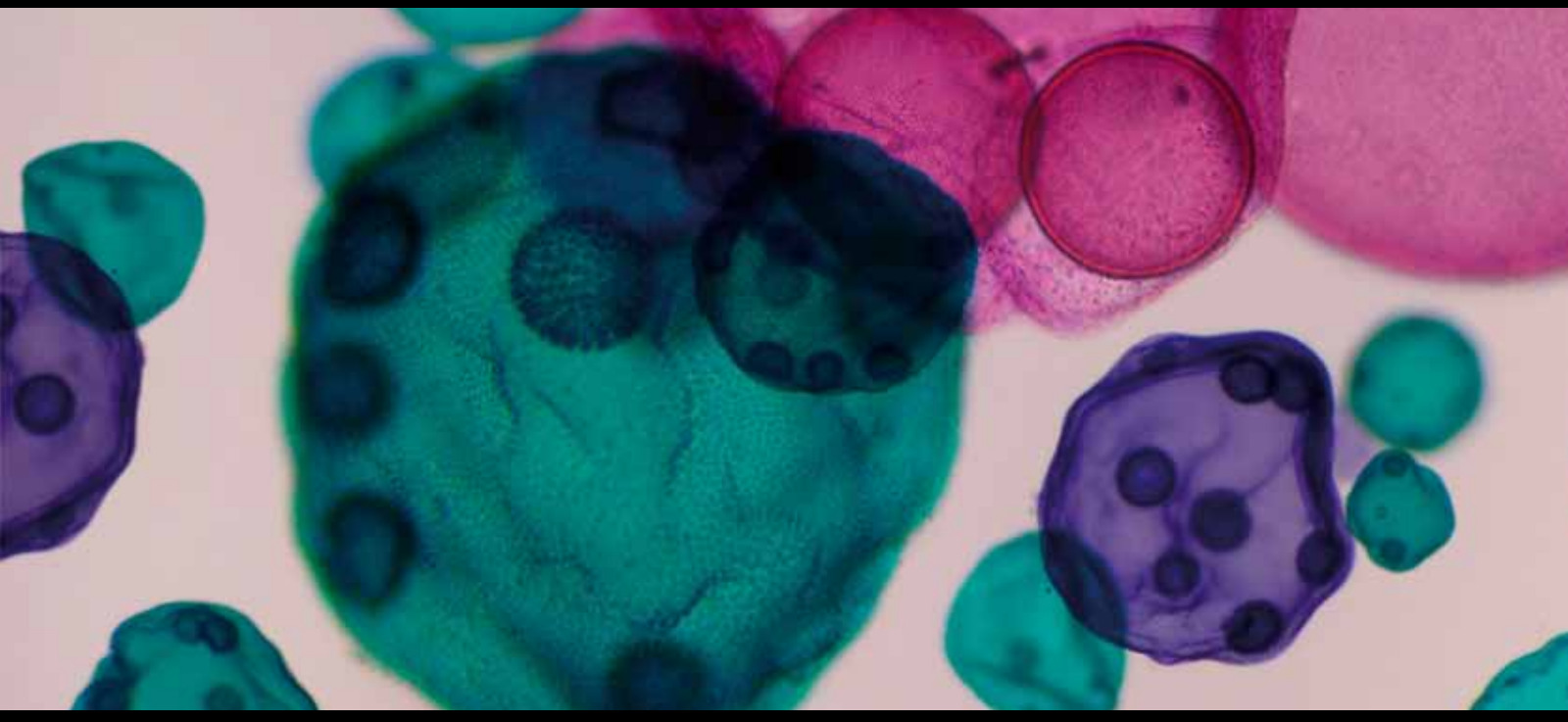
International Journal of Microbiology

Coping with the Environment: How Microbes Survive Environmental Challenges

Guest Editors: Haichun Gao, Tao Weitao, and Qiang He



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Editorial

Coping with the Environment: How Microbes Survive Environmental Challenges

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Microorganisms are exposed to constantly changing environments in their natural habitats either in planktonic form or microbial communities. Examples of these environmental changes encompass nutrient limitation, temperature, pH, and osmolarity fluctuations, radiation, and other harmful agents, such as excessive amount of superoxides and heavy metals. To respond and adapt to adverse environmental changes, microorganisms employ a striking combination of transcriptional regulatory circuits to sense and translate extracellular stimuli into specific cellular signals, resulting in altered gene expression and protein activities. Investigation of these underlying mechanisms and strategies could improve our understanding of microbial physiology in general and potentially lead to discoveries of practical value in the field of health care and environment protection. In this special issue, we have invited a few papers that explore this topic from various perspectives.

The first paper "*Exposure to glycolytic carbon sources reveals a novel layer of regulation for the MalT regulon*" presents the study on a synthetic lethal mutant *ompR malT*^{con} of *Escherichia coli* in which the constitutively expressed maltose system regulator MalT in its active form causes cell death in the absence of the osmoregulator OmpR. The authors propose that glycolysis provides a new layer of regulation to the maltose system on the basis that addition of glycolytic carbon sources promotes viability of the mutant.

The second paper "*The effect of sub-MIC β-lactam antibiotic exposure of Pseudomonas aeruginosa strains from people with cystic fibrosis in a desiccation survival model*" examines resistance of *Pseudomonas aeruginosa* strains to desiccation, which is an important environmental factor to the loss of viability of cells. Strains with a mucoid phenotype exhibit significantly improved desiccation survival when compared with nonmucoid ones, but effects of preincubation with sub-MIC beta-lactam antibiotics on desiccation resistance appear to be agent specific.

The third paper "*Detection of bacterial endospores in soil by terbium fluorescence*" of this special issue addresses the relationship of soil parameters (carbon-to-nitrogen ratio) on the occurrence of bacterial spores and distribution of spores in relation to sampling depth. The results demonstrate that the combination of microwave treatment of soil samples and measurement of terbium dipicolinate (DPA) photoluminescence is a rapid and reliable method for the assessment of bacterial spores.

The fourth paper "*The sulfate-rich and extreme saline sediment of the ephemeral Tirez lagoon: a biotope for acetoclastic sulfate-reducing bacteria and hydrogenotrophic methanogenic archaea*" is on the composition of methanogenic archaea, sulfate-reducing and sulfur-oxidizing prokaryotes in the extreme athalassohaline and particularly sulfate-rich sediment of Tirez Lagoon. The occurrence of hydrogenotrophic methanogenic and acetotrophic sulfate-reducing organisms and winter-summer community structures in Tirez sediment are analyzed by using the PCR-DGGE fingerprint technique for the functional adenosine-5′-phosphosulfate (*aprA*) and the methyl coenzyme M reductase (*mcrA*) gene markers.

The last paper "*Stress responses of Shewanella*" of this special issue focuses on stress responses of *Shewanella*, a group of facultative anaerobes capable of respiring on an array of compounds. As important and potential microorganisms for bioremediation, stress responses of *Shewanella* have been

subjected to an array of analyses, especially with high-throughput technologies such as whole-genome microarrays. This comprehensive review highlights the current understanding of mechanisms by which *Shewanella* survive and thrive within varied environments, as well as points out promising future research directions.

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Research Article

Exposure to Glycolytic Carbon Sources Reveals a Novel Layer of Regulation for the MalT Regulon

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Bacteria adapt to changing environments by means of tightly coordinated regulatory circuits. The use of synthetic lethality, a genetic phenomenon in which the combination of two nonlethal mutations causes cell death, facilitates identification and study of such circuitry. In this study, we show that the *E. coli ompR malT*^{con} double mutant exhibits a synthetic lethal phenotype that is environmentally conditional. MalT^{con}, the constitutively active form of the maltose system regulator MalT, causes elevated expression of the outer membrane porin LamB, which leads to death in the absence of the osmoregulator OmpR. However, the presence and metabolism of glycolytic carbon sources, such as sorbitol, promotes viability and unveils a novel layer of regulation within the complex circuitry that controls maltose transport and metabolism.

