and surface features; position and form of the hila (the botanical centre of the grain) and fissure; number and characteristics of pressure facets; and presence or absence of demonstrable lamellae. Manioc, arrowroot, *Dioscorea* spp. and maize contribute unique, individual grains (Fig. 1a, c, d). To confirm identifications of manioc and maize we used attribute combinations in a multiple grain analysis, which is the most conservative means to distinguish species because it provides population signatures for specific species and takes into account intra- and inter-species variation in grain attributes[15,16,19,27].

Starch grains in roots of modern domesticated *Manihot esculenta* are compound and predominantly bell-shaped with smooth and highly transparent surfaces, one to five unbanded pressure facets with rounded edges, hila that are centric and open, and no demonstrable lamellae. Many grains have fissures that are often crossed, winged to y-shaped, or stellated (Fig. 1a). This combination of characteristics distinguishes manioc from all other taxa, including the few others with bell-shaped starches such as *Pachyrrhizus* spp.[18], in which grains have banded and angled pressure facets, closed or semi-open hila, and no or simple fissures. Our results indicate that bell-shaped grains are primarily confined to subterranean organs of plants. Starch grains from maize kernels are predominantly spherical and smooth (flour corns) or mostly angular and four- to five-sided with a rough, grooved surface (popcorns and flint corns). In this and other studies[16,17,27], endosperm type is thus shown to exert considerable influence on starch morphology in maize. Our morphological and size determinations for different species incorporated sampling of multiple reference points within a single specimen, to account for within-sample variability.

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# Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal

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**Bacteria have been found associated with a variety of ancient samples[1], however few studies are generally accepted due to questions about sample quality and contamination. When Cano and Borucki[2] isolated a strain of *Bacillus sphaericus* from an extinct bee trapped in 25–30 million-year-old amber, careful sample selection and stringent sterilization techniques were the keys to acceptance. Here we report the isolation and growth of a previously unrecognized spore-forming bacterium (*Bacillus* species, designated 2-9-3) from a brine inclusion within a 250 million-year-old salt crystal from the Permian Salado Formation. Complete gene sequences of the 16S ribosomal DNA show that the organism is part of the lineage of *Bacillus marismortui* and *Virgibacillus pantothenticus*. Delicate crystal structures and sedi-**

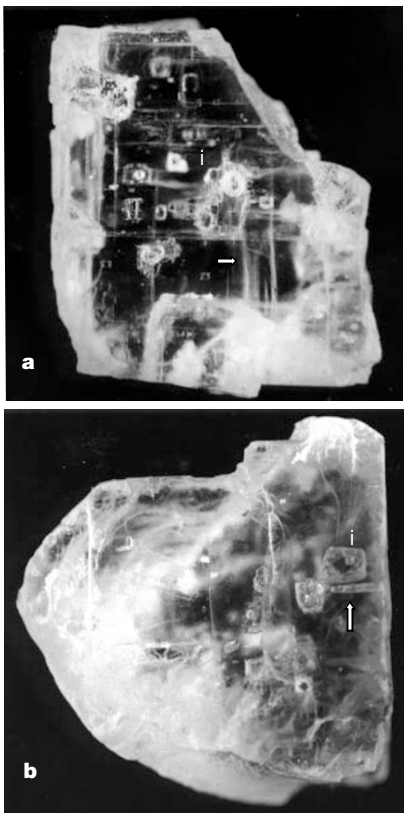


**Figure 1** Halite crystals taken from the dissolution pipe at the 569 m (1850 ft) level of the Salado Formation (air intake shaft[18] for the Waste Isolation Pilot Plant, Carlsbad, New Mexico) in October 1998. **a**, This crystal was rejected for sampling. The crystal has numerous fracture lines (arrow) and misshapen inclusions with gas bubbles (i). This crystal measured 3.2 × 2.8 × 0.9 cm. **b**, The sample that yielded *Bacillus* strain 2-9-3. This crystal measured 3.5 × 3.5 × 2.5 cm. The inclusion that contained the bacterium (below i) measured approximately 3 × 3 × 1 mm (9 cubic mm). The drill hole made during sampling of inclusion B (Table 1) is visible above the arrow. The thickness of this crystal obscures some of the internal details (the drill hole for inclusion A) in the photograph.

