Sucrose Metabolism in Haloarchaea: Reassessment Using Genomics, Proteomics, and Metagenomics

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ABSTRACT The canonical pathway for sucrose metabolism in haloarchaea utilizes a modified Embden-Meyerhof-Parnas pathway (EMP), in which ketohexokinase and 1-phosphofructokinase phosphorylate fructose released from sucrose hydrolysis. However, our survey of haloarchaeal genomes determined that ketohexokinase and 1-phosphofructokinase genes were not present in all species known to utilize fructose and sucrose, thereby indicating that alternative mechanisms exist for fructose metabolism. A fructokinase gene was identified in the majority of fructose- and sucrose-utilizing species, whereas only a small number possessed a ketohexokinase gene. Analysis of a range of hypersaline metagenomes revealed that haloarchaeal fructokinase genes were far more abundant (37 times) than haloarchaeal ketohexokinase genes. We used proteomic analysis of *Halohasta litchfieldiae* (which encodes fructokinase) and identified changes in protein abundance that relate to growth on sucrose. Proteins inferred to be involved in sucrose metabolism included fructokinase, a carbohydrate primary transporter, a putative sucrose hydrolase, and two uncharacterized carbohydrate-related proteins encoded in the same gene cluster as fructokinase and the transporter. Homologs of these proteins were present in the genomes of all haloarchaea that use sugars for growth. Enzymes involved in the semiphosphorylative Entner-Doudoroff pathway also had higher abundances in sucrose-grown *H. litchfieldiae* cells, consistent with this pathway functioning in the catabolism of the glucose moiety of sucrose. The study revises the current understanding of fundamental pathways for sugar utilization in haloarchaea and proposes alternatives to the modified EMP pathway used by haloarchaea for sucrose and fructose utilization.

IMPORTANCE Our ability to infer the function that microorganisms perform in the environment is predicated on assumptions about metabolic capacity. When genomic or metagenomic data are used, metabolic capacity is inferred from genetic potential. Here, we investigate the pathways by which haloarchaea utilize sucrose. The canonical haloarchaeal pathway for fructose metabolism involving ketohexokinase occurs only in a small proportion of haloarchaeal genomes and is underrepresented in metagenomes. Instead, fructokinase genes are present in the majority of genomes/metagenomes. In addition to genomic and metagenomic analyses, we used proteomic analysis of *Halohasta litchfieldiae* (which encodes fructokinase but lacks ketohexokinase) and identified changes in protein abundance that related to growth on sucrose. In this way, we identified novel proteins implicated in sucrose metabolism in haloarchaea, comprising a transporter and various catabolic enzymes (including proteins that are annotated as hypothetical).

KEYWORDS archaea

Sucrose is composed of a glucose unit linked to a fructose unit via an α -1,2-glycosidic linkage and is the most abundant disaccharide in terrestrial environments due to its presence in tissues of vascular plants (1). In addition to its role in plants, sucrose is a metabolite of the aquatic green-microalga *Dunaliella* (2), an inhabitant of hypersaline

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FIG 1 Known sucrose catabolism pathways in haloarchaea. The pathways show the separate fates of glucose degraded by the semiphosphorylative ED pathway and fructose degraded by the modified EMP pathway: purple, sucrose-specific steps; blue, spED pathway; red, modified EMP pathway; green, common shunt. Note that the conversion of glucose to gluconate in the spED pathway involves two steps: oxidation of glucose to gluconolactone, followed by spontaneous hydrolysis, or hydrolysis catalyzed by an unidentified gluconolactonase, to gluconate. 1-PFK, 1-phosphofructokinase; ABC, ATP-binding cassette; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KDG, 3-deoxy-2-oxo-D-gluconate; KDPG, 2-dehydro-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; PEP-PTS, PEP-dependent phosphotransferase system; S-layer, surface layer.

habitats, and a source of nutrients for haloarchaea (class *Halobacteria*). Sucrose can be synthesized by *Dunaliella* under both light and dark conditions (3, 4). Although *Dunaliella* synthesizes molar levels of glycerol as the major osmolyte (5), sucrose is also regarded as an osmotically active solute in this alga (3, 4).

The fates of glucose and fructose have been examined in a limited number of haloarchaea. In *Haloarcula vallismortis*, *Haloarcula marismortui*, and *Haloferax mediterranei* glucose and fructose are mostly degraded by different pathways: glucose is degraded by the semiphosphorylative Entner-Doudoroff (spED) pathway, and fructose is degraded by a modified Embden-Meyerhof-Parnas (EMP) pathway (6–12). These haloarchaeal glycolytic pathways are variants of the classical Entner-Doudoroff (ED) and EMP pathways, respectively (10, 12–15). In the haloarchaeal spED pathway, oxidation precedes phosphorylation: glucose is oxidized to gluconate (rather than being phosphorylated), and the phosphorylation step is deferred to later in the pathway using 3-deoxy-2-oxo-D-gluconate (KDG) as the substrate. The haloarchaeal EMP pathway involves the same conversions as the classical EMP pathway, except that fructose in the cytoplasm is phosphorylated to fructose 1-phosphate, rather than fructose 6-phosphate, by a ketohexokinase that is unique to haloarchaea (6–9) (Fig. 1 and 2a). Fructose 1-phosphate can also be generated during fructose uptake using a fructose phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (Fig. 1) (11).

(a) Modified EMP pathway
(e.g., *Haloarcula* spp., *Haloferax* spp.)

(b) Classical EMP pathway
(Bacteria & Eucarya)

(c) Classical EMP pathway
with phosphofructomutase
(e.g., *Aeromonas hydrophila*)

(d) Classical ED pathway
(e.g., Bacteria)

(e) Semi-phosphorylative ED pathway
with GFO domain protein

(f) Semi-phosphorylative ED pathway
with XI/TM domain protein

FIG 2 Alternative fructose catabolism pathways. The pathways shown include those ruled out for *Hht. litchfieldiae* based on genomic interrogation and proteomic data (indicated by a red cross). (a) Modified EMP pathway, known only for certain (Continued on next page)

However, the sucrose uptake mechanism has yet to be determined in haloarchaea, with studies on *Hfx. mediterranei* and *Har. vallismortis* showing no evidence for the involvement of a PEP-PTS for the uptake and concomitant phosphorylation of sucrose (7). Moreover, the associated cytoplasmic enzyme responsible for hydrolyzing sucrose to glucose and fructose has not been identified (7).