1. Introduction

Synthetic lethality, a phenomenon in which the combination of two nonlethal mutations causes death, is a powerful genetic tool that can, in an unbiased fashion, identify novel connections between cellular processes that function together to permit survival in a stressful environment. However, because the double mutant dies, investigating the process by which death occurs can be difficult. If, however, some permissive condition exists that permits survival of the double mutant, then the study of the death process is greatly facilitated, because genetic manipulations can be performed under permissive conditions and the consequences of those manipulations studied at nonpermissive conditions. Here, we explore one environmental condition (exposure to glycolytic carbon sources) that permits survival of the previously reported synthetic lethal mutant *ompR malT* ^{con} [1], which lacks the response regulator OmpR whilst harboring a constitutively active MalT^{con} protein.

As osmolality increases, the two-component response regulator OmpR becomes activated by the receipt of a phosphoryl group from its cognate sensor kinase EnvZ [2, 3]. Upon phosphorylation, OmpR controls more than 100 genes associated with outer membrane biogenesis, osmoregulation, and envelope stress [4, 5].

MalT is the central regulator of all *mal* genes [6, 7] (Figure 1). The *mal* genes encode proteins involved in transport and metabolism of maltose and maltodextrins. The outer membrane porin LamB facilitates the uptake of maltose and maltodextrins into the periplasm, where these sugars are bound by the maltose-binding protein MalE and delivered to the $MalFGK_2$ transporter [8]. Following transport into the cytoplasm, the sugars are metabolized [7]. MalT itself is activated by the maltose metabolite maltotriose and inhibited by MalK, MalY, Aes, and glucokinase [9–12]. The nucleoid proteins H-NS and StpA positively regulate MalT translation [13, 14]. *malT* transcription is activated by the cAMP-CRP complex, which renders it subject to catabolite repression [15, 16]. Finally, Mlc represses *malT* transcription [17].

Under non-permissive conditions, the *ompR malT* ^{con} mutant displays a set of striking phenotypes. Colonies on plates are translucent and form papillae [1]. In liquid medium, the culture loses turbidity upon entry into late exponential phase [1], because the inner membrane disintegrates [18]. The main cause for these phenotypes is elevated

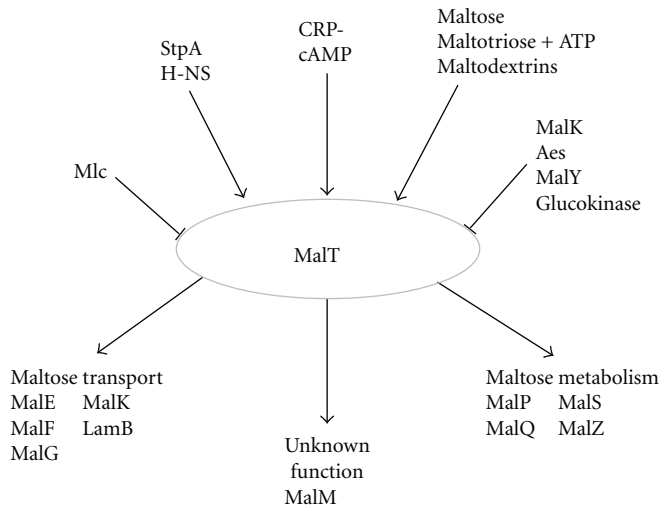

Figure 1: Regulation of MalT and its regulon. *malT* transcription is activated by the global regulator CRP-cAMP and repressed by Mlc. Translation of MalT is activated by H-NS and StpA. Activation of MalT activity can be attained by the binding of maltotriose, whereas it is inhibited by interaction with MalK, MalY, Aes, or glucokinase. Upon activation, MalT positively regulates expression of proteins that facilitate maltose uptake and metabolism.

LamB expression in the absence of OmpR: deletion of *lamB* permits survival [1], while genetic suppressors that reduce LamB expression also permit survival [18]. Similar to genetic suppressors, any environmental condition that reduces LamB levels should permit cell survival. For example, the *ompR malT* ^{con} mutant survives on minimal medium supplemented with glucose as the sole carbon source [1], almost certainly, because glucose causes catabolite repression of transcription from the *malT* and *malK* promoters and thus reduced expression of LamB [15, 19].

In this study, we identified an additional permissive environmental condition that supports survival of the *ompR malT* ^{con} mutant: the presence of noncatabolite repressing glycolytic carbon sources in the growth medium. Characterization of this condition allowed us to unveil a new regulatory layer of the MalT regulon. We hypothesize that this regulation requires metabolism of glycolysis-associated carbon sources.

2. Materials and Methods

2.1. Bacterial Strains, Bacteriophage, Transcriptional Fusions, and Plasmids. All bacterial strains used in this study are listed in Table 1. All strains evaluated were derivatives of *E. coli* AJW678 [20]. Derivatives were constructed by generalized transduction with P1*vir*, as described [21].

The transcription fusion *malE*pΔ92-*lac* was a generous gift from Winfried Boos (Universität Konstanz, Germany) and was described previously [11].

The *malT*^{con} allele (*malTc-1)* used in this study was a generous gift from Linda Kenney (University of Illinois at Chicago, IL, USA). It harbors a T949A base substitution and encodes the MalT^{con}W317R protein. Unless otherwise mentioned, deletion alleles were derived from the Keio collection [22].

2.2. Media and Growth Conditions. Because the *ompR malT* ^{con} mutant is conditionally lethal, cells were grown overnight under a permissive condition: 22°C in M63 minimal salts [21] with 22 mM sorbitol as the sole carbon source and supplemented with 100 μg mL^{-1} L-threonine, L-histidine, L-leucine, L-methionine, L-tryptophan, and 10 μg thiamine ml^{-1}. Whenever required, kanamycin (40 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}), or tetracycline (15 μg ml^{-1}) was added.

For tests of lethality, an inoculum from the overnight culture was subcultured at 37°C in LB (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl). LB agar plates also contained 1.5% (w/v) bacto agar. These growth conditions were considered non-permissive. Whenever required, carbon sources were added at a concentration of 22 mM. Cell growth was monitored spectrophotometrically (Beckman Instruments DU640) at 600 nm (OD_{600}).

2.3. Promoter Activity Assays. To monitor *malE*pΔ92-*lac* promoter activity, cells were grown aerobically with 250 rpm agitation at 37°C. At various time points during growth, 50 μL aliquots were harvested and added to 50 μL All-in-One β-galactosidase reagent (Pierce Biotechnology). β-galactosidase activity was determined quantitatively in a microtiter format, as described previously [23]. To avoid misleading results caused by lysing cells that spill β-galactosidase into the growth medium, we only considered β-galactosidase measurements before the onset of cell death.

2.4. Generation of Nonpolar Gene Deletions. To obtain nonpolar deletions, resistance cassettes were removed using flp-recombinase, according to the previously described protocol [24].

Table 1: Strains, plasmids, and reporter fusions used in this study.

	Relevant genotype	Reference
AJW678	*thi-1thr-1*(Am) *leuB6 metF159*(Am) *rpsL136 lacX74*	[20]
AJW2050	AJW678 *ompR*::Tn*10*	[44]
AJW3098	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A*	[1]
AJW3499	AJW678 *malT*^{con} *T949A*	[1]
AJW3732	AJW678 *ompR*::Tn*10* *malT*^{con} (S358I)	[1]
AJW3733	AJW678 *ompR*::Tn*10* *malT*^{con} (W317P)	[1]
AJW3734	AJW678 *ompR*::Tn*10* *malT*^{con} (R242C)	[1]
AJW3735	AJW678 *ompR*::Tn*10* *malT*^{con} (A244E)	[1]
AJW3736	AJW678 *ompR*::Tn*10* *malT*^{con} (A236S)	[1]
AJW3737	AJW678 *ompR*::Tn*10* *malT*^{con} (A236D)	[1]
AJW3738	AJW678 *ompR*::Tn*10* *malT*^{con} (P10Q)	[1]
AJW3739	AJW678 *ompR*::Tn*10* *malT*^{con} (R242S)	[1]
AJW3740	AJW678 *ompR*::Tn*10* *malT*^{con} (T38R)	[1]
AJW3741	AJW678 *ompR*::Tn*10* *malT*^{con} (S5L)	[1]
AJW3888	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*malZ*::Km	This study
AJW3902	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*glgP*::Km	This study
AJW3926	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*srlA*::Km	This study
AJW3927	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*srlD*::Km	This study
AJW3936	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*mlc*::Km	This study
AJW3943	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*malY*::Km	This study
AJW3947	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*aes*::Km	This study
AJW3967	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*malK*::frt	This study
AJW4023	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*hns*::Km	This study
AJW4028	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*stpA*::Km	This study
AJW4197	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A pstC*::frt *phoE*::km	This study
AJW4286	AJW678 *ompR*::Tn*10* *malT*^{con} *T949A* Δ*malK*::frt △*glk*::Km	This study
Reporter fusions		
*malE*pΔ92-*lac*	*trp*::[KanR-*malE*pΔ92-*lac*]$_{op}$	[30]

2.5. Outer Membrane Preparations. Outer membrane preparations were performed as described [25]. Outer membrane proteins were separated using 12% SDS-PAGE containing 4.8 M urea and stained with Coomassie brilliant blue [26].