**mentary features indicate the salt has not recrystallized since formation. Samples were rejected if brine inclusions showed physical signs of possible contamination. Surfaces of salt crystal samples were sterilized with strong alkali and acid before extracting brines from inclusions. Sterilization procedures reduce the probability of contamination to less than 1 in $10^9$.**

Bacteria associated with ancient samples include *Halococcus salifodinae*[3], and several other unnamed halophilic Archaea isolated from salt formations[4,5]. There are a variety of claims of isolation of eubacteria from salts (reviewed in ref. 1). Nevertheless, the most acceptable studies are of bacteria isolated from amber[2,6,7]. Before the ancient age of any organism can be firmly established and accepted, two basic criteria should be met: the geological age of the formation or period of isolation should be established; and clear laboratory data should show steps taken to maintain a low probability of contamination[1,8,9].

The Salado is generally accepted to be at least 250 Myr old. This age is supported by invertebrate fossils[10,11] and radiometric ages[12] from overlying formations, as well as radiometric ages of langbeinite minerals[13] taken directly from the Salado. Crystal features and original structures in the Salado beds are evidence of integrity over the last 250 Myr. Delicate fluid inclusions commonly trapped by halite crystals as they grow are one indicator that halite has not recrystallized since being deposited[8,14–20]. The Salado also includes dissolution pits and pipes that are filled with coarse, clear halite with fewer, but larger, fluid inclusions[18,20,21]. These pits and pipes are overlaid by undisturbed halite beds, signifying that they are the same age (Late Permian) as the formation[18,20,21]. These crystals also do not show boundary relationships of recrystallized halite[15] that would indicate a younger age. Because fluid inclusions in these crystals are much larger than in other primary halite, they are preferred for the type of sampling performed here (Fig. 1).

It is fair to note that the radiometric age for many potassium-bearing minerals in the Salado range from 210–215 Myr[22]. However, the $^{40}Ar$–$^{39}Ar$ plateau age for langebeinite crystals taken directly from the Salado is reproducibly 251 Myr[22]. The cause of the difference between the evident age of deposition and radiometric age is undetermined[22]. Although it could be argued that these younger dates represent a last stage of mineralization (and closing of the system at 210 Myr ago), textural data[18,20,21], langbeinite dates[13] and crystals of KCl in fluid inclusions in primary halite crystals[19] are strong evidence that our samples remained isolated for 250 Myr, not 210 Myr.

The crystal samples were taken using a hand-held electric drill and coring bit. The underground mining techniques used in this facility do not include blasting and the shaft was constructed by upreaming[18]. This limits mechanical damage to the wall rock compared with blasting. Only crystals from primary sedimentary features were removed. Each crystal used for microbiological sampling was examined, and crystals were rejected as damaged if inclusions showed rounded edges, elongation or stress-related roughness along the walls of the inclusion[15]. A large number of crystals were rejected, but this procedure helps ensure that inclusions were not contaminated during collection. The 250-Myr-old crystal sample that contained viable bacteria was found in a dissolution pipe 569 m below the surface, in the wall of the air intake shaft of the Waste Isolation Pilot Plant, Carlsbad, New Mexico (Fig. 1). The crystal measured 3.5 × 3.5 × 2.5 cm and was one of two primary crystals from this site that contained living bacteria (Table 1).

In addition to establishing that the crystals are primary, sterility is critical when attempting to find organisms trapped in the precipitating crystals[1,8,9]. The sample surface must be sterilized by a verifiable technique, and the sample must be extracted under aseptic conditions. The surface of samples like amber can be sterilized using glutaraldehyde, bleach and ethanol[2,6,7]. Unfortunately, the reactivity and solubility of NaCl means this type of sterilization cannot be applied to these rocks.

Recently, several new bacterial species and/or genera were cultured from unsterilized Salado halite dissolved in commonly used halophile medium[23]. The largest bacterial population found in the Salado proved to be less than $1 \times 10^5$ colony-forming units (c.f.u.) per gram of salt.

