged from 36.5 °C at VS7 to 60.2 °C at VS9sed, the highest value corresponding to a shallow pond containing sediment and microbial mat. Sample VS7, the source of the isolate characterised below, was thus the least saline, the most alkaline and the most reducing of the four, and also the coolest.

Table 1. Physicochemical characteristics of the four sampling points at the Hon Khoi solar saltern, June 2023.

Sample code	Location	Total salts (g L ⁻¹)	pH	Redox potential (mV)	Temperature (°C)	Sample type
VS1	Hon Khoi	340	6.9	+12	44.0	Brine
VS7	Hon Khoi	165	7.6	–50	36.5	Brine and sediment
VS8	Hon Khoi	320	6.9	+32	42.8	Brine
VS9sed	Hon Khoi	340	6.8	+243	60.2	Brine, microbial mat and sediment

3.2. Recovery and colony architecture of the isolates

Growth on the primary isolation plates was slow. No colonies were visible during the first 5–7 days of incubation, and colony outlines became clearly resolvable only after approximately 20 days; a further period of incubation to 30 days was required

before the smaller colonies could be scored reliably (Fig. 1). Progressive evaporation of the plates during this extended incubation caused visible salt crystallisation at the agar surface, yet colony expansion continued, indicating tolerance of salinities above the nominal 23% (w/v) of the medium.

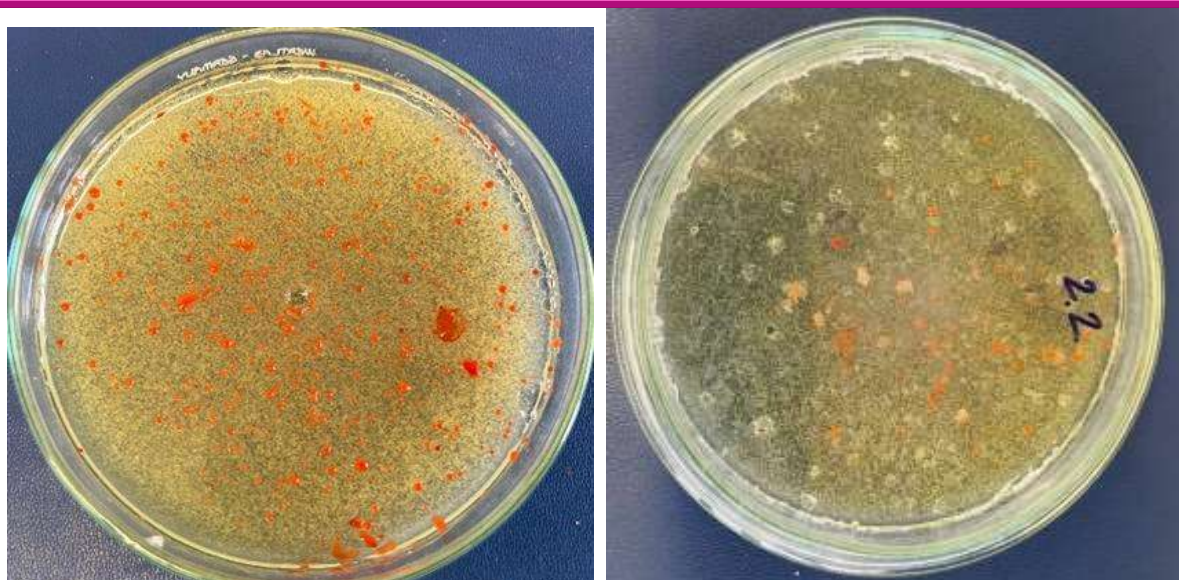


Fig. 1. Colonies on primary isolation plates of modified growth medium containing 23% (w/v) total salts, inoculated with brine from the Hon Khoi solar saltern. (A) After 20 days of aerobic incubation; (B) after 30 days, when the smaller colonies could be scored reliably. Salt crystallisation at the agar surface, resulting from evaporation during the extended incubation, is visible in (B).

