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Prevalence of Sucretolerant Bacteria in Common Soils and Their Isolation and Characterization

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Abstract

Sucretolerant microbes grow in the presence of sugar concentrations high enough to substantially lower water activities. Natural habitats high in sugars are mainly limited to dried fruit, floral nectar, honey, sugarcane, and associated soils. Organisms that tolerate extremes of solute concentration, high enough to lower water activities, might not be expected in common oligosmotic soils. We report on the isolation of sucretolerant bacteria from common soils using media supplemented with 50% sucrose (a_w 0.91) and their physiological characterization and identification by 16S rRNA gene phylogeny. Fifteen of these sucretolerant isolates from common soils were related to four *Bacillus* spp. A *Lysinibacillus* and a *Microbacterium* (actinomycete) also were collected. All grew at 50% sucrose and 13 grew at 60% sucrose. Most probable number counts were used to determine the abundance of sucretolerant microbes in several common soil types, including agricultural, managed turf, and native prairie. Microbial abundance (with fungicides) was about 10^5 and 10^3 cells g^{-1} soil in media containing 50 or 70% sucrose, respectively. The abundances of sucretolerant bacteria in common soils mirror those of halotolerant bacteria that grow at 10 and 20% NaCl. However, there is not a correlation between halotolerance and sucretolerance in our isolates, nor can predictions be made based on taxonomy. Specific solute effects may be at work, rather than biological responses to a single physicochemical parameter such as a_w . The occurrence of spore-forming sucretolerant bacteria in common soils has relevance to forward planetary protection and astrobiology. Extraterrestrial habitable regions are defined in part by tolerance to high solute concentrations and osmotolerant soil microbes may contaminate spacecraft.

Keywords

bacteria; extremophiles; halotolerance; hypersaline; soil; sucretolerance; sugar

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Introduction

Low water activity is a hallmark of environments with high concentrations of solutes. Microbes require liquid water for survival and the vast majority of bacteria grow poorly, or not at all, when the water activity of the medium is substantially below that of pure water (a_w 1.00) (Grant 2004; Schneegurt 2012). While microbes grow in seawater (a_w 0.97), even this salinity (~3%) is not tolerated by most microbes. The effect of high solute concentrations on microbial growth has mainly been studied using NaCl and microbes from hyperhaline environments. Ionic solutes other than NaCl, that dominate certain hypersaline environments, have been examined to a lesser extent, such as lakes rich in magnesium sulfate (Oren 1983a, b; Crisler et al. 2012; Cray et al. 2013; Kilmer et al. 2014). These studies have demonstrated that responses to salts can differ even at similar water activities. In addition, responses can differ between isolates that cluster together phylogenetically. While arguments have been made for the central role of water activity or chaotropicity in microbial growth, it appears that cellular responses are more complex than can be described by a single physical parameter (Williams and Hallsworth 2009; Cray et al. 2013; Fredsgaard et al. 2017). The concept of specific solute effects was proposed by Scott (1953) to explain the observation that the inhibition of microbial growth in highly osmotic media was not due to solute concentration and water activity alone (Scott 1953; Beuchat 1981; Chirife 1994; Chirife et al. 1995). Bacterial responses are then expected to vary by solute. For instance, responses to high concentrations of sugars are not the same as responses to high concentrations of salts for salinotolerant (and hence osmotolerant) bacteria. For many of our bacterial isolates from hypersaline environments, growth tolerance is greater to sugars than to salts (including *Nesterenkonia* and *Planococcus*), while other isolates are more tolerant to salts than sugars (including *Bacillus*, *Halomonas*, and *Planococcus*), or equally tolerant (or intolerant) to both (including *Marinococcus*) (Fredsgaard et al. 2017).