2.6. Semiquantitative RT-PCR. To compare *malT* transcript levels, cells were grown under the indicated conditions to an OD of 1. RNA was harvested using the RNeasy Mini kit (Quiagen). DNA contamination was removed by treatment with 5 U of RQ1 RNase-free DNase (Promega) in 1x RQ1 DNase buffer for 1 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The subsequent reverse transcription reaction was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). To exclude DNA contamination, we performed a mock cDNA reaction lacking reverse transcriptase. The resulting cDNA was diluted and PCR amplified in a reaction mixture containing 2 μL cDNA product, 1x PCR buffer, 0.2 μM dNTPs, 4 mM $MgCl_2$, 0.5 μM *malT*-specific primer malTfor2 (5′-ACTCAGCCC-ATAAGTCGGC-3′), 0.5 μM *malT*-specific primer malTrev2 (5′-CAAGACTTCAATCCCGCTAG-3′), and 1 U Taq polymerase in a total volume of 25 μL. Amplification conditions were 95°C for 30 sec, 54°C for 30 sec, and 72°C for 60 sec (30 cycles), followed by 72°C for 5 min. The PCR products were subsequently analyzed on a 1% agarose gel.

3. Results

3.1. Metabolism of Glycolytic Carbon Sources Promotes Viability. We previously reported that the *ompR malT* ^{con} double mutant (strain AJW3098, Table 1) exhibits a synthetic lethal phenotype caused by the increased expression of LamB [1].

Glucose catabolite represses *mal* transcription, and thus reduces LamB expression [15, 16]. Therefore, it should not be

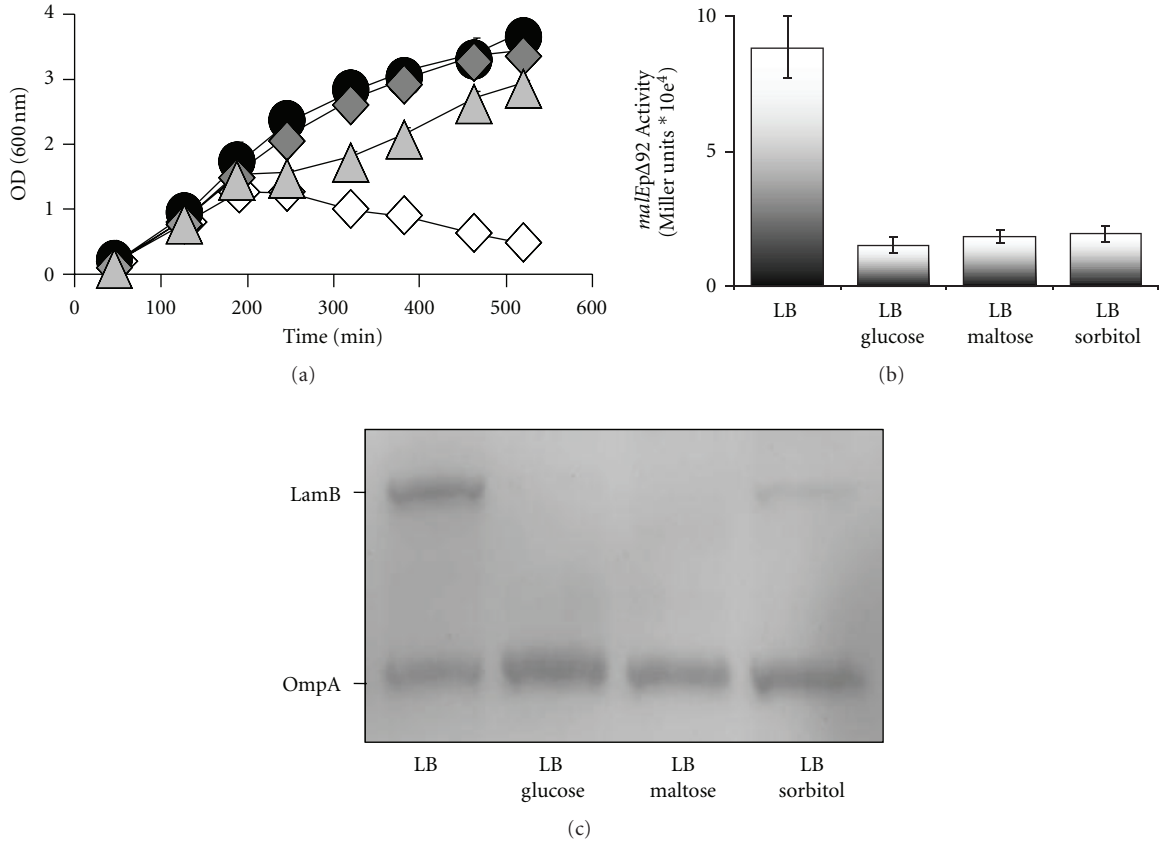

Figure 2: Effect of carbon sources on viability, *mal* gene transcription and LamB expression. (a) Growth curves of *ompR malT* ^{con} mutants (strain AJW3098) grown in LB at 37°C without sugars (white diamonds) or supplemented with 22 mM glucose (black circles), maltose (medium gray diamonds), or sorbitol (light gray triangles). Values represent the mean of triplicates. Error bars are only shown when greater than the symbol. (b) Effect of glycolytic carbon sources on *mal* gene transcription. β-galactoside activity was determined in *ompR malT* ^{con} mutants (strain AJW3098) carrying a *malE*pΔ92-*lac* reporter fusion. Cells were grown in LB without carbon source or LB supplemented with 22 mM glucose, maltose, or sorbitol. Cells were harvested at an OD_{600} of 1. Values represent the mean of triplicates. (c) Effect of glycolytic carbon sources on LamB expression. Addition of carbon sources reduces LamB levels in *ompR malT* ^{con} mutants as determined by outer membrane preparations. Cells were grown in LB at 37°C and harvested during late exponential phase. Gels were stained with Coomassie brilliant blue. Lane 1, LB no additional carbon source (LB); lane 2, LB 22 mM glucose (LB glucose); lane 3, LB 22 mM maltose (LB maltose); lane 4, LB 22 mM sorbitol (LB sorbitol).

surprising that exposure to glucose promoted survival of the *ompR malT* ^{con} mutant [1]. In contrast, the effect of maltose on *mal* gene expression can vary. Depending on expression or activity levels of the MalT protein, maltose can either enhance or inhibit MalT regulon expression [6, 27–29].

Here, we tested if exposure to maltose enhances or suppresses lethality of the *ompR malT* ^{con}mutant by growing it in LB at 37°C in the presence or absence of maltose and found that exposure to maltose suppressed death (Figure 2(a)). To test if this behavior is a general characteristic of *malT*^{con} alleles, we tested if other *malT*^{con} alleles behaved similarly. Several *ompR malT* ^{con} double mutants harboring a set of representative *malT*^{con} alleles (strains AJW3732-AJW3741) [1, 11] were grown in the presence or absence of maltose. As reported previously [1], each of these double mutants died in the absence of maltose. In contrast, they all survived in its presence (data not shown). We conclude that maltose can promote viability and that this behavior is a general characteristic of *malT*^{con} alleles.