Halohasta litchfieldiae strain tADL was isolated from Deep Lake, Antarctica (16), where it represents the numerically dominant species (17). Its ability to effectively compete has been linked to an ability to utilize sugars (17–19), and the laboratory isolate has been found capable of growth using sucrose, fructose, glucose, and glycerol as substrates (18, 20). Pyruvate, which is the main end product of sugar catabolism, also supports the growth of *Hht. litchfieldiae* tADL (18). When combined with sucrose or glycerol, pyruvate stimulates strong growth (18). Similar promotive effects of pyruvate have been observed for other haloarchaea (21).

In this study, we surveyed 27 genomes of sugar-utilizing or non-sugar-utilizing species of haloarchaea for genes associated with the degradation of sucrose and its cleavage products, glucose and fructose. After finding ketohexokinase absent but fructokinase present in the majority of genomes of saccharolytic species, with results mirrored in metagenome data of hypersaline environments, we explored sucrose/fructose metabolism by performing proteomic analysis of *Hht. litchfieldiae* tADL. By providing sucrose plus pyruvate as defined carbon sources and comparing proteomic profiles to those of cells grown with pyruvate alone, we assessed the potential pathways involved in sugar metabolism, focusing in particular on how fructose metabolism could occur in the absence of ketohexokinase.

RESULTS AND DISCUSSION

Genomic and metagenomic surveys. (i) Sucrose uptake and hydrolysis. Sucrose uptake in haloarchaea does not appear to involve a PEP-PTS (7); sucrose PEP-PTS genes were not identified in the haloarchaeal genomes. Sucrose permease genes associated with sucrose uptake in bacteria (1) were also absent. The most likely mechanism for sucrose uptake involves ATP-binding cassette (ABC) transporters (CUT1 family) (22); relevant genes were found associated with those encoding glucose- or fructose-specific enzymes (especially glucose dehydrogenase or fructokinase, respectively). A gene for the enzyme responsible for sucrose hydrolysis was proposed in *Har. marismortui* (*rrnAC1479*) (14), and homologs (glycosyl hydrolase family 32) were found in a total of nine genomes (Fig. 3; see also Table S1 in the supplemental material).

The sucrose ABC transporter gene cluster often included two experimentally uncharacterized genes with protein domains suggestive of a role in glucose and/or fructose metabolism: COG0673 (predicted dehydrogenase; glucose-fructose oxidoreductase [GFO] domain) and COG1082 (sugar phosphate isomerase/epimerase; xylose isomerase-like, triosephosphate isomerase [XI/TIM] barrel domain) (Fig. 3, Table S1, and Fig. S1) (see also “(ii) Fructose catabolism” below in “Proteomics”). One or both genes were often located next to genes involved in sucrose catabolism, including fructokinase (*Hht. litchfieldiae*, *Halostagnicola larsenii*, *Natronobacterium gregoryi*, and *Natronococcus occultus*) (Fig. S1) and the putative sucrose ABC trans-

FIG 2 Legend (Continued)

haloarchaea. The absence of an identifiable ketohexokinase precludes this pathway in the majority of fructose-utilizing haloarchaea. (b) Classical EMP pathway. The absence of an identifiable 6-phosphofructokinase precludes this pathway in fructose-utilizing haloarchaea. (c) Classical EMP pathway with phosphofructomutase, an enzyme not known in haloarchaea. (d) Classical ED pathway. The absence of an identifiable 6-phosphogluconate dehydratase precludes this pathway in fructose-utilizing haloarchaea. (e) Novel hypothetical pathway which entails the concomitant oxidation and reduction of glucose and fructose, providing an intermediate (gluconate) that can enter the haloarchaeal semiphosphorylative ED pathway. (f) Novel hypothetical pathway which entails the conversion of fructose to glucose, which can enter the spED pathway. Novel enzymes proposed for the pathways in panels e and f are shown in gold. Note that, for simplicity, the conversion of glucose to gluconate is represented by a single arrow. DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFO, glucose-fructose oxidoreductase; KDG, 3-deoxy-2-oxo-D-gluconate; KDPG, 2-dehydro-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; XI/TIM, xylose isomerase-like, triosephosphate isomerase barrel.

		sucrose uptake & hydrolysis		semi-phosphorylative ED pathway					modified EMP pathway					common spED/EMP shunt				gluconeogenesis only							
	utilisation of sugars (sucrose, glucose, fructose)	sucrose ABC transport system	β-fructo-furanosidase (GH32)	sucrose hydrolase (GH15)	glucose dehydrogenase	gluconate dehydratase	KDG kinase	KDPG aldolase (archaeal)	KDPG aldolase	fructokinase	ketohexokinase	fructose PEP-PTS	1-phospho-fructokinase	FBP aldolase class I	FBP aldolase class II	triose phosphate isomerase	phosphoglycerate kinase	phosphoglycerate mutase	enolase	pyruvate kinase	fructose-1,6-bisphosphatase	PEP synthase	GAPDH type II	GFO domain	XII/TIM domain
Har. vallismortis	S, G, F							x	x						x										
Har. marismortui	S, G, F							x	x						x										
Har. hispanica	S, G, F							x	x						x										
Hfx. volcanii	S, G, F		x					x	x					x											
Hfx. mediterranei	S, G, F		x					x	x					x											
Hmc. mukohataei	S, G			x				x	x			x			x										
Hht. litchfieldiae	S, G, F		x					x		x															
Hrr. lacusprofundi	S, G, F							x		x	x	x			x										
Hgm. borinquense	S, G, F		x					x		x	x	x	x	x											
Hst. larsenii	S, G, F		x					x		x					x		x						!		
Nbt. gregoryi	S, G, F	x	x	x				x	x	x	x	x	x		x										
Htg. turkmenica	S, G, F										x					x							!		
Hrd. utahensis	G, F	x	x		x						x	x		x							x	x	x	x	x
Hrd. tiamaea	G, F			x	x						x	x		x							x	x	x	x	x
Nnm. pellirubrum	G, F		x	x				x			x	x	x		x		x						!		
Ncc. occultus	S, G								x		x	x	x		x										
Hac. jeotgali	S, G			x				x			x														
Hpg. xanaduensis	G		x						x		x	x	x		x										
Nab. magadii			x					x			x	x	x		x										
Hqr. walsbyi		x	x						x		x	x	x	x										x	x
Hvx. ruber		x	x	x	x				x	x	x	x	x		x										
Nmn. pharaonis		x	x	x	x	x	x	x	x	x	x	x	x		x		x							x	x
Nmn. moolapensis		x	x	x					x		x	x	x		x		x							x	x
DL31		x	x	x				x		x	x	x	x		x		x							x	x
Hbt. salinarum		x	x	x				x		x	x	x	x		x		x							x	x
Hbt. sp. DL1		x	x	x	x	x	x	x	x	x	x	x	x		x		x							x	x
Haa. sulfurireducens		x	x	x	x	x	x	x	x	x	x	x	x		x		x							x	