Stable high-sugar environments are not common. Decaying fruit and cane, floral nectar, honey, and aphid exudates are examples of natural high-sugar environments (Ruiz-Argueso and Rodriguez-Navarro 1975; Gilliam et al. 1983; Iurlina and Fritz 2005; Olaitan et al. 2007; Álvarez-Pérez et al. 2012). Commercial food production generates high-sugar streams such as sugar thick juice and maple syrup (Hollaus and Klaushofer 1973; Belrami et al. 1991; Justé et al. 2008a; Thompson 2009). Osmotolerant and osmophilic yeasts (*Candida*, *Debaryomyces*, *Metschnikowia*, *Pichia*, *Zygosaccharomyces*) are known from high-sugar environments (Nishi 1963; Sandhu and Waraich 1985; Lachance et al. 2001; Herrera et al. 2008, 2009; Justé et al. 2008a, b). These are environments that can have very high osmotica, with sugar contents as high as 70%, thereby creating low water activities (Jermini and Schmidt-Lorenz 1987; Justé et al. 2008; Grant 2004). A few bacteria (*Gluconobacter*, *Lactobacillus*) have been described from nectars and syrups, but these have not been shown to be sucrotolerant in culture (Gilliam et al. 1983; Raguso 2004). Microbes found in honey are derived from the microbial community of bees, mainly gut organisms within the *Bacillus* (Ruiz-Argueso and Rodriguez-Navarro 1975; Sandhu and Waraich 1985; Brysch-Herzberg 2004; Herrera et al. 2008). Previous literature questions whether microbes even proliferate in honey. It does not appear that bacterial isolates from honey have been demonstrated to be sucrotolerant in culture.

The most extremely osmotolerant microbial isolates to-date are fungi from high-sugar environments that grow at the lowest water activities yet reported (Pitt and Christian 1968; Grant 2004; Schneegurt 2012). A few of these sucrotolerant (able to grow in high sugar) fungi appear to be osmophilic (requiring high osmotica for growth) and may be sucrophilic (requiring high sugar for growth). No equivalent bacterial osmophiles or sucrophiles have been reported, only halophilic microbes (requiring high NaCl for growth) and salinotolerant bacteria that grow in high concentrations of NaCl and other salts.

While it may be reasonable to assume that microbes tolerant of high salt concentrations, typically associated with hypersaline environments, would not be found in oligosaline environments, this does not appear to be the case (Nielsen et al. 1995; Eichigo et al. 2005; Chen et al. 2010). We have isolated bacteria from common soils that are capable of growth in 20% NaCl (Kilmer et al. 2010; Porazka et al. 2011). The abundance of salinotolerant microbes in common oligosaline soils appears to be substantial. The current study examines common soils for sucrotolerant microbes that grow in media supplemented with 50 or 70% sucrose. Sucrotolerant microbial isolates were obtained from common soils and then identified and characterized.

Methods

Media and Water Activity

The development of artificial nectar media with salts (ANS) has been described previously (Fredsgaard et al. 2017). ANS series media contained at least 0.5% (w/v) of each fructose, glucose, and sucrose. This was then supplemented with sucrose to produce a series of media with a designation indicating % sucrose (w/v), such as ANS50, which contains 50% sucrose. ANS media also were supplemented with amino acid sources (0.5% [w/v] of both yeast extract and tryptone). Sugar solutions and amino acid solutions were autoclaved separately to prevent browning, presumably due to Maillard reaction products. Media made this way were lightly colored and caramelization was not substantial. ANS media were further supplemented with salts: (per L) 2.0 g NH_4SO_4 , 0.36 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 1 mg $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 0.16 g MgCl_2 , 2.0 g KCl, and 0.5 ml trace minerals (per L; 2 mg CoCl_2 , 10 mg $\text{CuSO}_4 \cdot 6 \text{H}_2\text{O}$, 200 mg $\text{MnCl}_2 \cdot 6 \text{H}_2\text{O}$, 100 mg $\text{NaMoO}_4 \cdot 2 \text{H}_2\text{O}$, 100 mg $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$). A filter-sterilized antifungal cocktail was added to ANS series media from a 100X stock (per 100 ml; 1.0 g carbendazim, 1.0 g cycloheximide, and 0.16 g nystatin). Salt Plains (SP) artificial seawater media have been described previously (Caton et al. 2004), supplemented with 5 to 30% (w/v) NaCl or with MgSO_4 concentrations up to 60%.

The water activity of each medium was measured using an Aqualab Series 3 water activity meter (Decagon Devices, Inc., Pullman, WA) and are shown in Table 1. The instrument was calibrated with standard NaCl solutions and operated at room temperature.

Soil Collection and Most Probable Number (MPN) Counts

Composite soil samples were collected from a variety of habitats in the Wichita, KS region, as described in Table 2. This included managed turf areas and native prairie grassland, two

agricultural soils, and two soils associated with wetlands. None of these oligosaline environments had appreciable bulk salinities.