Our finding that all the *ompR malT* ^{con} double mutants survived when exposed to maltose, combined with our previous report that disruption of *lamB* permits survival [1], supports the earlier observation that maltose can reduce *mal* gene expression in cells carrying *malT*^{con} alleles [29]. Thus, we asked if exposure to maltose reduces MalT regulon transcription. First, we monitored MalT regulon expression of an *ompR malT* ^{con} double mutant carrying a transcriptional *malE*pΔ92-*lac* fusion [30]. We, then, directly monitored LamB expression using outer membrane preparations. Exposure of the *ompR malT* ^{con} double mutant to maltose resulted in repressed *malE*pΔ92-*lac* transcription (Figure 2(b)) and reduced LamB expression (Figure 2(c) and Supplemental Figure 1(a) which is available at doi:10.1155/2011/107023). Similarly, the *malT*^{con} single mutant displayed reduced LamB expression in the presence of maltose (Supplemental Figure 1(b)), indicating that this effect is independent of OmpR. In contrast, WT cells and the *ompR* single mutant showed an increase in LamB expression when maltose was

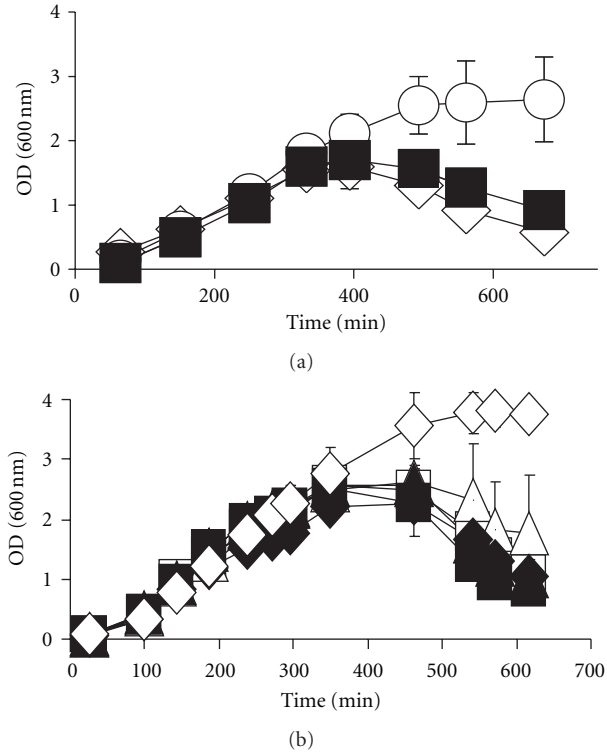

Figure 3: Effect of nonglycolytic carbon sources and sugar metabolism on viability. (a) Growth curves of *ompR malT* ^{con} mutants (strain AJW3098) grown in LB at 37°C without additional carbon source (white diamonds) or supplemented with serine (white circles) or succinate (black squares). Values represent the mean of triplicates. Error bars are only shown when greater than the symbol. (b) Growth curves of *ompR malT* ^{con}, (strain AJW3098, diamonds), *ompR malT* ^{con}*srlA* (strain AJW3926, squares) and *ompR malT* ^{con}*srlD* (strain AJW3927, triangles) mutants grown in LB at 37°C. Black symbols, cells grown in LB without sorbitol; white symbols, cells grown in LB supplemented with 22 mM sorbitol. Values represent the mean of triplicates. Error bars are only shown when greater than the symbol.

present (Supplemental Figure 2). We conclude that maltose supports survival of the *ompR malT* ^{con} double mutant by downregulating LamB expression.

The observation that maltose, a noncatabolite-repressing sugar, could reduce *mal* gene expression prompted us to ask if other noncatabolite-repressing sugars exert the same effect. We, therefore, grew the *ompR malT* ^{con} mutant in LB at 37°C in the presence or absence of diverse carbon sources. As expected, strong catabolite-repressing sugars (i.e., glucose, fructose, and mannitol) enabled survival (Figure 2(a) and Supplemental Table 1), whilst many noncatabolite-repressing carbon sources did not (Figure 3(a) and Supplemental Table 1). Surprisingly, some weaker or noncatabolite repressing carbon sources (i.e., sorbitol, serine, pyruvate, and mannose) promoted cell survival (Figures 2(a) and 3(a) and Supplemental Table 1). All the survival-supporting carbon sources are metabolized via glycolysis, whilst all the nonsurvival supporting carbon sources are not. Thus, some glycolysis-associated mechanism, in addition to catabolite repression, must be able to promote cell survival.

To determine why carbon sources like sorbitol promoted cell survival, we first tested if sorbitol exerts its effect by influencing *mal* gene expression. We monitored both *malE*pΔ92 transcription and LamB expression in the *ompR malT* ^{con} double mutant supplemented with sorbitol and found them to be reduced (Figures 2(b) and 2(c)). We conclude that sorbitol promotes cell survival by reducing LamB expression.

Since sorbitol requires a transport mechanism that is different from maltose or glucose, [7, 8, 31], we asked whether survival requires transport or metabolism of the sugar. We, therefore, constructed an *ompR malT* ^{con} *srlD* triple mutant (strain AJW3927), which can transport sorbitol but not metabolize it. We also constructed an *ompR malT* ^{con} *srlA* triple mutant (strain AJW3926), which can neither transport nor metabolize sorbitol due to a polar effect of the *srlA* deletion on *srlD*. Exposure to sorbitol did not permit survival of either mutant (Figure 3(b)). These results support the argument that the effect of sorbitol on the viability of the *ompR malT* ^{con} double mutant requires metabolism of the sugar.

3.2. Sorbitol Promotes Survival through a Novel Mechanism. The mechanism by which sorbitol or a metabolite exerts its effect could require either the known regulators of the maltose regulon (Figure 1), or the outer membrane porin PhoE, which has been shown to promote viability when expressed at high levels [18]. Our aim was to test whether any of these factors are required for sorbitol-promoted survival. If none of these regulators is involved, we reasoned that sorbitol must operate through a currently unknown mechanism.

In a previous report, we found that the increased expression of the PhoB regulon member PhoE, an outer membrane porin, can promote viability of the *ompR malT* ^{con} double mutant [18]. Since sugar metabolism is known to de-repress the PhoB regulon [32], we tested whether sorbitol exerts its effect by increasing PhoE abundance in the outer membrane. However, the *ompR malT* ^{con} *pstC phoE* mutant (strain AJW4197), which dies in LB [18], survived when we supplemented LB with sorbitol (data not shown). We conclude that sorbitol acts independently of PhoE.

To exert its effect on viability, products of sorbitol metabolism could act through a variety of regulators known to control *malT* expression or activity (Figure 1). For example, Mlc and CRP-cAMP affect *malT* transcription [15–17], H-NS and StpA stimulate MalT translation [13, 14], and the binding of maltotriose, MalK, MalY, Aes, or glucokinase modulates MalT activity [6, 7, 9–12, 28].

The death of the *ompR malT* ^{con} *mlc* triple mutant (strain AJW3936) in the absence of sorbitol and its survival when sorbitol was present (Supplemental Figure 3(a)) shows that sorbitol does not exert its effect by activating the transcriptional repressor Mlc (Figure 1) [17] or by increasing its concentration and thereby repressing *malT* transcription. That sorbitol did not influence *malT* transcription was confirmed by semiquantitative RT-PCR, which showed that exposure to sorbitol did not reduce *malT*^{con} mRNA (Figure 4). Exposure to maltose also did not reduce *malT*^{con} mRNA, confirming earlier reports that maltose affects MalT^{con}activity rather than affecting *malT*^{con} transcription [6, 28]. Glucose, on the other hand, caused a reduction of *malT*^{con} mRNA (Figure 4),

Figure 4: Effect of carbon sources on *malT*^{con} transcription. Semiquantitative RT-PCR of *malT*^{con} in the *ompR malT* ^{con} double mutant (strain AJW3098) grown under nonpermissive conditions in the absence (lane 1–5) or presence of glucose (lane 6–10), maltose (lane 11–15), or sorbitol (lane 16–20). PCR amplification was carried out with a dilution series of the cDNA: undiluted (lane 1, 6, 11, and 16), 1 : 10 dilution (lane 2, 7, 12, 17), 1 : 25 dilution (lane 3, 8, 13, and 18), 1 : 125 dilution (lane 4, 9, 14, 19), and mock control (lane 5, 10, 15, and 20).