A total of 27 isolates were purified from the four samples. Recovery was distinctly uneven: VS9sed yielded the largest number of isolates (12), followed by VS8 (7), VS7 (5) and VS1 (3). Colony morphology was recorded for all 27 isolates (Table 2). The collection was morphologically conservative in outline

and elevation — 26 of 27 colonies were circular with entire margins and a convex or umbonate profile, and only isolate M3.1 was irregular — but varied in opacity, with 6 isolates translucent and 21 opaque, and in surface texture, 26 being glossy and only M5.3 matt.

Table 2. Colony morphology of the 27 isolates purified from the Hon Khoi solar saltern on MGM containing 23% (w/v) total salts, recorded after 30 days at 40 °C.

No.	Isolate	Outline	Pigment	Opacity	Elevation	Margin	Surface
1	M1.1	Circular	White	Translucent	Convex	Entire	Glossy
2	M1.2	Circular	White	Opaque	Convex	Entire	Glossy
3	M1.3	Circular	White	Translucent	Convex	Entire	Glossy
4	M1.4	Circular	Pale yellow	Opaque	Convex	Entire	Glossy
5	M1.5	Circular	White	Translucent	Convex	Entire	Glossy
6	M2.1.1	Circular	White	Slightly opaque	Umbonate	Entire	Glossy
7	M2.1.2	Circular	Yellow	Opaque	Umbonate	Entire	Glossy
8	M2.1.3	Circular	Yellow	Opaque	Umbonate	Entire	Glossy
9	M2.1.4	Circular	Deep orange	Opaque	Umbonate	Entire	Glossy
10	M3.1	Irregular	Pale pink	Opaque	Umbonate	Entire	Glossy
11	M3.2	Circular	White	Opaque	Umbonate, white centre	Entire	Glossy
12	M3.3	Circular	White	Translucent	Convex	Diffuse	Glossy
13	M5.1	Circular	Yellow	Opaque	Convex	Entire	Glossy
14	M5.2	Circular	White	Opaque	Umbonate	Entire	Glossy
15	M5.3	Circular	Red-brown	Opaque	Umbonate, brown centre	Entire	Matt
16	M5.4	Circular	Yellow	Opaque	Convex	Entire	Glossy
17	M5.5	Circular	Yellow	Opaque	Convex	Entire	Glossy
18	M5.6	Circular	White	Opaque	Convex	Entire	Glossy

19	M5.7	Circular	Yellow	Opaque	Umbonate, deep-yellow centre	Entire	Glossy
20	M5.8	Circular	White	Opaque	Convex	Entire	Glossy
21	M5.9	Circular	White	Opaque	Convex	Entire	Glossy
22	M5.10	Circular	Pink	Opaque	Convex	Entire	Glossy
23	M5.11	Circular	Orange	Opaque	Umbonate	Entire	Glossy
24	M6.1	Circular	Pale yellow	Opaque	Convex	Entire	Glossy
25	M6.2	Circular	White	Translucent	Convex	Entire	Glossy
26	M6.3	Circular	Yellow	Opaque	Convex	Entire	Glossy
27	M6.4	Circular	White	Translucent	Convex	Entire	Glossy

3.3. Colony pigmentation

Pigmentation was the most informative morphological character (Fig. 2). Thirteen isolates (48%) formed white, unpigmented colonies. The remaining 14 (52%) were pigmented: 9 yellow (M1.4, M2.1.2, M2.1.3, M5.1, M5.4, M5.5, M5.7, M6.1, M6.3), 2 orange (M2.1.4, M5.11), 2 pink (M3.1, M5.10) and 1 red-

brown (M5.3). The pink and orange morphotypes, characteristic of bacterioruberin-accumulating haloarchaea, were recovered from three of the four samples. Isolate M5.3, from sample VS7, was the only strain forming a dark red-brown, matt colony with a raised centre, and was thus morphologically unique within the collection.