At each location, a 10-m² area was staked and three equivalent bare spots (that may include a rhizosphere component) were selected haphazardly for sampling. Within a 1-m² area, five points were haphazardly selected, and ~10 g of soil was collected from the top 2 cm at each point after brushing away leaf litter. Thus, composite samples included a total of 15 subsamples, in an effort to dampen heterogeneity (nuggets) among small samples to obtain a representative soil sample. Sampling was performed using sterile instruments and containers. The composite samples were mixed in bags by hand and aliquots were taken for microbiology and physical analyses.

For each MPN array, 10 g of the composite sample were diluted with 90 ml of the appropriate medium supplemented with 0.1% Na pyrophosphate (a chaotropic agent), then placed on an orbital shaker (150 rpm) for 1 h to dislodge microbes. After the dilution bottle had settled for a few minutes, 0.2 ml was transferred to the first tube of each MPN trial. The MPN analyses were designed with five repetitions of six 10-fold serial dilutions in 13 × 100-mm culture tubes with a volume of 2 ml and then maintained on an orbital shaker (150 rpm). Positive tubes were scored after two weeks visually or by measuring turbidity at 600 nm using a Genesys 10S spectrophotometer (ThermoFisher). A threshold value of 0.2 OD units was used to score positive growth. Scores were compared to a statistical table to determine the MPN and the 95% confidence interval factor was 3.3 (Woomer 1994).

The dry weight of soils were determined gravimetrically after heating at 110 °C to constant weight. Salinity was tested on a filter-sterilized 1:10 aqueous dilution of dry soil using a handheld salinity refractometer with automatic temperature compensation (Fisher). A 50:50 soil:water slurry was used for pH measurements with an Accumet 25 instrument (Fisher).

Microbial Isolation and Characterization

The highest dilution, positive tubes from MPN analyses with ANS50 medium were used for microbial isolation. Aliquots of positive cultures were directly plated or serially diluted for spread plating. Colonies arising on plates were selected for isolation based on gross morphological and physiological features, including pigmentation, size, margin, or rate of growth. Separated colonies were then purified by 6 repetitive-streaking isolations. Bacterial isolates were curated as 50% glycerol stocks at –80 °C and as agar slants.

Salinity and sugar tolerances were determined using isolates grown as triplicate shake-tubes in 2 ml of media in a moist box on a rotating platform (150 rpm). Culture density was determined visually or as absorbance at 600 nm using a medium blank. Salinity testing was performed in SP medium supplemented with NaCl or MgSO₄. Temperature tolerance from 4 to 60 °C was measured using ANS 50 plates held at the appropriate temperature for two weeks. Isolates were Gram-stained using the Protocol Gram-staining kit (Fisher Diagnostics) following the manufacturer's instructions. Acid-fast staining was done using the Ziehl-Neelsen (ZN) method and nitrate reduction tests were done as previously described (Caton et al. 2004).

Isolate Identification

Crude DNA extracts from each isolate were prepared using a freeze-thaw technique as described in Caton et al. (2004). Genomic DNA in the clarified supernatant was the target of PCR amplification of nearly complete 16S rRNA gene fragments using bacterial primers (EUBPA: 5′-AGAGTTTGATCCTGGCTCAG-3′ and EUBPH: 5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989). PCR was performed in a thermal cycler (Eppendorf Mastercycler) as 25-μL reactions containing 0.2 μM of each primer, 1 U of ExTaq DNA polymerase and associated master mix (Takara), and 5 μL of cell extract. DNA was denatured at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final 5-min extension at 72 °C. PCR amplicons were single-pass sequenced using the EUBPA primer by a commercial vendor. Negative controls using sterile water instead of DNA extracts produced no amplicons.

Sequences were automatically aligned using Clustal-W (Thompson et al. 1994) and then manually examined and trimmed in MacClade v4.08 (Sinauer Associates). Contextual 16S rRNA gene sequences were identified in GenBank or RDP using BLAST (Altschul et al. 1990) or from comparison to relevant literature. PAUP 4.0 b10 (Swofford 1998) generated phylogenetic trees using distance analysis, with Jukes-Cantor rules and the neighbor-joining algorithm. Sequences were trimmed to equal lengths and positions with gaps and ambiguous bases ignored, leaving 826 positions for analysis. Bootstrap analysis was used to assess the relative support for each branch with a total of 100 replicates conducted heuristically using the distance-based, neighbor-joining algorithm and the nearest-neighbor-interchange algorithm in PAUP. The trees were rooted using *Aquifex pyrophilus* as the functional outgroup. No putative chimeras were identified through iterative analyses using Pintail within MOTHUR (Schloss et al. 2009). All sequences appear in GenBank with accession numbers KX021844 to KX021860.