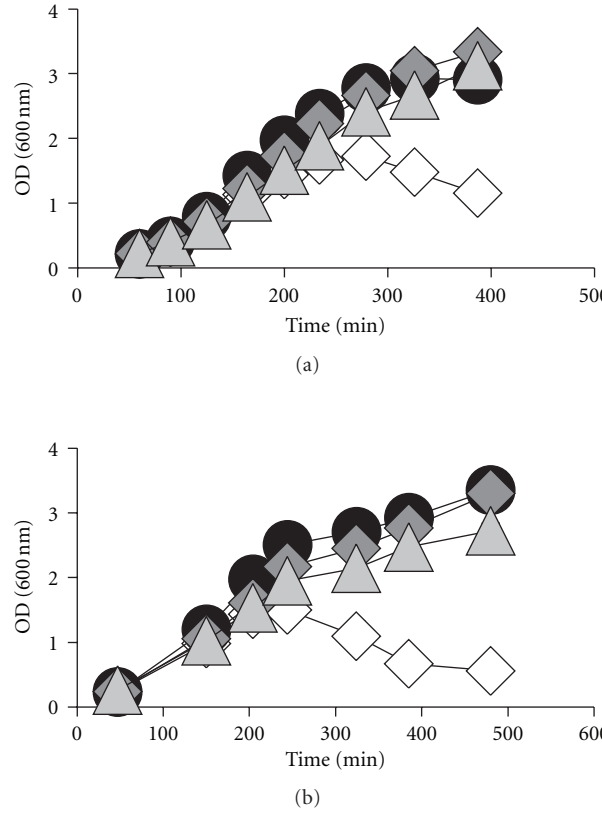

Figure 5: Effect of maltotriose on viability. (a) Growth curves of the *ompR malT* ^{con} *glgP* mutant (strain AJW3902) grown in LB at 37°C without sugars (white diamonds) or supplemented with glucose (black circles), maltose (dark gray diamonds), or sorbitol (light gray triangles). Values represent the mean of triplicates. Error bars are only shown when greater than the symbol. (b) Growth curves of the *ompR malT* ^{con} *malZ* mutant (strain AJW3888) grown in LB at 37°C without sugars (white diamonds) or supplemented with glucose (black circles), maltose (dark gray diamonds), or sorbitol (light gray triangles). Values represent the mean of triplicates. Error bars are only shown when greater than the symbol.

which can be explained by its catabolite-repressing effect on *malT* transcription [15, 16]. Thus, sorbitol permits viability of the *ompR malT* ^{con} double mutant by a mechanism that does not involve altered transcription of *malT*^{con}.

Since sorbitol does not affect *malT*^{con} transcription, we asked if it exerts its effect through any of the regulators that affect MalT^{con} activity. StpA is reported to exert a weak, activating effect on MalT regulon expression by modulating MalT translation [13]. Accordingly, we found that deletion of *stpA* in the *ompR malT* ^{con} mutant (strain AJW4028) did not promote survival (Supplemental Figure 3(b)). We further determined that sorbitol did not exert its effect through StpA (Supplemental Figure 3(b)). Since deletion of *hns* in the *ompR malT* ^{con} double mutant promoted viability [18], we could not determine whether sorbitol exerts its effect through H-NS.

Next, we tested if sorbitol could affect MalT^{con} activity by altering maltotriose levels. We presumed that the excess carbon might be converted to glycogen and that the subsequent degradation of that glycogen might increase the intracellular maltotriose concentration, and cause endogenous induction of MalT (Figure 1) [33, 34]. Whilst glycogen phosphorylase (GlgP) is instrumental in the production of maltotriose from glycogen, maltodextrin glucosidase (MalZ) has been reported to remove maltotriose by hydrolyzing it to maltose and glucose [34]. Deletion of either *malZ* or *glgP* in the *ompR malT* ^{con} background (strains AJW3888 and AJW3902, respectively) did not rescue viability and did not diminish the ability of sorbitol to support growth (Figure 5). We conclude that the *ompR malT* ^{con} mutant is largely insensitive to maltotriose and that sorbitol likely does not suppress lethality by altering maltotriose concentrations.

MalY, Aes, MalK, and glucokinase inhibit MalT activity (Figure 1) [9–12]. We, therefore, constructed the triple mutants *ompR malT* ^{con} *malY* (strain AJW3943) and *ompR malT* ^{con} *aes* (strain AJW3947), and an *ompR malT* ^{con} *malK* triple mutant carrying a nonpolar *malK* allele (strain AJW3967) to avoid disruption of LamB expression. We further constructed an *ompR malT* ^{con} *malK glk* quadruple mutant (strain AJW4286). We monitored growth of the first two mutants under non-permissive conditions in the presence or absence of glucose, maltose, or sorbitol. Since null mutations of *malK* and/or *glk* cause defects in the importation or metabolism of maltose, mutants carrying these alleles were only grown in the presence of glucose or sorbitol. In response to all tested sugars, the mutants survived (Supplemental Figures 4(a)–4(d)). Thus, none of the sugars, including sorbitol, act through MalY, Aes, MalK, or glucokinase.

Since sorbitol-dependent survival of the *ompR malT* ^{con} mutant depends on none of the known regulatory mechanisms, we hypothesize that a novel regulatory mechanism exists, which involves posttranscriptional modulation of MalT activity.

4. Discussion

4.1. Glycolysis Provides a New Layer of Regulation to the Maltose System. A highly complex network integrates numerous diverse signals to precisely regulate the expression and function of the maltose transport and metabolism system [6, 7, 35] (Figure 1). We now hypothesize that an additional regulatory layer exists that involves glycolysis. We base this hypothesis on the observation that the synthetic lethality of the *ompR malT* ^{con} mutant can be inhibited by growth in the presence of several glycolysis-associated carbon sources (Figures 2(a) and 3(a) and Supplemental Table 1). That a sugar like glucose or fructose can inhibit lethality is easily explained by its capacity to catabolite-repress *malTp* and *malKp* transcription and, hence, limit LamB expression [15, 16]. That sugars like maltose and sorbitol also can inhibit lethality, however, is both surprising and telling.

4.2. Inhibition by Maltose. Depending on MalT levels or MalT activity, the effect of maltose on MalT regulon expression can vary. In cells expressing WT MalT in large excess, it is reported that exposure to maltose causes a slight reduction (~2-fold) in *mal* gene expression [29]. However, in cells moderately overexpressing WT MalT, exposure to maltose has been reported to induce MalT regulon expression [29]. The same is true when cells express WT MalT from the endogenous gene [6, 28]. We confirmed this observation by showing that LamB levels increase in WT cells and *ompR* mutants when maltose is present (Supplemental Figure 2). If the same were true of cells that carry the *malT*^{con} allele, then the resulting increase in LamB levels would be expected to lead to an even more premature death of the *ompR malT* ^{con} double mutant. Instead, maltose reduced *malE* transcription and LamB expression, and thus permitted survival (Figures 2(b) and 2(c) and Supplemental Figure 1).

In cells carrying *malT*^{con} alleles, exposure to maltose has been reported to induce expression at the *malE* promoter, with the notable exception of highly constitutive MalT^{con} proteins [27, 29]. In contrast, we found that exposure to maltose causes reduced *malE* promoter activity in the *ompR malT* ^{con} double mutant (Figure 2(b)) and reduced LamB levels in both the *malT*^{con} single and *ompR malT* ^{con} double mutants (Figure 2(c) and Supplemental Figure 1), permitting the *ompR malT* ^{con} double mutant to survive. Since survival was observed in all 10 *ompR malT* ^{con} mutants tested, representing each location cluster and inhibition class [11], it is likely that the observed inhibitory response is a general characteristic of MalT^{con} proteins. The discrepancy between this and the previous reports could be due to utilization of different strain backgrounds (MC4100 versus AJW678) or of different media (minimal medium with glycerol as the base carbon source and supplemented with maltose versus LB supplemented with maltose).

In a previous study, exposure to maltose in the context of high expression of MalT^{WT} caused a moderate 2-fold reduction of *malE* transcription [29]. To explain this result, a model was proposed in which overproduction of MalT^{WT} results in a large number of MalT^{WT} oligomers that substitute for CRP. This would result in formation at the *malEp* and *malKp* promoters of a less active nucleoprotein complex containing only MalT [36]. A further development of this model proposed unlimited aggregation of MalT to be responsible for the inhibition of *malEp* transcription at high concentrations of MalT [37]. For these models to explain our observations, the native gene would have to express enough MalT^{con} to permit successful competition with CRP for DNA binding. Furthermore, those constitutively active proteins would have to become more active in response to maltose. Finally, the combination would have to be able to exert a 2-fold larger effect (4-fold inhibition) than did the wild-type protein expressed from a multicopy plasmid (2-fold inhibition). We think it is more likely that maltose acts upon MalT^{con} in a manner similar to that of sorbitol.