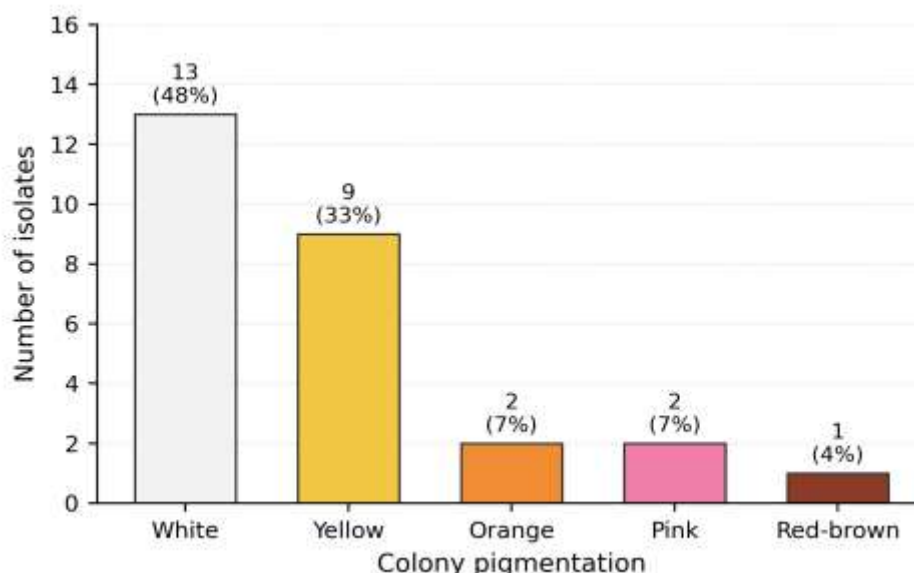


Fig. 2. Distribution of colony pigmentation among the 27 isolates purified from the Hon Khoi solar saltern. Counts and the corresponding percentage of the collection are given above each bar.

3.4. Obligate halophily

None of the 27 isolates produced colonies after transfer to MGM containing less than 20% (w/v) total salts, whereas parallel 23% (w/v) control plates supported normal growth. All subsequent purification and maintenance was therefore performed at or above 23% (w/v) salts. The collection accordingly consists exclusively of obligate halophiles rather than halotolerant organisms, consistent with the salinity of the source brines.

3.5. Anisomycin-susceptible subset

Five of the 27 isolates — M2.1.4 from VS1 and M5.1, M5.3, M5.7 and M5.11 from VS7 — produced inhibition zones around

the anisomycin disks and were scored as susceptible (Table 3). Zones were faint rather than sharply demarcated and became discernible only after approximately 20 days of incubation, consistent with the slow growth of the isolates. All five susceptible isolates were pigmented, spanning yellow (M5.1, M5.7), orange (M2.1.4, M5.11) and red-brown (M5.3) colonies; no unpigmented isolate was susceptible. The concordance between anisomycin susceptibility and carotenoid pigmentation supports the assignment of these five isolates to the haloarchaea and, by implication, of the unpigmented, anisomycin-resistant majority to halophilic Bacteria. These five isolates were carried forward, and their polysaccharide-hydrolysing phenotypes have been reported separately [14]. On plate assays with chitin,

carboxymethyl cellulose and hyaluronic acid, VS7M5.3 was the only isolate scoring the highest grade on two substrates, strong on carboxymethyl cellulose and on hyaluronic acid and moderate on chitin; VS7M5.1 was strong on carboxymethyl

cellulose only, VS7M5.7 moderate on chitin and on carboxymethyl cellulose, and VS1M2.1.4 and VS7M5.11 showed no detectable activity on carboxymethyl cellulose [14]. VS7M5.3 was selected for sequencing on that basis.

Table 3. Isolates susceptible to anisomycin (30 µg per disk).

No.	Source sample	Isolate	Colony pigment	Anisomycin susceptibility
1	VS1	M2.1.4	Deep orange	+
2	VS7	M5.1	Yellow	+
3	VS7	M5.3	Red-brown	+
4	VS7	M5.7	Yellow	+
5	VS7	M5.11	Orange	+

Note: +, inhibition zone present. All 22 remaining isolates produced no inhibition zone and were scored as resistant.