Immersing crystals in 10M NaOH, washing, then immersing in 10M HCl provides a minimal sterility assurance level of at least 1 in $10^{-11}$(ref. 24). Steam sterilization of all extraction equipment (see Methods) provides a sterility assurance level of 1 in $10^{-9}$. Consequently, we assume that 1 in $10^{-9}$ is the overall minimum sterility assurance level for the experiment. In addition to the sterility assurances, contamination controls were used during all drilling operations. These included open plates of media distributed at random in the hood, streaking every sterilized crystal face before drilling, and an open plate of medium placed to catch the salt fines produced by the drill bits. These controls have been consistently negative for all salt crystals sampled.

The inclusion (crystal 1850-3A, Table 1) that yielded strain 2-9-3 measured about 3×3×1 mm (~9 μl of fluid). This fluid was inoculated into two different media: caesin-derived amino acids medium (CAS)[25] and glycerol–acetate medium (GA) (Table 1) (R.H.V., W.D.R. & M. Kamekura, manuscript in preparation). Only the CAS enrichment yielded any bacteria. Streak plates made directly from the enrichment tube yielded only one type of colony. Another bacterial strain was present in a second inclusion (1850-3C) in this same crystal. A third strain was present in crystal 1850-2. Both of these strains are currently being compared with isolate 2-9-3 and other bacilli. The *Bacillus* species (designated 2-9-3) was first isolated on CAS agar supplemented with 20% (w/v) NaCl. The original growth conditions were 37 °C with aeration on a rotating drum.

The complete 16S rDNA sequence of 2-9-3 (GenBank accession number AF166093) was compared with all other known rDNA sequences through a GenBank BLAST search. Similar rDNA sequences were downloaded from the database Internet site using GenBank accession numbers and aligned using ClustalW 1.7. The phylogeny of the new isolate was then determined using programs in

**Table 1 Crystal sampling within the 569 m (1850 ft) region of the Salado Formation**

| Crystal number | Inclusion size (mm) | Fluid recovered (μl) | Medium type* | | |
|---|---|---|---|---|---|
| | | | CAS | CG | GA |
| 1850-1 | 2 × 3 × 3 | 2 | – | – | n.i.† |
| 1850-2 | 2.5 × 3 × 4.5 | 10 | + | – | – |
| 1850-3A‡ | 3 × 3 × 1 | 9 | + | n.i. | – |
| 1850-3B | 3 × 2 × 2 | 10 | – | – | n.i. |
| 1850-3C | 4 × 3 × 3.5 | 12 | + | – | n.i. |

All of the crystals listed were sampled on the same day. Sixty-one additional inclusions in 50 crystals have also been sampled on additional days. A total of 365.5 μl of brine fluid have been inoculated into over 70 different test tubes. to date, only the three tubes listed have produced growth.
* CAS, caesin-derived amino acids medium; CG, cellulose glucose medium[23]; GA, glycerol-acetate medium.
† n.i., none inoculated.
‡ A, B and C refer to different inclusions within crystal-3.

**Table 2 Contamination control: sterilization procedures eliminate the 250-Myr-old isolate from crystal-sampling equipment**

| 2-9-3-contaminated materials | Pre-sterilization | | Post-sterilization | |
|---|---|---|---|---|
| | Day 3 | Day 7 | Day 3 | Day7 |
| Stock culture | +* | n.d.† | –‡ | – |
| 21 drill bits | + | n.d. | – | – |
| 4 plastic forceps | + | n.d. | – | – |
| 4 metal forceps | + | n.d. | – | – |
| 5-microlitre syringes | + | n.d. | – | – |

* 100% of plates positive for growth.
† n.d., not determined as all tests were positive at day 3.
‡ 100% of plates negative for growth.