4.3. Inhibition by Sorbitol. Sorbitol has never been reported to influence the maltose system or inhibit MalT regulon transcription; thus, the inhibitory mechanism through which it operates must be novel. With the notable exceptions of H-NS and the CRP-cAMP complex, we tested the involvement of each known MalT regulator (Figure 1) and found that none are required (Figures 4 and 5 and Supplemental Figures 3 and 4). We ruled out the CRP-cAMP complex, because sorbitol causes only weak catabolite repression [8, 38]. This argument is further supported by the observation that exposure to sorbitol did not alter *malT*^{con} mRNA levels (Figure 4). We also excluded H-NS, since, to our knowledge, the global regulator has never been reported to respond to glycolysis. Thus, we hypothesize that sorbitol exerts its effect on *mal* gene transcription through a novel mechanism that is independent of the currently reported regulators and signals.

Our studies show that the lethal phentoype caused by the MalT^{con} protein used in this study is insensitive to both the inducer maltotriose (Figure 5) and the inhibitor MalK [1, 39] (data not shown). Although MalK can exert a small effect on the activity of this MalT^{con} protein, an observation made when we tested media that does not contain the MalT inducer trehalose, this small effect was insufficient to influence the lethal phenotype (Reimann and Wolfe, unpublished data). Thus, stripped of the two primary layers of regulation provided by maltotriose and MalK, the *ompR malT*^{con} double mutant exposes an otherwise undetectable layer of regulation. That sorbitol must be metabolized to inhibit MalT regulon transcription (Figure 3(b)) suggests the existence of a central metabolite that modulates MalT regulon expression.

The identity of this central metabolite remains unknown. However, recent reports that CRP and other transcription factors can become acetylated [40–42] coupled with the knowledge that the protein deacetylase CobB depends on NAD^+ for its function [43] raises the exciting possibility that increased glycolytic flux due to metabolism of the excess sorbitol results in acetylation of CRP, MalT, or some other component of the nucleoprotein complex that modulates *malE* and *malK* transcription, resulting in inhibition and thus survival.

The concept of a glycolytic metabolite opens up the possibility that maltose, glucose, fructose, and other glycolytic

carbon sources could work through the same mechanism. In the case of glucose and fructose, however, the effect is normally concealed by their strong catabolite-repressing effect. Likewise, in cells harboring a $MalT^{WT}$ protein, the strong regulatory effects of maltotriose and MalK would normally counterbalance the regulatory effect of maltose metabolism.

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References

[1] S. A. Reimann and A. J. Wolfe, "A critical process controlled by MalT and OmpR is revealed through synthetic lethality," *Journal of Bacteriology*, vol. 191, no. 16, pp. 5320–5324, 2009.

[2] M. M. Igo, J. M. Slauch, and T. J. Silhavy, "Signal transduction in bacteria: kinases that control gene expression," *New Biologist*, vol. 2, no. 1, pp. 5–9, 1990.

[3] T. Mizuno, A. Shinkai, K. Matsui, and S. Mizushima, "Osmoregulatory expression of porin genes in *Escherichia coli*: a comparative study on strains B and K-12," *FEMS Microbiology Letters*, vol. 68, no. 3, pp. 289–293, 1990.

[4] T. Oshima, H. Aiba, Y. Masuda et al., "Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12," *Molecular Microbiology*, vol. 46, no. 1, pp. 281–291, 2002.

[5] B. M. Pruss, C. A. Besemann, A. M. Denton, and A. J. Wolfe, "A complex transcription network controls the early stages of biofilm development by *Escherichia coli*," *Journal of Bacteriology*, vol. 188, no. 11, pp. 3731–3739, 2006.

[6] W. Boos and A. Bohm, "Learning new tricks from an old dog: MalT of the *Escherichia coli* maltose system is part of a complex regulatory network," *Trends in Genetics*, vol. 16, no. 9, pp. 404–409, 2000.

[7] W. Boos and H. A. Shuman, "Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 1, pp. 204–229, 1998.

[8] M. Ehrmann, R. Ehrle, E. Hofmann, W. Boos, and A. Schlosser, "The ABC maltose transporter," *Molecular Microbiology*, vol. 29, no. 3, pp. 685–694, 1998.

[9] R. Peist, A. Koch, P. Bolek, S. Sewitz, T. Kolbus, and W. Boos, "Characterization of the aes gene of *Escherichia coli* encoding an enzyme with esterase activity," *Journal of Bacteriology*, vol. 179, no. 24, pp. 7679–7686, 1997.

[10] N. Joly, A. Bohm, W. Boos, and E. Richet, "MalK, the ATP-binding cassette component of the *Escherichia coli* maltodextrin transporter, inhibits the transcriptional activator MalT by antagonizing inducer binding," *Journal of Biological Chemistry*, vol. 279, no. 32, pp. 33123–33130, 2004.

[11] A. Schlegel, O. Danot, E. Richet, T. Ferenci, and W. Boos, "The N terminus of the *Escherichia coli* transcription activator MalT is the domain of interaction with MalY," *Journal of Bacteriology*, vol. 184, no. 11, pp. 3069–3077, 2002.

[12] C. Lengsfeld, S. Schonert, R. Dippel, and W. Boos, "Glucose- and glucokinase-controlled *mal* gene expression in *Escherichia coli*," *Journal of Bacteriology*, vol. 191, no. 3, pp. 701–712, 2009.

[13] J. Johansson, B. Dagberg, E. Richet, and B. E. Uhlin, "H-NS and StpA proteins stimulate expression of the maltose regulon in *Escherichia coli*," *Journal of Bacteriology*, vol. 180, no. 23, pp. 6117–6125, 1998.

[14] H. S. Park, Y. Ostberg, J. Johansson, E. G. H. Wagner, and B. E. Uhlin, "Novel role for a bacterial nucleoid protein in translation of mRNAs with suboptimal ribosome-binding sites," *Genes and Development*, vol. 24, no. 13, pp. 1345–1350, 2010.

[15] C. Chapon, "Role of the catabolite activator protein in the expression of the maltose regulon of *Escherichia coli*," *Annales de Microbiologie*, vol. 133, no. 1, pp. 77–80, 1982.

[16] C. Chapon and A. Kolb, "Action of CAP on the *malT* promoter in vitro," *Journal of Bacteriology*, vol. 156, no. 3, pp. 1135–1143, 1983.

[17] K. Decker, J. Plumbridge, and W. Boos, "Negative transcriptional regulation of a positive regulator: the expression of MalT, encoding the transcriptional activator of the maltose regulon of *Escherichia coli*, is negatively controlled by Mlc," *Molecular Microbiology*, vol. 27, no. 2, pp. 381–390, 1998.

[18] S. A. Reimann and A. J. Wolfe, "Constitutive expression of the maltoporin LamB in the absence of OmpR damages the cell envelope," *Journal of Bacteriology*, vol. 193, no. 4, pp. 842–853, 2011.

[19] E. Richet, D. Vidal-Ingigliardi, and O. Raibaud, "A new mechanism for coactivation of transcription initiation: repositioning of an activator triggered by the binding of a second activator," *Cell*, vol. 66, no. 6, pp. 1185–1195, 1991.

[20] S. Kumari, C. M. Beatty, D. Browning et al., "Regulation of acetyl coenzyme A synthetase in *Escherichia coli*," *Journal of Bacteriology*, vol. 182, no. 15, pp. 4173–4179, 2000.

[21] T. J. Silhavy, M. L. Berman, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 1984.

[22] M. Baba, T. Ara, M. Hasegawa et al., "Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection," *Molecular Systems Biology*, vol. 2, article 2006.0008, 2006.

[23] C. M. Beatty, D. Browning, S. J. W. Busby, and A. J. Wolfe, "Cyclic AMP receptor protein-dependent activation of the *Escherichia coliacs*P2 promoter by a synergistic class III mechanism," *Journal of Bacteriology*, vol. 185, no. 17, pp. 5148–5157, 2003.

[24] K. A. Datsenko and B. L. Wanner, "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 12, pp. 6640–6645, 2000.