Results

Abundance of Sucretolerant Microbes by MPN Counts

Most probable number analysis combines serial dilution arrays and statistical tables to estimate the abundance of microbes in discrete samples that can grow under the chosen culture conditions. ANS media supplemented with 50 or 70% sucrose was used for MPN analyses of common soils, those that are oligoosmotic, low in salt and other solutes. The base medium for ANS includes the complex ingredients (yeast extract and tryptone) and broad assemblage of salts that are commonly found in general purpose media. Maintaining aerobic cultures in ANS medium selects for certain microbes and excludes others. Growing all viable cells from environmental samples is not possible, since the conditions for optimal microbial growth vary so widely among taxa and microbes may be present in a viable but non-culturable state. The values reported here should be interpreted as mainly reflecting the abundance of culturable aerobic heterotrophs, rather than total viable cell (or spore) counts. A fungicide mixture was routinely added to the media to inhibit the growth of fungi and provide a better estimate of the abundance of sucrotolerant bacteria (and potentially archaea). Agricultural soils, creek and lake margins, and turf soils, both managed and native, were examined using fresh composite samples (Table 2).

There were appreciable numbers of sucrotolerant organisms in common soils that grew in the presence of 50% sucrose (Table 3). About 10^5 growing cells per g^{-1} soil were observed for these common soils. With a water activity of 0.91, only specialized organisms can survive and grow in ANS50. The Great Plate Anomaly is a term used to describe the large differences in microbial abundance observed by culture-based and direct microscopic examination of environmental samples, and is believed to be the result of selective culture media and incubation conditions and cells that are viable but non-culturable (Olsen and Bakken 1987). Typical soil harbors $\sim 10^8$ cells g^{-1} soil observable by growth on a single medium, with values 10- to 100-fold greater based on direct microscopic cell counts. On this basis, $<0.1\%$ of the bacteria present in common soils grow in culture in the presence of 50% sucrose. The absence of fungicides did not appreciably change the abundance of sucrotolerant microbes observed on ANS50. The abundance of bacteria from common soils capable of growth in the presence of 70% sucrose was a small fraction ($\sim 0.1\%$) of the abundance of bacteria capable of growth in the presence of 50% sucrose.

Isolation and Identification of Sucrotolerant Bacteria from Common Soils

Enrichment cultures in ANS50 supplemented with fungicides were used to isolate sucrotolerant bacteria from common soils. Serial dilution, spread-plating, and repetitive streak-plating of isolated colonies were used to purify bacterial strains. Seventeen sucrotolerant isolates were obtained from enrichments of common soils at 50% sucrose. The isolates were identified by phylogenetic analysis of 16S rRNA gene sequences (Fig. 1). Only Gram-positive bacteria were isolated, with no representatives from the Gram-negative or CFB clusters. All of the isolates, except ST3, were within the *Bacillus* clade of Gram-positive bacteria. This included examples of *B. megaterium*, *B. methylotropicus*, *B. pumilus*, *B. thuringiensis*, and *Lysinibacillus sphaericus*. A sucrotolerant example of *Microbacterium arthrosphaerae* was the only high-GC Gram-positive Actinobacteria isolated.

Characterization of Sucrotolerant Bacteria from Common Soils

All of the isolates were capable of growing in ANS media supplemented with 50 or 60% sucrose. While sucrotolerance in these isolates was high, salinotolerance also was substantial (Table 4). All of the isolates grew in the presence of 60% MgSO_4 , near saturation ($a_w = 0.91$). All but 4 of the isolates grew at 10% NaCl and many grew at 15% NaCl ($a_w = 0.88$). None grew at 20% NaCl ($a_w = 0.85$). It should be noted that water activities for MgSO_4 brines are higher than those of corresponding concentrations of NaCl, since MgSO_4 does not dissociate effectively in water.

All of the isolates stained Gram-positive as expected given their identification by rRNA gene sequencing. All of the isolates were negative for mycolic acids by acid-fast staining. Nitrate reduction to nitrite was observed for ST1 and ST3 (Table 4). Temperature tolerance screening found that all of the isolates grew in the range of 25 to 45 °C (Table 4). Growth was observed for all but two isolates at 50 °C, while 7 isolates grew at 55 °C. None of the isolates grew at 60 °C and none grew at 4 °C.