FIG 3 Genes related to sucrose metabolism encoded in haloarchaeal genomes. Data for utilization of sugars (sucrose [S], glucose [G], and fructose [F]) is based on previous reports (16, 18, 25, 26, 29, 33, 34, 62–76). Filled boxes indicate the presence of the gene(s), and colors are used to highlight related genes (e.g., genes from the same pathway); the absence of gene(s) is indicated by an x. GAPDH type I is associated with glycolysis whereas GAPDH type II is associated with gluconeogenesis, except in three species (*Hst. larsenii*, *Htg. turkmenica*, and *Natrinema pellirubrum*) in which GAPDH type II is assumed to operate in both directions (indicated by an exclamation point). ABC, ATP-binding cassette; ED, Entner-Doudoroff; FBP, fructose-1,6-bisphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFO, glucose-fructose oxidoreductase; GH15, glycoside hydrolase family 15; GH32, glycoside hydrolase family 32; KDG, 3-deoxy-2-oxo-D-gluconate; KDPG, 2-dehydro-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; PEP-PTS, phosphoenolpyruvate-dependent phosphotransferase system; spED, semiphosphorylative ED pathway; XI/TIM, xylose isomerase-like, triose phosphate isomerase barrel domain. *Har. vallismortis*, *Haloarcula vallismortis*; *Har. marismortui*, *Haloarcula marismortui*; *Har. hispanica*, *Haloarcula hispanica*; *Hfx. volcanii*, *Haloferax volcanii*; *Hfx. mediterranei*, *Haloferax mediterranei*; *Hmc. mukohataei*, *Halomicrobium mukohataei*; *Hht. litchfieldiae*, *Halohasta litchfieldiae*; *Hrr. lacusprofundi*, *Halorubrum lacusprofundi*; *Hgm. borinquense*, *Halogeometricum borinquense*; *Hst. larsenii*, *Halostagnicola larsenii*; *Nbt. gregoryi*, *Natronobacterium gregoryi*; *Htg. turkmenica*, *Haloterrigena turkmenica*; *Hrd. utahensis*, *Halorhabdus utahensis*; *Hrd. tiamaea*, *Halorhabdus tiamaea*; *Nnm. pellirubrum*, *Natrinema pellirubrum*; *Ncc. occultus*, *Natronococcus occultus*; *Hac. jeotgali*, *Halalkalicoccus jeotgali*; *Hpg. xanaduensis*, *Halopiger xanaduensis*; *Nab. magadii*, *Natrialba magadii*; *Hqr. walsbyi*, *Haloquadratum walsbyi*; *Hvx. ruber*, *Halovivax ruber*; *Nmn. pharaonis*, *Natronomonas pharaonis*; *Nmn. moolapensis*, *Natronomonas moolapensis*; *Hbt. salinarum*, *Halobacterium salinarum*; *Hbt. sp. DL1*, *Halobacterium* sp. strain DL1; *Haa. sulfurireducens*, *Halanaeroarchaeum sulfurireducens*. DL31 is an undescribed Antarctic haloarchaeal genus (17).

porter (*Hht. litchfieldiae*, *Hst. larsenii*, *Haloterrigena turkmenica*, *Ncc. occultus*, and *Natrialba magadii*) (Fig. S1).

(ii) Haloarchaeal spED pathway. The essential enzymes for the degradation of glucose to pyruvate via the haloarchaeal spED pathway were encoded in most genomes of haloarchaeal species known to utilize sucrose and glucose: glucose dehydrogenase, gluconate dehydratase, KDG kinase, 2-dehydro-3-deoxy-phosphogluconate (KDPG) aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase (Fig. 1 and 3; Table S1). The last five enzymes listed comprise the lower shunt for the conversion of glyceraldehyde 3-phosphate to pyruvate that is common to both the ED and EMP pathways (including the modified versions of both in haloarchaea) and were present in all the surveyed haloarchaeal genomes (Fig. 3 and Table S1). There are two distinct and unrelated types of KDPG aldolases in haloarchaea: the bacterial-type (COG0800), and an archaeal-type (COG0329) (23). Both were represented among the surveyed haloarchaeal genomes, with some species encoding both types (*Halorhabdus utahensis*,

Halorhabdus tiamatea, and *Htg. turkmenica*) and certain nonsaccharolytic species lacking both types (*Natronomonas pharaonis*, *Halobacterium* sp. strain DL1, and *Halanaerobacterium sulfurreducens*) (Fig. 3 and Table S1).

While the genes involved in the spED pathway were generally identifiable, there are a number of noteworthy clarifications. First, the immediate product of glucose dehydrogenase is gluconolactone, which can spontaneously hydrolyze to gluconate. However, this occurs slowly (especially at low temperatures), so a gluconolactonase may be required to catalyze this conversion (12). Lactonases can belong to multiple unrelated families and are therefore difficult to identify (24). The gluconolactonase provisionally identified in *Haloferax volcanii* (HVO_B0083) (15) does not have recognizable homologs in the majority of haloarchaeal genomes, including Antarctic *Hht. litchfieldiae*, which may be expected to have the gene as it grows in a cold environment. As such, the precise cellular mechanisms leading to hydrolysis of gluconolactone in the spED pathway remain to be determined.