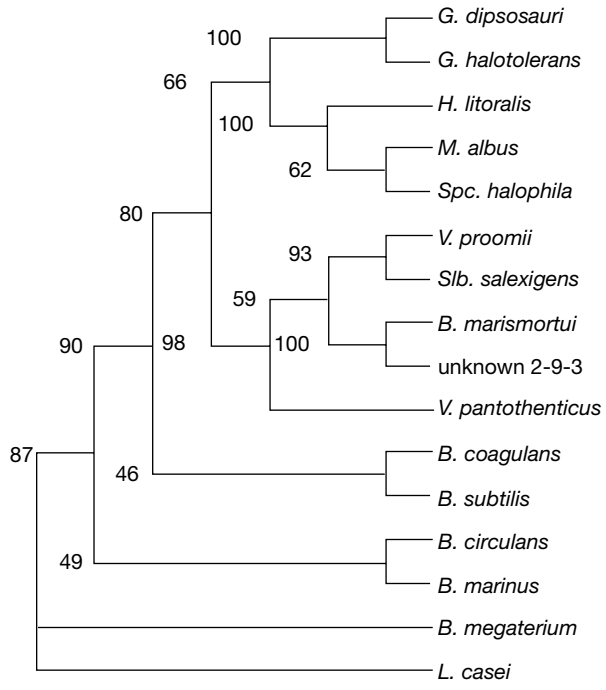


**Figure 2** Phylogenetic tree developed from the 16S rDNA sequence of unknown 2-9-3 compared with several other species of the family *Bacillaceae*. The bootstrap values from 100 repetitions for each grouping are shown to the left of the branch. The Genbank accession numbers for the organisms used in this analysis are *Bacillus megaterium* X60629; *B. subtilis* X60646; *B. marismortui* AJ009793; Isolate 2-9-3 AF166093; *B. coagulans* X60614; *B. circulans* X60613; *B. marinus* AJ237708; *Virgibacillus pantothenticus* X60627; *V. proomii* AJ0122667; *Halobacillus littoralis* X94558; *Gracilibacillus halotolerans* AF036922; *G. dipsosauri* X82436; *Salibacillus salexigens* (*Slb.*) Y11603; *Sporosarcina halophila* (*Spc.*) X62174. *Lactobacillus casei* D16553 served as the outgroup.

the Phylip phylogenetic software package (http://evolution.genetics.washington.edu/phylip.html). These analyses showed that the organism was most similar to *Bacillus marismortui* (99% similarity (S)[26] and *Virgibacillus pantothenticus* (97.5% S)[27]. Phylogenetic analysis showed that isolate 2-9-3, *B. marismortui*[26], *V. pantothenticus*[27], *Salibacillus salexigens*[28,29] and *V. proomii*[27] form a distinct lineage within the larger *Bacillus* cluster (Fig. 2).

The sterilization technique that we used[24] was initially developed using a non-spore-forming, Gram-negative, halotolerant eubacterium and a halophilic Archeon. As the first isolate found with these techniques proved to be a spore-forming, Gram-positive *Bacillus,* re-testing the procedures using 2-9-3 seemed warranted. Exposing a culture of 2-9-3 containing $10^8$ cells and spores to 10 M NaOH for 1 min reduced the population by at least $10^3$ c.f.u. $ml^{-1}$. Exposing the culture to 10 M HCl for 1 min reduced the number of viable cells and spores to undetectable levels (a reduction greater than $10^8$ c.f.u. $ml^{-1}$). In addition, all sampling equipment was first contaminated with cells and spores of 2-9-3 then autoclaved. The testing showed that the sterilization cycle used for this work eliminated the organism (Table 2). None of the plates exposed in the laminar flow hood for 50 min, or those used to sample the disinfected clamp used to hold crystals for drilling, produced viable colonies following 7 days of incubation.

As the crystal containing 2-9-3 was exposed to the 10 M NaOH for 5 min, washed in sterile brine, then exposed to 10 M HCl for 5 min, we believe that the crystal experienced the full effect of both sterilizing agents. Consequently, the probability that 2-9-3 was a surface contaminant on the crystal is less than 1 in $10^{-11}$. The data in Table 2 and that of Rosenzweig *et al.*[24] show that this bacterium could not have survived as a contaminant on any equipment or crystal used during this isolation. We conclude that strain 2-9-3 was isolated from the fluid inclusion contained within the 250-Myr-old primary crystal. The crystal sampled showed no sign of having been damaged or penetrated before this work. There is strong support for an age of 250 Myr for the crystal. Therefore, we believe that strain 2-9-3 is a bacterium that was present in a hypersaline environment during the late Permian, trapped inside a crystal at that time and survived within the crystal until the present.