Discussion

It is natural to associate organisms that can live under extreme chemical or physical conditions with environments that are defined by these extremes. However, microbes that can grow under extreme conditions can be isolated from common environments that do not appear to be extreme. Unlike hypersaline environments, which cover large areas of the Earth's surface, there are precious few natural high-sugar environments, including: aphid exudates, dried fruit, floral nectar, honey, and sugarcane. We have found sucrotolerant bacteria in common oligoosmotic soils, apart from these sugary environments. No previous study has collected or counted sucrotolerant microbes found in common soils. From the discussion below it appears that our collection more resembles the halotolerant microbes in common soils (mainly Bacilli) rather than the wider range of bacteria found in natural high-sugar environments.

Flower nectars vary greatly in sugar content and quality, with sugar contents of 70% in some instances (Pleasants 1983; Jermini et al. 1987; Jermini and Schmidt-Lorenz 1987; Tokouka and Ishitani 1991). Nearly all microbiological studies of floral nectars have focused on osmophilic yeasts (Sandhu and Waraich 1985; Lachance et al. 2001; Mushtaq et al. 2006, 2008; Herrera et al. 2009). A study of floral nectars from a variety of plants in the Arizona desert, recovered no yeasts, but isolated a few unidentified bacteria that were in low abundance (Gilliam et al. 1983). A more comprehensive study of nectar bacteria from wild flowers found low species diversity (Álvarez-Pérez et al. 2012). Most of the isolates among the 18 taxa observed were within the *Gammaproteobacteria*, including *Pantoea* and *Pseudomonas*. Nearly all of the isolates grew in media containing 30% sucrose, although it should be noted that this concentration does not change water activity (0.96) enough to be restrictive for many bacteria (Fredsgaard et al. 2017).

Honey is produced in two stages: an initial evaporation carried out by the honeybee that decreases the water content to 40%–50%, and a second evaporation which occurs as the honey ripens in the honeycomb, which reduces the water content to 15%–18% (Ruiz-Argueso and Rodriguez-Navarro 1975). Microorganisms found in honeycombs are bacteria and yeasts that primarily derive from honeybee guts (Olaitan et al. 2007). Two groups of bacteria, *Gluconobacter* and *Lactobacillus*, were primarily detected in ripening honey (Ruiz-Argueso and Rodriguez-Navarro 1975). *Zymomonas* also were observed in honey. Once the moisture content reached 18%, no viable bacteria were detected.

The food industry produces manufacturing streams with very high sugar contents. Extraction juice from sugar beets was the source of 60 bacterial isolates, mainly *Bacillus* spp., with three of these growing at 40% sucrose (Belamri 1991). *Bacillus* spp. also seemed to be metabolically active in sugar beet magma (67 °Bx), but no microbes were recovered from thick juice in this study (Klaushofer et al. 1971; Hollaus 1978; Klaushofer et al. 1998). In a contrasting study, sugar beet thick juice (66 to 72 °Bx) produced 53 isolates, but sugar tolerances were not measured, except for a *Tetragenococcus* isolate which grew at 66% sucrose or 25% NaCl (Justé et al. 2008a, b). Sugar beet extracts also have yielded thermotolerant bacterial isolates of unknown sucrotolerance (Haskä and Nystrand 1982; Nystrand 1984).

A few studies have specifically enriched for halotolerant microbes in common oligohaline soils (Echigo et al. 2005; Usami et al. 2007; Chen et al. 2010; Chen et al. 2011a, b; Mendpara et al. 2013; Jhala et al. 2015). The most extensive project isolated over 500 strains of bacteria from low-salt ($<20 \text{ mg NaCl g}^{-1}$ soil) soils near Tokyo Bay by direct plating onto medium containing 20% NaCl (Echigo et al. 2005, 2007, 2010). Identified isolates were mainly *Bacillus*, *Filobacillus*, *Gracibacillus*, *Halobacillus*, and *Virgibacillus*, with some growing at 2.6 M ($\sim 15\%$) NaCl. A similar study using a variety of non-saline soils isolated over 100 strains of halotolerant *Bacillus* (Nielsen et al. 1995). Two isolates grew at 20% NaCl, while nearly all grew at 8% NaCl. A wider taxonomic range of halotolerant bacteria (5 to 20% salinity tolerance) was obtained from enrichment cultures of non-saline forest soils (Chen et al. 2010). Among the 61 isolates identified were Actinomycetes, Firmicutes (mainly *Bacillus*), and a few *Proteobacteria*. Finally, high-salt fungi (*Aspergillus* and *Penicillium*) have been isolated from common Kansas soils, with several growing in saturated NaCl, saturated sucrose (79%), or 33% CaCl_2 (Chen 1964).