3.6. Yield and purity of genomic DNA

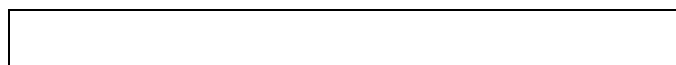
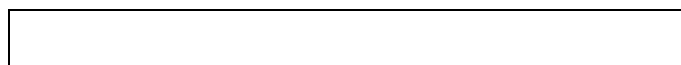
Genomic DNA of usable quality was obtained from four of the five susceptible isolates; extraction from VS1M2.1.4 failed repeatedly, most plausibly because of incomplete lysis of the cell envelope. Yields for the four successful extractions ranged from 85.2 to 92.3 ng µL⁻¹ (Table 4). A_{260}/A_{280} ratios of 1.8–2.0

indicated negligible protein carry-over, and A_{260}/A_{230} ratios of 2.0–2.2 indicated low residual polysaccharide and salt. Agarose gel electrophoresis resolved a single high-molecular-weight band above 10 kb for each sample, with no smearing or low-molecular-weight trail, confirming that the DNA was intact and suitable for amplification (Fig. 3).

Table 4. Concentration and purity of genomic DNA extracted from the anisomycin-susceptible isolates.

Isolate	DNA concentration (ng µL ⁻¹)	A_{260}/A_{280}	A_{260}/A_{230}
VS7M5.1	85.2	1.9	2.1
VS7M5.3	92.3	1.8	2.0
VS7M5.7	88.7	2.0	2.2
VS7M5.11	87.4	1.8	2.0

Note: Extraction from isolate VS1M2.1.4 did not yield DNA of usable quality and this isolate was not carried forward.



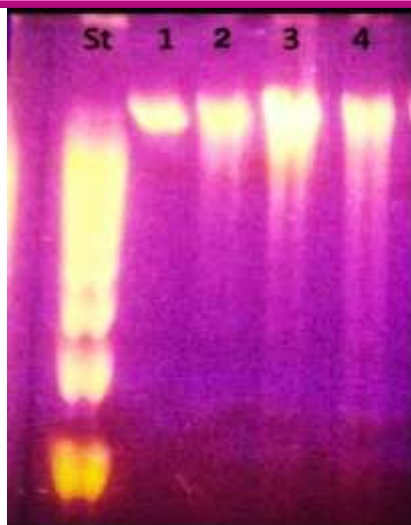


Figure 3. Agarose gel electrophoresis (1% w/v, ethidium bromide) of genomic DNA extracted from the anisomycin-susceptible isolates. St, 1 kb DNA ladder; lane 1, VS7M5.1; lane 2, VS7M5.3; lane 3, VS7M5.7; lane 4, VS7M5.11. All samples resolve as a single high-molecular-weight band above 10 kb with no evidence of fragmentation.

3.7. Amplification of the 16S rRNA gene

Amplification with the archaea-specific primer pair 4F/1492R yielded a product of the expected size, approximately 1.5 kb, from all four templates, with no secondary bands above or below the target, indicating specific amplification (Fig. 4). Yields were, however, markedly unequal. VS7M5.3 gave a single sharp, intense band, whereas the bands obtained from VS7M5.1, VS7M5.7 and VS7M5.11 were comparatively faint. Only the VS7M5.3 amplicon met the intensity and purity criteria for sequencing, and it alone was taken forward. The differential amplification efficiency is most simply explained by sequence variation in the primer-binding regions among the isolates, an expected consequence of haloarchaeal 16S rRNA gene diversity, although differences in template quality cannot be excluded.



Figure 4. Agarose gel electrophoresis (1.5% w/v, TAE) of 16S rRNA gene PCR products amplified with primers 4F and 1492R. L, DNA ladder; lane 1, VS7M5.3; lane 2, VS7M5.1; lane 3, VS7M5.7; lane 4, VS7M5.11. All lanes show a single product of approximately 1.5 kb with no secondary bands; the band from VS7M5.3 is markedly more intense than those of the other three isolates.