Second, a glucose dehydrogenase gene is absent in the two *Halorhabdus* spp. even though they reportedly grow on glucose and fructose (but not sucrose) (25, 26). The two *Halorhabdus* sp. genomes were also unique among the 27 genomes in encoding a bacterial-like xylose isomerase (23), and in bacteria xylose isomerase functions in the interconversion of glucose and fructose (27). The growth properties of these species might be explained by fructose being converted to glucose and then entering the spED pathway via an unidentified glucose dehydrogenase or by glucose converted to fructose and catabolized via a pathway involving fructokinase (see "Haloarchaeal fructokinase" below).

Third, the inability of certain haloarchaea to metabolize glucose (*Nmn. pharaonis*, *Halobacterium* sp. DL1, and *H. sulfurreducens*) likely reflects the lack of an spED pathway (Fig. 3 and Table S1). However, despite encoding a complete spED pathway, some haloarchaea have been reported to lack the ability to utilize glucose as a carbon and energy source. This was observed for *Halobacterium salinarum* NRC-1 (28, 29) even though the enzyme activity of glucose dehydrogenase was found to be induced by glucose (30); this has been attributed to the absence of a catabolic GAPDH type I in *Hbt. salinarum* (31) (see the paragraph "Glucose catabolism and gluconeogenesis" in "Proteomics" below).

(iii) Haloarchaeal EMP pathway involving ketohexokinase. For fructose degradation, the haloarchaeal EMP pathway involves the same conversions as the classical EMP pathway, except that fructose is phosphorylated to fructose 1-phosphate rather than fructose 6-phosphate. In addition, fructose 1-phosphate is further phosphorylated to fructose 1,6-bisphosphate, which is an intermediate in the classical EMP pathway (12). The haloarchaeal EMP pathway for fructose catabolism is initiated by either ketohexokinase or fructose PEP-PTS. Cytoplasmic fructose is phosphorylated to fructose 1-phosphate in an ATP-dependent manner by ketohexokinase (7, 9), while fructose PEP-PTS generates fructose 1-phosphate during uptake of fructose (11) (Fig. 1).

The activity of haloarchaeal ketohexokinase has been characterized although the enzyme has not been identified (6–9). A candidate gene in *Halomicrobium mukohataei* has been proposed (23), and homologs are present in six genomes (Fig. 3 and Table S1), including the species with demonstrated ketohexokinase activity (*Har. vallismortis*, *Har. marismortui*, and *Hfx. mediterranei*) (6, 7, 9, 32). Pair-wise comparison of the experimentally derived, estimated amino acid composition of *Har. vallismortis* ketohexokinase (8) with the amino acid sequence of the *Har. vallismortis* candidate ketohexokinase showed a statistically significant positive correlation ($r = 0.91$) (Table S2), providing supporting evidence that they are the same protein. Overall, the above data support the hypothesis that the gene identified in *Hmc. mukohataei* (23) is responsible for producing ketohexokinase activity. The gene is present in six genomes that represent only three genera: *Haloferax*, *Haloarcula*, and *Halomicrobium* (Fig. 3 and Table S1). A total of nine of the 27 haloarchaeal genomes encode the components of a fructose PEP-PTS uptake system, including the three species with demonstrable ketohexokinase activity. *Hmc. mukohataei* is the only

FIG 4 Maximum-likelihood phylogenetic tree of PFK-B sugar kinase family proteins. The tree includes fructokinase, KDG kinase, and 1-phosphofructokinase (84 proteins in total). Sulfofructokinase from *Escherichia coli* was used as the outgroup, and bootstrap values greater than 50% are reported. For the expanded tree, see Fig. S2 in the supplemental material.

haloarchaeon surveyed here that encodes a putative ketohexokinase but lacks identifiable fructose PEP-PTS genes.

The enzyme that catalyzes the phosphorylation of fructose 1-phosphate to fructose 1,6-bisphosphate is 1-phosphofructokinase (7, 9, 32). For *Har. marismortui*, 1-phosphofructokinase enzyme activity was shown to be very high for cultures growing on fructose (9). Twelve of the 27 genomes, including the majority of sugar-utilizing species, encode 1-phosphofructokinase (Fig. 3 and Table S1). All six species that encode ketohexokinase also encode 1-phosphofructokinase. Genes for 1-phosphofructokinase are present in six other species, four of which encode a fructose PEP-PTS (the *Halorhabdus* spp. do not) adjacent to the 1-phosphofructokinase gene (Fig. S1), suggesting that these species employ PEP-PTS and 1-phosphofructokinase for consecutive phosphorylations of fructose. A number of haloarchaea are reported to have the ability to grow on sucrose and fructose (18, 33, 34) but lack an identifiable 1-phosphofructokinase (Fig. 3 and Table S1), as has been noted for *Halogeometricum borinquense* (23). Collectively, these growth, enzymatic, and genomic data indicate that additional fructose degradation pathways are likely to exist in addition to the modified EMP pathway used by haloarchaea.

(iv) Haloarchaeal fructokinase. The genomes of 14 haloarchaeal species, including most sugar-utilizing haloarchaea, encode a gene annotated as fructokinase (COG0524). Haloarchaeal genomes that encode this putative fructokinase do not encode ketohexokinase and vice versa; the two genes are mutually exclusive among the surveyed genomes. Haloarchaeal fructokinases form a phylogenetic cluster with known fructokinases from bacteria and hyperthermophilic archaea (Fig. 4 and Fig. S2), providing circumstantial support for their functionality. A survey of metagenomes from hypersaline environments revealed that haloarchaeal fructokinase genes are far more abundant than haloarchaeal ketohexokinase genes: 1,467 versus 40 across 15 metagenomes representing five hypersaline habitats (Deep Lake, Antarctica; Cahuil Lagoon, Chile; Santa Pola, Spain; Isla Christina, Spain; Lake Tyrrell, Australia) (Table 1). Thus, despite the key role of ketohexokinase in the only characterized fructose degradation pathway in haloarchaea (12, 14, 23), ketohexokinase is not the dominant protein for fructose phosphorylation in haloarchaeal-dominated hypersaline environments.