Microbial growth tolerances to different solutes vary in a manner that is not easily described by a single physicochemical property such as water activity (Fredsgaard et al. 2017). The concept of specific solute effects is valuable in this context, where physiological responses to solutes reflect a more complex interplay of parameters that are specific to individual solutes (Scott 1953; Beuchat 1981; Chirife 1994; Chirife et al. 1995). At the same degree of saturation, bacterial responses to salts and sugars differ (Fredsgaard et al. 2017). Isolates in the current study were enriched at high sugar concentrations and all grew in ANS50 (a_w 0.91). Substantial salinity tolerance also was observed for these isolates with growth at 10 or 15% NaCl (a_w 0.92 or 0.88, respectively), and all isolates grew at 60% MgSO_4 (a_w 0.91). However, several isolates did not grow at 10% NaCl. None of the sucrotolerant isolates grew at 20% NaCl or in saturated brine, with no growth seen below an a_w of 0.88. In contrast, we have isolated bacteria from common soils by enrichment at 10% NaCl that can tolerate 20% NaCl (Kilmer et al. 2010; Porazka et al. 2011). Even with the limited set of solutes and isolates in the current study, it is clear that cellular responses to salts and sugars vary in a way not described by a_w alone. Our previous survey of salinotolerant bacteria for sucrotolerance also suggests that taxonomic assignment is not a good predictor of growth tolerances (Fredsgaard et al. 2017). The current study found sucrotolerant isolates mainly in the Bacilli, but salinotolerant Bacilli were not especially sucrotolerant.

Our initial studies of oligosaline turf soils found that 1.7×10^5 and 7.1×10^3 cells g^{-1} soil were tolerant to 10 and 20% NaCl, respectively (Kilmer et al. 2010; Porazka et al. 2011). The abundance of sucrotolerant microbes in these soils appears to be in a similar range. In common soils, bacterial abundance measured in ANS50 medium (with fungicides) was 0.64 to 3.9×10^5 cells g^{-1} soil. Microbial abundance measured in ANS70 medium was substantially lower (0.12 to 1.2×10^3 cells g^{-1} soil), even though its measured a_w is not appreciably lower than that of ANS50. While abundance values are similar among media containing 70% sucrose and 20% NaCl, note that the a_w for 20% NaCl (0.85) is substantially lower than the a_w for 70% sucrose (0.90). It is interesting to note that the abundance of sucrotolerant bacteria in soils from Hot Lake, BC, an environment saturated with MgSO_4 ($a_w \sim 0.9$), were about 100X lower than in common soils, with fungi making up a greater

proportion of sucrotolerant microbes, however overall biomass in Hot Lake soils is greatly lower than in common soils (Fredsgaard and Moore, unpublished observations).

The ecological significance of finding extremely tolerant microbes in common soils is unclear. Tolerance to high solute concentrations may offer no particular reproductive advantage in common soils and at the same time may not have substantial costs to tolerant organisms. This would suggest that sucro- and salinotolerance are fortuitous, with neutral selection pressure in these environments. In contrast, sucro- or salinotolerance may enhance survival should hyperosmotic microhabitats exist in common soils. For example, if water vapor was present inside of pores within soil crumbs, salts (or sugars) might dissolve creating saturated brine, within a bulk soil that is oligosmotic. Finally, it has been suggested by Eichigo et al. (2005) that extremely tolerant microbes are transient members of the community in common soils. The tolerant microbes might be transported as spores that may persist for long periods of time in soils under conditions that do not permit growth. We did not distinguish between spores and vegetative cells in the current study and none of the isolates were sucrophilic, requiring high sugar concentrations for growth. Furthermore, while *Bacillus* and *Lysinibacillus* spp. form spores, *Microbacterium* spp. do not. It seems likely that the presence of different sucro- and salinotolerant microbes in soils represents a range of survival strategies and natural histories.