[25] R. Morona and P. R. Reeves, "The *tolC* locus of *Escherichia coli* affects the expression of three major outer membrane proteins," *Journal of Bacteriology*, vol. 150, no. 3, pp. 1016–1023, 1982.

[26] J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular Cloning—A Laboratroy Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 1989.

[27] B. Dardonville and O. Raibaud, "Characterization of malT mutants that constitutively activate the maltose regulon of *Escherichia coli*," *Journal of Bacteriology*, vol. 172, no. 4, pp. 1846–1852, 1990.

[28] O. Raibaud and E. Richet, "Maltotriose is the inducer of the maltose regulon of *Escherichia coli*," *Journal of Bacteriology*, vol. 169, no. 7, pp. 3059–3061, 1987.

[29] M. Schwartz, "The maltose regulon," in Escherichia coli *and* Salmonella typhimurium. *Cellular and Molecular Biology*, F. C. Neidhardt and J. L. Ingraham, Eds., American Society for Microbiology, Washington, DC, USA, 1987.

[30] E. Richet, "On the role of the multiple regulatory elements involved in the activation of the *Escherichia colimalEp* promoter," *Journal of Molecular Biology*, vol. 264, no. 5, pp. 852–862, 1996.

[31] F. C. Grenier, E. B. Waygood, and M. H. Saier Jr., "The bacterial phosphotransferase system: kinetic characterization of the glucose, mannitol, glucitol, and N-acetylglucosamine systems," *Journal of Cellular Biochemistry*, vol. 31, no. 2, pp. 97–105, 1986.

[32] A. Hartmann and W. Boos, "Mutations in *phoB*, the positive gene activator of the *pho* regulon in *Escherichia coli*, affect the carbohydrate phenotype on MacConkey indicator plates," *Research in Microbiology*, vol. 144, no. 4, pp. 285–293, 1993.

[33] K. Decker, R. Peist, J. Reidl, M. Kossmann, B. C. Brand, and W. Boos, "Maltose and maltotriose can be formed endogenously in *Escherichia coli* from glucose and glucose-1-phosphate independently of enzymes of the maltose system," *Journal of Bacteriology*, vol. 175, no. 17, pp. 5655–5665, 1993.

[34] R. Dippel, T. Bergmiller, A. Bohm, and W. Boos, "The maltodextrin system of *Escherichia coli*: glycogen-derived endogenous induction and osmoregulation," *Journal of Bacteriology*, vol. 187, no. 24, pp. 8332–8339, 2005.

[35] A. Schlegel, A. Bohm, S. J. Lee, R. Peist, K. Decker, and W. Boos, "Network regulation of the *Escherichia coli* maltose system," *Journal of Molecular Microbiology and Biotechnology*, vol. 4, no. 3, pp. 301–307, 2002.

[36] O. Raibaud, D. Vidal-Ingigliardi, and E. Richet, "A complex nucleoprotein structure involved in activation of transcription of two divergent *Escherichia coli* promoters," *Journal of Molecular Biology*, vol. 205, no. 3, pp. 471–485, 1989.

[37] V. Schreiber and E. Richet, "Self-association of the *Escherichia coli* transcription activator MalT in the presence of maltotriose and ATP," *Journal of Biological Chemistry*, vol. 274, no. 47, pp. 33220–33226, 1999.

[38] J. W. Lengeler and E. Lin, "Reversal of the mannitol-sorbitol diauxie in *Escherichia coli*," *Journal of Bacteriology*, vol. 112, no. 2, pp. 840–848, 1972.

[39] M. Reyes and H. A. Shuman, "Overproduction of MalK protein prevents expression of the *Escherichia coli* mal regulon," *Journal of Bacteriology*, vol. 170, no. 10, pp. 4598–4602, 1988.

[40] S. Thao, C.-S. Chen, H. Zhu, and J. C. Escalante-Semerena, "Nepsilon-lysine acetylation of a bacterial transcription factor inhibits its DNA-binding activity," *PLoS One*, vol. 5, no. 12, article, 2010.

[41] B. J. Yu, J. A. Kim, J. H. Moon, S. E. Ryu, and J. G. Pan, "The diversity of lysine-acetylated proteins in *Escherichia coli*," *Journal of Microbiology and Biotechnology*, vol. 18, no. 9, pp. 1529–1536, 2008.

[42] J. Zhang, R. W. Sprung, J. Pei et al., "Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*," *Molecular and Cellular Proteomics*, vol. 8, no. 2, pp. 215–225, 2009.

[43] V. J. Starai, I. Celic, R. N. Cole, J. D. Boeke, and J. C. Escalante-Semerena, "Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine," *Science*, vol. 298, no. 5602, pp. 2390–2392, 2002.

[44] M. Berman, L. Enquist, and T. J. Silhavy, *Advanced Bacterial Genetics*, Cold Spring Harbor Lab Press, Cold Spring Harbor, NY, USA, 1981.

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Research Article

The Effect of Sub-MIC β-Lactam Antibiotic Exposure of *Pseudomonas aeruginosa* Strains from People with Cystic Fibrosis in a Desiccation Survival Model

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Prior to modern typing methods, cross-infection of *P. aeruginosa* between people with cystic fibrosis (CF) was felt to be rare. Recently a number of studies have demonstrated the presence of clonal strains of *P. aeruginosa* infecting people with CF. The aim of this study was to determine whether strains of *P. aeruginosa* demonstrated differences in resistance to desiccation and whether preincubation in subminimum inhibitory concentrations (MICs) of β-lactam affected desiccation resistance. The experimental data were modelled to a first-order decay model and a Weibull decay model using least squares nonlinear regression. The Weibull model was the preferred model for the desiccation survival. The presence of a mucoid phenotype promoted desiccation survival. Preincubation with antibiotics did not have a consistent effect on the strains of *P. aeruginosa*. Meropenem reduced desiccation resistance, whereas ceftazidime had much less effect on the strains studied.

1. Introduction

Pseudomonas aeruginosa is a gram-negative, nonfermentative, aerobic bacillus belonging to the family Pseudomonadaceae. The organism is ubiquitous within the environment and is particularly isolated from moist areas such as water and soil. *P. aeruginosa* causes chronic respiratory infections in people with cystic fibrosis (CF) and acts as opportunistic pathogen causing bacteraemia, urinary tract infections, and hospital acquired pneumonia in patients with burns, urinary catheters, and those on invasive ventilation [1]. Although *P. aeruginosa* is a nonfermentative aerobe, it can grow under anaerobic conditions using nitrate as an electron receptor. Its ability to survive in a wide range of environmental conditions is partially explained by its versatile nutritional abilities and its ability to resist high concentrations of salt, dyes, disinfectants, and many common antibiotics. The bacteria has been commonly found in the drains of wash basins in hospital wards [2], and aerosols containing *P. aeruginosa* can be detected when opening a tap [3, 4]. Isolation of *P. aeruginosa* from tap water is due to contamination of the tap itself, rather than the mains water supply [5].

Prior to the advent of modern genetic typing methods cross-infection of *P. aeruginosa* between people with CF was felt to be a rare event. More recently a number of studies have been undertaken that demonstrate the presence of clonal strains of *P. aeruginosa* infecting multiple patients in CF clinics [6–9].

P. aeruginosa is intrinsically resistant to most commonly used antibiotics. Antibiotic resistance is achieved through a combination of restricted antibiotic uptake through the outer membrane and a variety of energy-dependent mechanisms. The energy-dependent mechanisms through which *P. aeruginosa* achieves antibiotic resistance include efflux pumps and β lactamase-production. The energy-dependent mechanisms are usually under close regulation, and antibiotic resistance is often a result of mutations in the regulatory genes of these mechanisms [10]. Preincubation with antibiotics has been demonstrated to have a number of effects on *P. aeruginosa* including induction of a biofilm form of

Table 1: Details of *Pseudomonas aeruginosa* strains.

Pseudomonas aeruginosa strain		MIC (μg mL^{-1})	
		Ceftazidime	Meropenem
Environmental strain	NCIMB 10848	1.0	0.32
Unique CF	4412061	256	32
Unique CF mucoid	4390364-1	2.0	0.50
Manchester [7]	2003/492	256	32
Liverpool/Seacroft	4390416-2	8	32
Seacroft	4390195	256	32
Liverpool [18]	2003/493	2.0	3.0
Liverpool mucoid [18]		8	0.5
Leeds Paediatric [6]	4410030	256	32
Leeds Paediatric mucoid [6]	7175611-1	1.5	32

growth [11], improved heat and osmotic stress response [12], changes to hydrophobicity [13], and reduced bacterial adherence [14].