Fructokinase catalyzes the ATP-dependent phosphorylation of fructose to fructose 6-phosphate (35–37). However, from genomic interrogation, a route by which fructose

TABLE 1 Number of ketohexokinase and fructokinase genes in metagenomes from global hypersaline environments that contain haloarchaea

Metagenome identification no. ^a	Total no. of reads	Location	No. of ketohexokinase genes	No. of fructokinase genes	Reference
DL2008_5mRS_0.1um	1,007,472	Deep Lake, Antarctica	1	95	17
DL2008_13_0.1um	1,158,857	Deep Lake, Antarctica	1	87	17
DL2008_24_0.1um	1,108,448	Deep Lake, Antarctica	4	124	17
DL2008_24_0.8um	1,063,876	Deep Lake, Antarctica	6	131	17
DL2008_24_3um	931,207	Deep Lake, Antarctica	3	132	17
DL2008_36_pooled	2,787,468	Deep Lake, Antarctica	6	245	17
SRR1549536	222,074	Cahuil Lagoon, Central Chile	2	8	77
SRR328983	740,891	Santa Pola, Spain	1	105	78
SRR979792	970,656	Santa Pola, Spain	5	81	79
SRR316684	293,368	Santa Pola, Spain	0	39	78
SRR328982	1,315,367	Santa Pola, Spain	0	56	78
SRR944625	1,494,771	Santa Pola, Spain	1	7	80
SRR988245	1,223,923	Isla Christina, Spain	5	34	79
SRR5637210	1,085,431	Lake Tyrrell, Australia (sample HAT)	4	161	81
SRR5637211	1,167,998	Lake Tyrrell, Australia (sample HBT)	1	162	81
Total no. of genes			40	1,467	

^aDeep Lake (DL) metagenomes and metagenomes deposited in the NCBI Sequence Read Archive (SRR prefix).

6-phosphate could enter the EMP pathway in haloarchaea is not obvious. The enzyme 6-phosphofructokinase, which generates fructose 1,6-bisphosphate from fructose 6-phosphate, has not been identified in any haloarchaeal genome (14, 23, 28, 38) (Fig. 2b); this includes the classical ATP-dependent 6-phosphofructokinase, as well as ADP- and pyrophosphate-dependent archaeal 6-phosphofructokinases (14). One possibility is that fructose 6-phosphate could enter the EMP pathway using a novel haloarchaeal 6-phosphofructokinase, perhaps with broad specificity, as found for many phosphofructokinase B (PFK-B) family enzymes (39, 40). As a precedent in archaea, a PFK-B family phosphofructokinase/nucleoside kinase from *Aeropyrum pernix* exhibited a broad range of phosphoryl acceptors that included fructose 6-phosphate, fructose, and various nucleosides (40).

It has been proposed that fructose 6-phosphate may be converted to fructose 1-phosphate to enter the EMP pathway (23) (Fig. 2c); but a phosphofructomutase, an inducible enzyme in the bacterium *Aeromonas hydrophila* (41), has not been documented in archaea, and we did not identify obvious candidate genes in haloarchaeal genomes. In bacteria, fructose 6-phosphate can also be directed to the classical ED pathway (42) (Fig. 2d). However, again, there is no evidence that any haloarchaea have a complete classical ED pathway. Although haloarchaea encode the necessary enzymes for the conversion of fructose 6-phosphate to 6-phosphogluconate (glucose 6-phosphate isomerase and glucose 6-phosphate dehydrogenase) as part of the oxidative pentose phosphate pathway, haloarchaea lack an identifiable 6-phosphogluconate dehydratase gene for the conversion of 6-phosphogluconate to 2-keto-3-deoxy-D-gluconate (KDPG), and no 6-phosphogluconate dehydratase activity has been found in the haloarchaea for which this has been tested (*Hal. vallismortis* and *Hfx. mediterranei*) (7). In the absence of an obvious path for fructose catabolism, we utilized proteomics to explore the possible fate(s) of fructose released from sucrose catabolism.

Proteomics. *Hht. litchfieldiae* was used as a model for haloarchaea that lack an identifiable ketohexokinase and can grow on sucrose and/or fructose. Forty-eight proteins were found to be differentially abundant (significantly higher or lower by 1.5-fold) in sucrose-grown cells (Table S3), out of a total of 1,096 detected proteins (Table S4). Fourteen of the differentially abundant proteins were noted for their potential relevance in sucrose metabolism (Fig. 5 and Table S3) and are discussed below.

(i) Sucrose uptake and hydrolysis. Sucrose-containing cultures had higher abundances of the solute-binding lipoprotein (halTADL_1911) and ATPase (halTADL_1908)

FIG 5 Possible sucrose metabolism pathways in *Hht. litchfieldiae* based on proteomic data. Proteins are indicated as follows: pink, sucrose-specific proteins involved in sucrose uptake and hydrolysis into glucose and fructose; blue, glucose-specific proteins associated with the semiphosphorylative ED pathway for glucose catabolism; orange, fructose-specific and oxidative pentose phosphate proteins; green, common semiphosphorylative ED pathway and EMP pathway proteins; red, gluconeogenesis proteins. Hypothetical reactions are indicated in gold. Enzymes that exhibited at least a 1.5-fold change in abundance in sucrose-containing medium have an arrow beside their names showing increased (upward arrow) or decreased (downward arrow) abundance. Note that of the two GFO domain proteins in the gene cluster, only halTADL_1905 was differentially abundant; halTADL_1905 and halTADL_1907 share 27% identity. ABC, ATP-binding cassette; DHAP, dihydroxyacetone phosphate; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFO, glucose-fructose oxidoreductase domain protein; KDG, 3-deoxy-2-oxo-D-gluconate; KDPG, 2-dehydro-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; TrmB, putative TrmB transcriptional regulator; XI/TIM, xylose isomerase-like/triosephosphate isomerase barrel domain protein.

components of a CUT1 family ABC transporter (23), which we infer to be the sucrose importer in *Hht. litchfieldiae* (see “(i) Sucrose uptake and hydrolysis” in “Genomic and metagenomic surveys” above). The components are most closely related to characterized archaeal and bacterial carbohydrate import ABC transporters (43, 44), with sequence identities of 28% for the solute-binding lipoprotein and 45% to 46% for the ATPase.