3.8. Sequence identity and phylogenetic placement of VS7M5.3

Sequencing of the VS7M5.3 amplicon returned a high-quality read of 1,453 nucleotides, within the length range conventionally used for 16S rRNA gene-based identification. BLASTn analysis returned exclusively members of the genus *Halorubrum* among the closest matches, with query coverage of 99–100% and E-values of 0 throughout (Table 5). The top-ranked entries were *Halorubrum luteum* strain CGSA15 (100%) and *Halorubrum luteum* strain CECT 7303 (99.78%), followed by *Halorubrum salsamentum* strain Y69 (98.97%) [19] and *Halorubrum aethiopicum* strain SAH-A6 (97.13%) [20]. All identity values exceeded the 94.5% threshold conventionally applied for genus-level circumscription, placing the strain unambiguously in *Halorubrum*, a genus circumscribed on four species transferred from *Halobacterium* [21].

Table 5. BLASTn comparison of the 1,453-nucleotide 16S rRNA gene sequence of strain VS7M5.3 against the NCBI database: the four closest type-strain entries.

No.	Closest reference strain	GenBank accession	RefSeq accession	Identity (%)	Query coverage (%)	E-value
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1	<i>Halorubrum luteum</i> strain CGSA15	DQ987877	NR_115925.1	100	100	0
2	<i>Halorubrum luteum</i> strain CECT 7303	AB663417	NR_113216.1	99.78	100	0
3	<i>Halorubrum salsamentii</i> strain Y69	—	NR_177340.1	98.97	99	0
4	<i>Halorubrum aethiopicum</i> strain SAH-A6	—	NR_159273.1	97.13	100	0

Note: Identity, query coverage and E-value are as returned by BLASTn. Entries are the four closest type strains, ranked by bit score. All identity values exceed the 94.5% threshold conventionally applied for genus-level circumscription.

Neighbour-joining analysis (Fig. 5) recovered a *Halorubrum* clade containing VS7M5.3, *H. aethiopicum* SAH-A6 and the two *H. luteum* strains at 98% bootstrap support, clearly separated at 100% support from a cluster of uncultured Nanohaloarchaeota clones obtained from polysaccharide enrichments of the same saltern system, a lineage whose members depend on haloarchaeal hosts for polysaccharide breakdown products [22]. Within the *Halorubrum* clade, the two *H. luteum* strains formed a tight pair supported at 100%, while VS7M5.3 occupied a comparatively long, separate branch outside that pair rather than nesting within it. An identity of 100% at full query coverage would ordinarily indicate

conspecificity with *H. luteum*. The topology, however, did not place VS7M5.3 within the *H. luteum* pair, and the internal nodes separating it from the named species received no bootstrap support above the reporting threshold. The two lines of evidence are therefore not concordant, which is itself informative: near-identical 16S rRNA gene sequences are shared by *Halorubrum* strains that are distinct at the genomic level, as in *H. sfaxense* ETD6, which returned 99.77% identity to *H. xinjiangense* but only 24.5% DNA–DNA hybridisation [23]. Species assignment in this genus consequently cannot rest on this marker, and the strain is designated *Halorubrum* sp. VS7M5.3, pending genome-based analysis.