The abundance of sucrotolerant microbes in common soils has direct relevance to astrobiology from two perspectives, habitability and forward planetary protection. Spacecraft visiting celestial bodies invariably carry microbes deposited during their assembly. The clean dry environments in spacecraft assembly facilities (SAF) have been shown to harbor salinotolerant microbes (Venkateswaran et al. 2001; La Duc et al. 2003). The assemblage of microbes in SAFs appears to derive from common soils and human microflora (Foster and Winans 1975; Puleo et al. 1977). Initial studies have found that common soils near JPL SAFs exhibit about the same abundance of microbes ($\sim 10^5$ cells g^{-1} soil) capable of growing in 50% sucrose media as found in the common soils examined in the current report (Al Soudi and Zayed, unpublished observations). Terrestrial environments on Mars are arid and liquid water may only be present in the form of heavy brines, saturated with salts that depress their freezing points (Zorzano et al. 2009; Davila et al. 2010). Finding microbes in common soils that might grow in dense brines has relevance to the refinement of planetary protection protocols. Special Regions on Mars and the icy worlds that may be habitable have been defined in part based on a_w , using the growth of yeasts in high-sugar media at record-low a_w as the standard (Pitt and Christian 1968; Rummel et al. 2014). We have shown in the current report and previously that sucrotolerance and salinotolerance in bacteria are not correlated and that taxonomic assignment is not a good indicator of tolerance. The current study demonstrates that sucrotolerant bacteria are present in common soils that might contaminate SAFs and spacecraft with organisms that can survive at low water activities.

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Figure 1. Phylogenetic tree based on 16S rRNA gene sequences for sucrotolerant bacterial isolates from common soils. Bootstrap values greater than 50% are shown.

Table 1

Water activities of media supplemented with various concentrations of sugars or salts.

Concentration (% w/v)	Solute		
	Sucrose	NaCl	MgSO ₄
1	0.98	0.95	0.99
10	–	0.92	0.98
15	–	0.88	–
20	–	0.85	0.98
25	–	0.80	–
30	0.96	0.76	0.97
40	0.94	–	0.95
50	0.91	–	0.94
60	0.90	–	0.91
70	0.90	–	–

Table 2

Description of soil samples used for most probable number counts and microbial isolations.

Soil	pH	Salinity	Moisture	Location
Cornfield	7.1	0	3.6	37°46′43″N/97°24′27″W
Creek margin	6.7	0	30.1	37°44′31″N/97°16′21″W
Managed turf	7.4	0	25.9	37°43′04″N/97°17′20″W
Pond margin	6.9	0	33.5	37°44′36″N/97°16′10″W
Prairie grassland	6.0	0	20.5	37°44′33″N/97°16′30″W
Soybean field	7.1	0	4.2	37°46′36″N/97°24′38″W

Table 3

Abundance of sucrotolerant microbes determined by most probable number counts of soils in media containing 50 or 70% sucrose, with or without fungicides. The 95% CI factor was 3.3 for these assays.

Soil	Abundance of Sucrotolerant Microbes (cells g⁻¹ soil)			
	Sucrose with Fungicides		Sucrose without Fungicides	
	50%	70%	50%	70%
Sweet corn	3.91×10^5	5.35×10^2	3.91×10^5	6.38×10^3
Soybean	3.91×10^5	1.15×10^3	3.91×10^5	6.38×10^3
Hot Lake	1.15×10^3	1.15×10^2	3.90×10^3	1.65×10^2
Creek	1.64×10^5	1.15×10^2		
Pond	2.26×10^5	1.15×10^2		
Golf	2.46×10^5	1.15×10^2		
Prairie	6.38×10^4	1.15×10^3		

Table 4

Characteristics of sucrotolerant bacterial isolates from common soils.

Isolate	Solute Tolerance (% w/v)			Maximum Growth Temperature (°C)	Nitrate Reduction
	Sucrose	NaCl	MgSO ₄		
1	60	<10	60	45	+
2	60	15	60	50	-
3	60	<10	60	45	+
4	60	15	60	55	-
5	60	<10	60	55	-
6	60	15	60	55	-
7	60	15	60	55	-
8	60	15	60	50	-
9	50	15	60	50	-
10	60	15	60	50	-
11	60	15	60	55	-
12	60	15	60	55	-
13	60	15	60	55	-
14	60	<10	60	50	-
15	50	15	60	50	-
16	50	10	60	50	-
17	50	10	60	50	-