Sucrose degradation may involve halTADL_0141 (glycosyl hydrolase family 15) because its abundance was higher in sucrose-grown cells. The homologs present in 14 other haloarchaea (Fig. 3 and Table S1) may play a similar functional role. Six of these genomes (not including *Hht. litchfieldiae*) also contained the β -fructofuranosidase (glycosyl hydrolase family 32) gene first proposed for sucrose hydrolysis in *Har. marismortui* (14) (Fig. 3 and Table S1). These hydrolase proteins lack signal peptides, which is consistent with cytoplasmic cleavage of sucrose (7). These data suggest that haloarchaea possess multiple mechanisms for sucrose hydrolysis, with some species possibly utilizing two different hydrolases.

(ii) Fructose catabolism. Glucose was detected in the supernatant of sucrose-grown *Hht. litchfieldiae* cultures soon after cultures began exponential growth; glucose concentrations (average of three replicates) were 28 μM , 52 μM , and 330 μM for 2, 4, and 6 days, respectively, after cultures entered exponential phase. In contrast, cultures grown only on pyruvate showed no glucose in the medium. The identification of a putative primary transporter and cytoplasmic hydrolase for sucrose in the proteome (see “(i) Sucrose uptake and hydrolysis” above) is consistent with nonphosphorylated glucose being leaked from cells into the medium rather than with extracellular sucrose breakdown.

However, the fate of fructose in *Hht. litchfieldiae* is unclear. Whereas the genomic and proteomic data point to a key role for fructokinase (halTADL_1913) in fructose metabolism, there is less evidence to describe how fructose 6-phosphate (the product of fructokinase) could enter glycolysis. It is possible that fructose 6-phosphate enters the oxidative pentose phosphate pathway. The requisite enzymes for the synthesis of ribulose 5-phosphate from fructose 6-phosphate were detected in the *Hht. litchfieldiae* proteome: glucose-6-phosphate isomerase (halTADL_0801), glucose-6-phosphate dehydrogenase (halTADL_0298), and 6-phosphogluconate dehydrogenase (halTADL_2122) (Table S4). Because we supplied both sucrose and pyruvate in the growth medium, it is possible that cells were able to direct fructose toward anabolic processes via the pentose phosphate pathway rather than to glycolysis (to make pyruvate). Thus, fructose could be fed directly into the pentose phosphate pathway using fructokinase, rather than indirectly via gluconeogenesis using fructose 1,6-bisphosphatase.

If fructose is used catabolically, it could possibly enter the spED pathway via an undescribed mechanism. In *Har. marismortui*, ^{13}C -labeling studies showed that glucose was degraded exclusively via the spED pathway, whereas fructose was degraded predominantly via the haloarchaeal EMP pathway (96%), with a small fraction (4%) degraded via the spED pathway (9, 12). The *Hht. litchfieldiae* genome encodes two 1-phosphofructokinase proteins (Table S1), but neither was detected in the proteome. Although the absence of protein detection in proteomics does not rule out that the protein was synthesized by the cell, the nondetection of 1-phosphofructokinase is consistent with *Hht. litchfieldiae* not utilizing the haloarchaeal EMP pathway for degradation of fructose in the cytoplasm. Other proteins of the EMP pathway that were detected (Fig. 5 and Table S4) were not differentially abundant, and their presence is explained by the role they play in gluconeogenesis (see “(iii) Glucose catabolism and gluconeogenesis” below).

Fructose could potentially enter the spED pathway via a mechanism that involves the protein halTADL_1905 (COG0673; GFO domain protein) or the protein halTADL_1906 (COG1082; xylose isomerase-like, TIM barrel domain) (see “(i) Sucrose uptake and hydrolysis” above in “Genomic and metagenomic surveys”). While these proteins are not exclusively associated with haloarchaea possessing fructokinase (Fig. 3, Table S1, and Fig. S1), for *Hht. litchfieldiae* it is conspicuous that both proteins are encoded in the same gene cluster as the sucrose ABC transporter system and fructokinase, and both had higher abundances in sucrose-grown cells. In general, the COG0673 and COG1082 proteins are encoded in haloarchaeal genomes that also encode either fructokinase or ketohexokinase (Fig. 3 and Table S1), which suggests that they play roles in carbohydrate metabolism that are independent of fructose phosphorylation. The GFO domain of halTADL_1905 invites comparison with the enzyme GFO, which catalyzes the concomitant oxidation and reduction of glucose and fructose, respectively, to generate gluconolactone (an spED pathway intermediate) and sorbitol (glucitol) (45, 46) (Fig. 2e). However, we are doubtful that halTADL_1905 functions as a GFO. First, in the bacterium *Zymomonas mobilis*, which lives in sugar-rich environments, the sorbitol produced by periplasmic GFO is not metabolized but accumulates in the cytoplasm as an osmoprotectant in response to high extracellular sugar concentrations (45–47). Second, aside from *Z. mobilis* GFO, the GFO domain is present in other characterized enzymes, including a specific type of haloarchaeal xylose dehydrogenase (48) and the *Bacillus subtilis* glucose-6-phosphate 3-dehydrogenase (49). halTADL_1905

shows 25% to 27% sequence identity to the aforementioned enzymes and 27% identity to another GFO domain protein (halTADL_1907) in the same gene cluster as halTADL_1905 (Fig. 5) that was detected in the proteome but was not differentially abundant. Thus, GFO domains are found in diverse NAD(P)-binding enzymes associated with carbohydrate metabolism.

halTADL_1906 has a xylose isomerase-like, TIM barrel domain, which may mean that the enzyme catalyzes the isomerization of fructose to glucose; the glucose could enter the spED pathway (Fig. 2f), which would obviate a dedicated fructose degradation pathway involving fructokinase or ketohexokinase. Thus, both glucose and fructose moieties of sucrose could conceivably be degraded by the same pathway (spED pathway), separate from exogenous fructose (EMP pathway, initiated by concomitant uptake and phosphorylation by fructose PEP-PTS). In support of this possibility, a precedent for different glycolytic fates of endogenous and exogenous fructose is evident in certain bacteria. For example, in *Rhodobacter capsulatus* both the glucose and the fructose moieties of sucrose are catabolized via the classical ED pathway (35), whereas exogenously supplied fructose is imported by fructose PEP-PTS and degraded via fructose 1-phosphate and the classical EMP pathway (50).