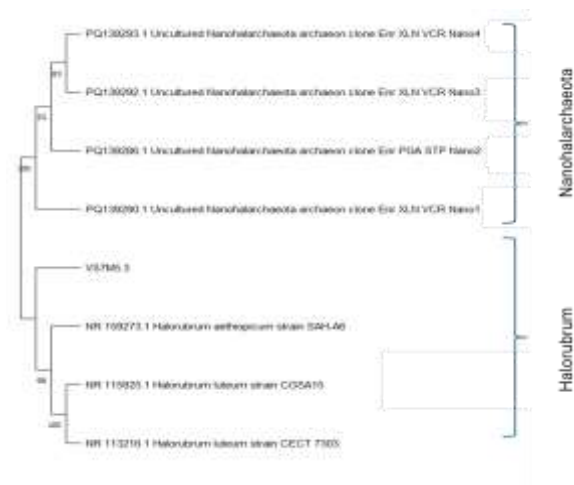


Fig. 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain VS7M5.3 relative to *Halorubrum* type strains and to uncultured Nanohaloarchaeota clones recovered from polysaccharide enrichments of the same saltern system. Bootstrap values (%) from 1,000 replicates are shown at the nodes. The tree was inferred in MEGA 11 and rendered with ChiPlot.

4. Discussion

The 27 isolates recovered here are far fewer than the roughly 500 obtained by Yavari-Bafghi et al. from three samples of Iranian hypersaline lakes [24]. The most likely reason is the salinity of the source material: the Hon Khoi brines contained 165–340 g L⁻¹ total salts, one to two orders of magnitude above the 2.3–2.8 g L⁻¹ reported in that study, and the recovery gradient across our own samples is consistent with this interpretation, the least saline point (VS7, 165 g L⁻¹) nonetheless yielding the only isolate that could be sequenced. Low colony counts under such conditions should be read as a property of the habitat rather than as a deficiency of the method; the corollary is that the strains obtained are genuine extreme halophiles, a conclusion reinforced by the complete absence of growth below 20% (w/v) salts and by continued colony expansion as the plates dried and salt crystallised.

The pigmentation profile provides a useful, if approximate, first partition of the collection. The 48% of isolates forming unpigmented colonies most plausibly represent moderately halophilic *Bacteria*, which are routinely co-recovered on high-salt media from saline springs and salterns [25], whereas the orange, pink and red-brown morphotypes are consistent with bacterioruberin accumulation, which is characteristic of the *Halobacteria* and serves both membrane rigidification and antioxidant functions in high-light, high-ionic-strength brines [3,26,27]. Two caveats should be stated. Colony colour is not a diagnostic character: unpigmented haloarchaea are well documented, and carotenogenic *Bacteria* such as *Salinibacter* also occur in salterns. Furthermore, yellow colonies — the largest pigmented group here — may reflect β -carotene or lycopene rather than bacterioruberin, and cannot be assigned on colour alone. The convergence of anisomycin susceptibility with pigmentation in all five selected isolates is therefore the more informative observation, although anisomycin sensitivity is itself imperfect: it is a physiological proxy rather than a phylogenetic marker, and interpretation was complicated here by faint, slowly developing inhibition zones [15,16].

Placement of VS7M5.3 in *Halorubrum* is consistent with metagenomic surveys of Vietnamese saltworks, in which *Halorubrum* is among the dominant haloarchaeal genera [13], and digital DNA–DNA hybridisation as the operative criteria [35]. The conservative designation *Halorubrum* sp. VS7M5.3 is the defensible conclusion from the present data.

4.1. Limitations

This study has several limitations. First, sequence data were obtained for a single isolate: three of the four amplifiable strains yielded amplicons too faint to sequence, so the taxonomic composition of the culture collection as a whole remains unresolved. Second, identification rests on one phylogenetic marker; no genome sequence, average nucleotide identity value or digital DNA–DNA hybridisation estimate is available, and the strain therefore cannot be assigned to a species. Third, the phylogenetic reconstruction included a small reference set and