The distribution of living microbes in salt crystals is not known at present. There may be regions of these ancient formations containing significant numbers of live bacteria[23]. Specific crystals may harbour several inclusions containing live organisms, although other inclusions and/or crystals from the same sample appear sterile (Table 1, ref. 23). However, only three enrichments grew from inclusions in 2 crystals out of 53 crystals sampled so far, indicating that survival may be a rare occurrence. At this point, we cannot address the mechanisms of survival, which may require a complex interaction between abiotic conditions in specific inclusions and biotic capability. In the case of *Bacillus* 2-9-3, sporulation would be a potential mechanism of survival as high ionic strength media does stimulate spore formation *in vitro*. However, we have no direct evidence of the presence of spores in the brine extracted from the inclusions.

The presence of 2-9-3 in these crystals is consistent with the basic sedimentary patterns in the Salado. Dissolution pits show that less saline waters covered exposed salt beds, dissolved salt to form pipes and began another round of deposition. The water with a lower salt content probably carried a variety of microorganisms into the evaporating salt pan. Bacteria with an ability to adapt to the increasing salt survived, and a bacterial spore in the brine may have been trapped in the crystals filling the pipes. Once the organism was fully encased, evolutionary pressures would have been relieved. Strains remaining in the standing brine may have continued along divergent evolutionary pathways.

Whether or not *Bacillus* strain 2-9-3 is an ancestor to these other organisms cannot be determined through the study of a single highly conserved gene such as the 16S rDNA[30]. It is interesting to note that there are 45 unequivocal sequence differences (in 1,560 base pairs) existing between the 16s rDNA of 2-9-3 and *Virgibacillus pantothenticus.* Assuming that these two organisms have been separated since 2-9-3 was trapped in the crystal (250 Myr), this becomes a base-pair substitution rate equal to $1 \times 10^{-10}$ substitutions per site per year. This substitution rate is slower than that found for *Bacillus sphaericus*[2] but identical to that calculated for enteric microbes[2] and Aphid symbionts[2]. The reasons for minimal sequence differences between 2-9-3 and *Bacillus marismortui* are unknown.

Previous reports of bacteria isolated from salt have met with resistance, partly because of concerns for contamination. Samples from many studies have been described as brines, rock salt, efflorescences, recrystallized salt or wet salt. These terms provide little or no assurance of the provenance of the sample or geological history. Flowing brines and salt efflorescences, for example, may be contaminated and young, and recrystallized salts may be of indeterminate age. In our study, however, we support the discovery by geological evidence of the overall age and stability of both the formation and the individual crystals, as well as the documented sterility maintained during the sampling and lab work. This research doubles the length of time bacteria can survive trapped inside stable geological materials. □

## Methods

Once selected, crystals were gently cleaned with a rotary grinder, and polished using distilled water. This process removed any adhering soils and eliminated small fissures that might prevent the sterilant from reaching all portions of the crystal surface. The polishing step also increased the ability to see the inclusion and monitor the progress of the drill bit. The cleaned crystals were moved into a Biological Level 3 clean-room facility physically separated from our main laboratory. Personnel working with crystals wore clean room

coveralls, boots, hats and latex gloves. Drill bits, media, washes and syringes were sterilized by autoclaving. All autoclaving was carried out for 40 min at 121 °C in a double door pass through autoclave, continually serviced under annual maintenance contracts with quarterly inspection and testing. Drill bits, forceps and syringes used for samplings were individually packaged before sterilization. All media were placed into test tubes (no more than 8 ml per tube) and autoclaved. The manufacturer's specifications indicate that all of the autoclaved materials were sterilized to a sterility assurance level equal to $1 \times 10^{-9}$, or 1 chance of contamination in $10^9$. In addition, all media to be used for samples were placed into an incubator at 37 °C for 3 days before use. If any medium batch contained even a single contaminated tube, the entire batch was discarded.