(iii) Glucose catabolism and gluconeogenesis. Proteins involved in the breakdown of glucose to pyruvate via the spED pathway were detected in the *Hht. litchfieldiae* proteome, and the majority had higher abundances in sucrose-containing cells: glucose dehydrogenase (halTADL_0397), gluconate dehydratase (halTADL_0374), GAPDH type I (halTADL_0817), phosphoglycerate kinase (halTADL_0816), and pyruvate kinase (halTADL_3014) (Fig. 5 and Table S3).

All gluconeogenesis enzymes were also detected in the *Hht. litchfieldiae* proteome, including the irreversible enzymes fructose-1,6-bisphosphatase and PEP synthase (Table S4). Two PEP synthase homologs (halTADL_0698 and halTADL_1210) were among the most abundant proteins in cultures grown in sucrose-free medium compared to levels in sucrose-containing medium (Fig. 5 and Table S3), which accords with increased gluconeogenesis under these conditions. A carboxylate tripartite ATP-independent periplasmic (TRAP) transporter solute receptor of the TAXI family (halTADL_0243) (Table S3) also showed higher abundance in sucrose-free cultures, and we infer this protein to be involved in pyruvate uptake. The tricarboxylic acid (TCA) cycle proteins citrate synthase (halTADL_0686), aconitate hydratase (halTADL_2902), and succinate dehydrogenase SdhD subunit (halTADL_0444) showed lower abundances in sucrose-containing cultures (Table S3); this is consistent with a decreased contribution of the oxidative TCA cycle to energy conservation as a result of the increased generation of ATP and reducing equivalents through glycolysis.

The two GAPDH homologs showed contrasting abundances in response to carbon source: GAPDH type I (halTADL_0817) exhibited higher abundance in *Hht. litchfieldiae* grown in sucrose-containing medium, whereas GAPDH type II (halTADL_1211) exhibited lower abundance in these cultures; GAPDH type I and GAPDH type II showed the highest (increased 3.5-fold) and lowest (decreased 2.7-fold) abundances, respectively, of all detected proteins (Fig. 5 and Table S3). Similarly, in *Hfx. volcanii* growth on glucose resulted in higher expression of GAPDH type I but repression of GAPDH type II (12, 31, 51). This indicates inverse responses of the two GAPDH proteins, with GAPDH type I involved in glycolysis and GAPDH type II involved in gluconeogenesis (12, 31). An anabolic role for GAPDH type II is consistent with the absence of GAPDH type II in *Halorhabdus*, which lacks other identifiable genes for gluconeogenesis (23, 31). Most of the haloarchaea that possess GAPDH type II but lack GAPDH type I do not utilize carbohydrates and so would not be expected to perform glycolysis. However, three sugar-utilizing haloarchaeal species encode only GAPDH type II (no type I), which is presumably used in both directions (amphibolic) in these species, illustrating that dual GAPDH homologs (type I and type II) are not essential in all sugar-utilizing haloarchaea (Fig. 3 and Table S1).

Conclusion. The study showed that the fate of the glucose moiety of sucrose in haloarchaea is better defined than that of the fructose moiety. As has been previously described (9, 11, 15), evidence supports glucose being degraded by the haloarchaeal spED pathway. The fate of the fructose moiety in many haloarchaea remains equivocal, and it has become apparent that fructose degradation does not always conform to the modified EMP pathway described for haloarchaea (6–9, 11, 32). That is, the latter pathway, initiated by ATP-dependent fructose phosphorylation by ketohexokinase, is not present in the majority of available fructose- and sucrose-utilizing haloarchaeal genomes. Furthermore, based on metagenomic analysis, haloarchaeal ketohexokinase genes are far less abundant than haloarchaeal fructokinase genes in hypersaline habitats. As a result, we infer that ketohexokinase is not as important for the assimilation of sucrose and fructose as fructokinase. Fructose 6-phosphate, the product of fructokinase, could be diverted to the pentose phosphate pathway and/or enter glycolysis via an unknown route. The proteomic data for *Hht. litchfieldiae* emphasized the roles of previously known proteins involved in sucrose uptake and hydrolysis and provided evidence for the involvement of two proteins that had not previously been ascribed functions (halTADL_1905, COG0673; halTADL_1906, COG1082) that cluster with the fructokinase and sucrose ABC transporter genes. From the proteogenomic analyses we speculate that both monosaccharides derived from sucrose hydrolysis could possibly be catabolized by the spED pathway. By studying a strain of haloarchaea that lacks ketohexokinase but possesses fructokinase, we gained insight and generated important questions about the main pathway for sucrose degradation in haloarchaea. For several proteins (ketohexokinase, fructokinase, sucrose hydrolase, and the sucrose ABC transporter system), specific functions have been predicted in the current and previous analyses (23) that can provide avenues for future experimental characterization.

MATERIALS AND METHODS

Genomic analyses. Twenty-seven haloarchaeal genomes were interrogated for the presence of genes encoding enzymes and transporters associated with the catabolism of sucrose via the spED and EMP pathways, as well as gluconeogenesis. The genomes include both sugar-utilizing and non-sugar-utilizing haloarchaeal species, based on the reported abilities of these species to metabolize sucrose, fructose, or glucose. Protein annotations were based on reported enzyme characterizations, transcriptional analyses, and genomic investigations of haloarchaea (6–11, 14, 15, 18, 51, 52). Where possible, annotations in the 27 genomes were based on sequence identities of at least 35% to experimentally characterized proteins in haloarchaea. However, protein sequences have not been identified for all enzymes (e.g., ketohexokinase and β -fructofuranosidase); thus, for certain proteins, previous haloarchaeal genome annotations (14, 23, 28) were incorporated into the genomic interrogations. For novel proteins implicated in haloarchaeal sucrose metabolism that were identified through *Hht. litchfieldiae* proteomics, orthologous proteins were determined in other haloarchaeal genomes based on at least 35% sequence identity to the respective *Hht. litchfieldiae* proteins. The genome sequence of *Hht. litchfieldiae* was recently reannotated; the original locus tags (used in this study), the new locus tags, and UniProt identifiers for *Hht. litchfieldiae* proteins are listed in Table S5 in the supplemental material.