and with the broader literature on solar salterns worldwide [28]. It also matters functionally. *Halorubrum* species are established sources of polyextremophilic enzymes, including an extracellular α -amylase from *H. xinjiangense* [29] and a β -galactosidase from the Antarctic species *H. lacusprofundi* [30], so a *Halorubrum* strain active on chitin, carboxymethyl cellulose and hyaluronic acid [14] is a plausible rather than an anomalous finding, particularly as chitinolytic systems are already well characterised in *Halobacterium salinarum* [31] and *Haloferax mediterranei* [32]. It is notable that the only haloarchaea so far shown to grow on hyaluronic acid as sole carbon source, obtained by enrichment from Vietnamese salt fields among other sites, belong to *Natronoarchaeum* and *Haloarcua* rather than *Halorubrum* [11]. Plate screening of a culture collection and substrate-driven enrichment thus sample different fractions of the same community, and the two approaches should be regarded as complementary. The same metagenomic survey found the VS7 community to be atypical within this saltern, with *Nanohaloarchaeota* accounting for 78.5% of the reads at that point [13,14]. VS7 was also the least saline and most reducing of the four ponds and the source of four of the five anisomycin-susceptible isolates, so the point departing most from the rest of the system in community composition is also the one from which the culturable haloarchaeal fraction was most readily recovered.

The principal interpretive limit is the marker itself. Genus-level assignment is secure, but *Halorubrum* is notorious for extensive intragenomic and intergenomic 16S rRNA gene recombination [33], and near-identical 16S sequences are shared by strains with markedly different physiology — *H. luteum* CGSA15, for example, was described from an alkaline soda lake with a pH optimum of 9.5–10.0 [34], whereas VS7M5.3 originates from a circumneutral brine at pH 7.6. The point is made directly by *H. sfaxense* ETD6, isolated from the Sfax solar saltern, whose 16S rRNA gene is 99.77% identical to that of *H. xinjiangense* while DNA–DNA hybridisation between the two is only 24.5% [23]. Species delineation in this genus consequently requires whole-genome comparison, with average nucleotide identity

no outgroup, and the internal nodes separating VS7M5.3 from named *Halorubrum* species were poorly supported; a reanalysis incorporating all validly named *Halorubrum* type strains, an appropriate outgroup and maximum-likelihood inference alongside neighbour-joining is required. Fourth, the isolates were not characterised physiologically — salinity, temperature and pH optima, polar lipid composition and substrate utilisation profiles were not determined — so the assignment remains phylogenetic rather than polyphasic. Fifth, pigmentation was scored visually and not verified spectrophotometrically or by pigment extraction, so the inferred presence of bacterioruberin

is provisional. Sixth, sampling was confined to four points on a single date, and seasonal variation in the saltern community was not addressed. Finally, anisomycin susceptibility was assessed qualitatively from a single disk concentration without a minimum inhibitory concentration determination or defined archaeal and bacterial reference strains.

5. Conclusion

The culturable prokaryotic community of the Hon Khoi solar saltern was characterised across a gradient of 165–340 g L⁻¹ total salts, pH 6.8–7.6 and in-situ temperatures of 36.5–60.2 °C, yielding 27 obligately halophilic isolates, none of which grew below 20% (w/v) salts. Pigmentation partitioned the collection into an unpigmented fraction of 48% and a carotenoid-pigmented fraction of 52%, and the five isolates susceptible to anisomycin were all pigmented, a concordance that supports their assignment to the haloarchaea. Genomic DNA of high purity was recovered from four of these five isolates, and near-complete 16S rRNA gene sequencing of the multi-substrate strain VS7M5.3 returned 1,453 nucleotides that place it firmly within the genus *Halorubrum*, sister to but distinct from *Halorubrum luteum* and *Halorubrum aethiopicum*. The strain is accordingly designated *Halorubrum* sp. VS7M5.3. The wide physicochemical spread of the sampled ponds, and the recovery of the only sequenceable isolate from the least saline and most reducing of them, indicate that within-saltern heterogeneity

rather than mean salinity governs which taxa can be brought into culture. To our knowledge this is the first taxonomic assignment of a culturable, enzymatically characterised haloarchaeon from a Vietnamese solar saltern, and it converts a screening hit into a defined biological resource that can be deposited, redistributed and compared. Because 16S rRNA gene data cannot resolve species boundaries in this recombination-prone genus, whole-genome sequencing with average nucleotide identity analysis, together with a polyphasic physiological and chemotaxonomic description, is the necessary next step, and would also establish whether the hydrolytic activities of the strain are encoded by carbohydrate-active enzyme families already known from haloarchaea.

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