Crystals were sterilized as described[24]. All sterilants and brine washes were placed into sterile covered beakers. The cleaned crystals were immersed in 10 M NaOH for 5 min, washed in sterile saturated salt brine for 2 min, then immersed in 10 M HCl for 5 min. Following the HCl, each crystal was washed in sterile saturated salt brine buffered with sodium carbonate. This protected the stainless steel of the laminar flow hoods and drill bits from the HCl. All washes and sterilants were changed after each crystal. Sterilized crystals were removed from the buffered brine inside a dedicated Class IIA laminar flow hood fitted with an HEPA filter. The laminar flow hood was disinfected with germicidal ultraviolet light for 2 h before use. All hood surfaces and non-autoclavable equipment were disinfected with a commercial disinfectant between each drilling[24]. Sterile crystals were tightly held in a pinch clamp and penetrated with a sterile 0.5 mm wire drill operated with a micromanipulator. The inclusion samples were extracted from the crystals using sterile 25- or 250-μl syringes. The brine samples were inoculated into the sterilized growth medium.

To be sure that the spore-forming isolate was not a contaminant, the various materials used in the handling, drilling and extraction of fluids from salt crystals were deliberately contaminated with a 36-h culture of isolate 2-9-3 containing a mixture of cells and spores. All contaminated materials were streaked onto plates of tryptic-soy agar (TSA), and CAS agar amended with either 8% (CAS-8) or 20% (CAS-20) NaCl. One plate of each medium was used. All materials were then packaged as normal for crystal work, autoclaved and, after cooling, were again streaked onto TSA, CAS-8 and CAS-20 plates. All plates were incubated at 37 °C and scored for the presence of growth after 3 and 7 days. The clamp used to hold crystals during drilling in the biosafety hood, was disinfected with Wescodyne and streaked onto plates of TSA, CAS-8 and CAS-20 medium. Two plates each of TSA, CAS-8 and CAS-20 were opened and placed into the running biosafety hood following 1 h of ultraviolet light. The plates were exposed for at least 50 min, equal to the time needed to sample three crystals. The stock culture of 2-9-3 used for contamination was also streaked onto TSA, CAS-8 and CAS-20 medium before and after autoclaving.

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# The proteins of linked genes evolve at similar rates

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**Much more variation in the rate of protein evolution occurs than is expected by chance[1]. But why some proteins evolve rapidly but others slowly is poorly resolved. It was proposed, for example, that essential genes might evolve slower than dispensable ones[2], but this is not the case[3]; and despite earlier claims[4], rates of evolution do not correlate with amino-acid composition[5]. A few patterns have been found: proteins involved in antagonistic co-evolution (for example, immune genes[3,6], parasite antigens[7] and reproductive conflict genes[8–10]) tend to be rapidly evolving, and there is a correlation between the rate of protein evolution and the mutation rate of the gene[1,11,12]. Here we report a new highly statistically significant predictor of a protein's rate of evolution, and show that linked genes have similar rates of protein evolution. There is also a weaker similarity of rates of silent site evolution (see ref. 13), which appears to be, in part, a consequence of the similarity in rates of protein evolution. The similarity in rates of protein evolution is not a consequence of underlying mutational patterns. A pronounced negative correlation between the rate of protein evolution and a covariant of the recombination rate indicates that rates of protein evolution possibly reflect, in part, the local strength of stabilizing selection.**

To examine the effects of linkage on rates of evolution, we established a data set of rates of evolution at both non-synonymous ($K_a$) and synonymous ($K_s$) sites of mouse–rat orthologues with confirmed genomic location. Rates of evolution of genes were compared with those less than 1 centiMorgan (cM) apart, this being equivalent to on average about 2,000 kilobases (kb) in the mouse genome[14]. We calculated the modular difference ($\Delta K$) between the $K$ values (that is, either $K_a$ or $K_s$, or $K_a/K_s$ depending