Metagenomic analyses. Custom hidden Markov model (HMM) profiles constructed from the set of manually identified haloarchaeal fructokinase (17 proteins) and ketohexokinase (6 proteins) genes were used in the MetAnnotate pipeline (53) with HMMER, version 3.1b2 (<http://hmmerr.org/>), and Usearch, version 9.0.2132 (54), to identify homologs from available hypersaline metagenomes that contain haloarchaea (Table 1). Only metagenomes sequenced using 454 technology were selected, as the longer read lengths (cf. Illumina) were required for stringent matching to the HMMs. As fructokinase proteins have similarity to the wider PfkB sugar kinase family, which also includes 2-dehydro-3-deoxygluconokinase and 1-phosphofructokinase (and other PfkB domain proteins), stringent criteria were set for the reads identified by MetAnnotate to be considered true fructokinase hits. Translated reads were trimmed to obtain the region which matched the fructokinase HMM profile and analyzed by BLAST (BLAST+, version 2.6.0, and blastp) against a custom database containing manually identified fructokinase (17 proteins), 2-dehydro-3-deoxygluconokinase (38 proteins), 1-phosphofructokinase (19 proteins), and other PfkB domain proteins (10 proteins). Manual inspection and validation of the custom database results against the wider NCBI and ExPasy protein databases was performed. Only sequences with the best BLAST match to one of the 17 manually identified fructokinases and also meeting stringent cutoffs (E value of $<e-18$; minimum length of 50 amino acids) were retained as genuine fructokinase matches; this underestimated the number of true fructokinases present but minimized the possibility of false positives. A similar manual inspection and validation of the haloarchaeal ketohexokinase results were performed, showing that reads matching the HMM profile with an E value of $<e-12$ and minimum length of 50 amino acids were genuine, stringent matches.

Phylogenetic analyses. Multiple alignments of PFK-B sugar kinase family proteins created in MEGA6 (55) using MUSCLE (56) were used to construct a phylogenetic tree by the maximum-likelihood method. Amino acid positions with less than 80% site coverage were eliminated, resulting in 295 amino acid positions in the final data set.

Hht. litchfieldiae cultures. *Hht. litchfieldiae* tADL cultures were grown in batch cultures in DBCM2 basal salt medium (57) containing 10 mM sucrose, 10 mM pyruvate, and 5 mM NH₄Cl (sucrose-containing medium) or 10 mM pyruvate and 5 mM NH₄Cl (sucrose-free medium) at 30°C and 120 rpm. Sucrose-containing medium contained pyruvate because *Hht. litchfieldiae* exhibits weak growth on sucrose as the sole defined carbon source, whereas cometabolizing pyruvate and sucrose generated strong growth (18). Cultures were monitored at an optical density of 600 nm. Glucose concentrations in *Hht. litchfieldiae* cultures were assayed with a glucose oxidase (GO) assay kit (Sigma-Aldrich, Castle Hill, Australia) using duplicate cultures grown under the same conditions as cultures used for proteomics.

LC-MS/MS and proteomics. *Hht. litchfieldiae* tADL cultures were harvested at mid-logarithmic phase (Fig. S3), and proteins were extracted from cells and the supernatant fraction as described previously (20, 58) and labeled using an eight-plex iTRAQ labeling system (59) according to manufacturer's instructions (Sciex, Framingham, USA). Peptides were prepared as described previously (60), except that a C₁₈ macrotrap (Michrom Bioresources, Auburn, USA) was used, followed by passage through an Oasis HLB cartridge (Waters, Milford, USA); peptide eluents (C₁₈ and Oasis HLB) were then pooled for analysis. Peptides were analyzed by nano-liquid chromatography (nano-LC) consisting of a Dionex UltiMate 3000 RSLCnano pump system, Switchos valve unit, and Famos autosampler (ThermoFisher Scientific, Waltham, USA). Samples were injected onto a C₁₈ precolumn cartridge (Acclaim PepMap 100, 5 μm, 0.3 by 5 mm, and 100-Å pore size; ThermoFisher Scientific), washed for 4 min, then switched in-line to a capillary column (~12.5 cm, C₁₈ reverse-phase packing material; ReproSil-Pur, 1.9-μm particle size and 200-Å pore size [Mäisch GmbH, Ammerbuch-Entringen, Germany]). Peptides were eluted using a linear gradient of H₂O:CH₃CN (98:2, 0.1% formic acid) to H₂O:CH₃CN (64:36, 0.1% formic acid) at 200 nl/min over 90 min. High voltage (2.3 kV) was applied to a low-volume union (Valco, Houston, USA), and the column tip was positioned at ~1.0 cm from the orifice. Data acquisition was performed using a TripleTOF 5600+ hybrid tandem mass spectrometer (MS/MS) (ABSciex, Foster City, USA). Positive ions were generated by electrospray and the 5600+ instrument was operated in the information-dependent acquisition (IDA) mode. A time of flight (TOF) MS survey scan was acquired (*m/z* 350 to 1,750; 0.2 s), and the 10 largest multiply charged ions (counts of >250; charge states of +2 and +4) were sequentially selected by the first quadrupole, Q1, for MS/MS analysis. Nitrogen was used as collision gas, advanced iTRAQ was enabled, and an optimum collision energy was automatically chosen (based on charge state and mass). Tandem mass spectra were accumulated for a maximum of 0.5 s (*m/z* 100 to 2,000) before dynamic exclusion for 20 s. Each labeling experiment was run twice to provide two technical replicates, which resulted in a total of four data sets for each fraction. These four data sets were combined, and MS data were searched using ProteinPilot software, version 4.5 (AB Sciex), against the local protein *Hht. litchfieldiae* tADL FASTA database to identify proteins; only proteins that were identified by at least two peptides were included. The proteins that had average weighted abundance ratios of 1.5-fold or more with a *P* value of less than 0.05 and error factor of less than 2 were considered further. The complete experimental procedures, statistical analysis for protein abundance data, and protein annotation method were described previously (20, 58).

Accession number(s). The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (61) under the data set identifier PXD010137.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02935-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

SUPPLEMENTAL FILE 2, XLS file, 0.3 MB.

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We declare that we have no conflicts of interest.

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