The aim of this study was to determine whether clonal strains of *P. aeruginosa*, identified as part of routine clinical sampling, demonstrated differences in resistance to desiccation and whether preincubation in subminimum inhibitory concentrations (MICs) of β-lactam antibiotics had an effect on the ability of the bacteria to resist desiccation. Both ceftazidime and meropenem are anti-pseudomonal antibiotics that are commonly used in the care of people with CF.

2. Materials and Methods

2.1. Bacterial Strains. The environmental strain of *P. aeruginosa* (NCIMB 10848) was obtained from the National Collection of Industrial and Marine Bacteria. Mucoid and nonmucoid variants of the Liverpool strain [15] and a nonmucoid variant of the Manchester strain [16] were obtained from the Centre for Infectious Disease, University of Edinburgh. All the other *P. aeruginosa* strains were obtained from clinical samples of sputa from people with CF and were genotyped by the Microbiology Department of Leeds General Infirmary using Pulsed-Field Gel Electrophoresis (PFGE) (See Table 1).

2.2. Antimicrobial Sensitivity Testing. MICs of ceftazidime and meropenem were performed on Iso-Sensitest (ISA) agar by Etest (AB Biodisk, Sweden) according to the manufacturer's guidelines [17]. MICs were read after 24 h incubation at 37°C.

2.3. Desiccation Survival Assay

2.3.1. Preparation of Controlled Relative Humidity Chamber. Controlled conditions of relative humidity were established and maintained by the presence of saturated salt solution in an air tight plastic box. 30 ± 3% relative humidity (RH) was maintained by the presence of saturated solution of $CaCl_2 \cdot 6H_2O$ (Sigma Chemicals, UK) [19, 20]. The temperature of the room during the experiment was 22 ± 2°C. A digital thermohygrometer (Extech Instruments, USA) was used to monitor relative humidity and temperature.

2.3.2. Inoculation of Glass Coverslips with Bacteria. After determination of the viable cell concentration curve, strains were grown to their maximum stationary phase cell concentration, and these cultures were used in the subsequent experiments for determining the survival on a dry surface. Strains were also grown for 24–48 h in nutrient broth or nutrient broth containing 0.25 × MIC concentrations of ceftazidime or meropenem at 37°C in air, with shaking at 110 rpm.

A 1.0 mL aliquot of the nutrient broth culture was placed in a 1.5 mL Eppendorf tube and centrifuged for five minutes in a microcentrifuge (Eppendorf Centrifuge 5140) at 13000 g. The supernatant fluid was discarded and the cell pellet resuspended in 1.0 mL of distilled water. 20 μL of the cell suspensions was deposited onto the sterile coverslips, and they were then placed in the controlled relative humidity chamber.

2.3.3. Determination of Viable Count. Each glass coverslip was placed in 2 mL of sterile distilled water under aseptic conditions using a sterile pair of forceps. Following appropriate serial dilutions using sterile distilled water the cell suspension was inoculated onto Columbia blood agar plates using the spread plate method. After overnight incubation in air at 37°C, the number of colony forming units was counted using a colony counter. Three glass coverslips were used separately for each count and three different dilutions were made for each coverslip. Viable counts were determined at time 0 h, 1 h, 6 h, and 24 h.

2.4. Mathematical Models of Bacterial Inactivation

2.4.1. First-Order Decay Model. The first-order decay model assumes that all the bacterial cells have an equal resistance to lethal treatment. This results in a linear relationship between

Table 2: Comparison of first order decay and Weibull survival models.

Bacterial strain	Model					Preferred model	P
	First order decay		Weibull				
	k	R^2	δ	p	R^2		
Environmental	0.16	0.6599	0.15	0.30	0.9397	Weibull	<0.0001
Liverpool	0.03	0.4678	0.003	0.18	0.6918	Weibull	0.0001
Liverpool mucoid	0.12	0.5336	0.003	0.15	0.9618	Weibull	<0.0001
Manchester	0.10	0.8418	1.46	0.37	0.9478	Weibull	<0.0001
Paediatric	0.19	0.6104	0.16	0.34	0.8550	Weibull	<0.0001
Paediatric mucoid	0.14	0.5564	0.03	0.22	0.9635	Weibull	<0.0001
Seacroft	0.02	0.8168	0.31	0.35	0.9836	Weibull	<0.0001
Seacroft/Liverpool	0.14	0.7438	0.32	0.30	0.9107	Weibull	<0.0001
Unique CF	0.15	0.9026	1.73	0.51	0.9746	Weibull	<0.0001
Unique CF mucoid	0.10	0.8527	2.03	0.41	0.9402	Weibull	<0.0001

the logarithm of the number of survivors and the treatment time, as described in the following first-order decay kinetics equation:

$$\log(N_t) = \log(N_o) - kt, \quad (1)$$

where N_0 = concentration at time 0, N_t = concentration at time t, k = inactivation rate, and t = time.

2.4.2. Weibull Model. The Weibull model of bacterial decay is a nonlinear model. It assumes that lethal events are probabilities and that the corresponding survival curves are cumulative forms of a distribution of lethal event, as described in (2) [21]. The shape of the survival curve is determined by p; when $p < 1$, the curve has a concave upwards appearance, when $p > 1$, the curve has a concave downwards appearance, and when $p = 1$, the survival curve is linear. The value δ represents the time to the first decimal reduction [22]. The scale and shape parameters are not independent; therefore an error in δ will be balanced by an error in p. Comparisons between survival curves were undertaken by comparing the value for δ with a fixed value for the shape parameter determined from the mean of the initial values for p [22]. See the following Weibull distribution equation [21]:

$$\log(N_t) = \log(N_0) - \left(\frac{t}{\delta}\right)^p, \quad (2)$$

where N_0 = concentration at time 0, N_t = concentration at time t, t = time, δ = scale parameter, and p = shape parameter.

2.5. Statistical Analysis. The two models of bacterial decay were modelled to the experimental data by least squares error analysis using GraphPad Prism (GraphPad Inc, San Deigo, USA). Comparisons between curves were made using the F-Test. A P value of <0.05 was deemed significant.

Comparisons between Weibull survival curves were made with a fixed value for the shape parameter p determined by the mean of the values p for the strains studied.

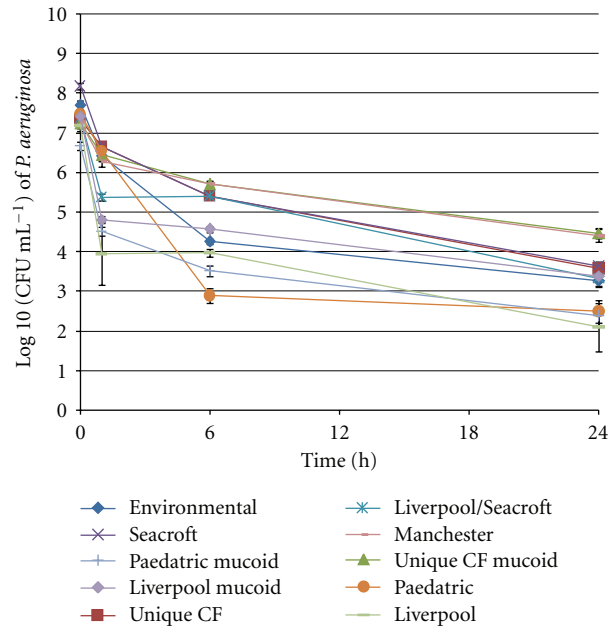

Figure 1: Mean viable counts of different strains of *P. aeruginosa* within the desiccation survival model. Error bars represent standard error of mean.

3. Results

Within the first hour following inoculation of all the different strains of *P. aeruginosa* onto a dry glass surface, there was a rapid fall in viable counts of bacteria. All strains of *P. aeruginosa* were still able to be recovered at 24 hours but only at very low counts (Figure 1).

3.1. Comparison of Mathematical Survival Curves Models in the Desiccation Model. The Weibull survival distribution was the preferred model for all the strains of *P. aeruginosa* examined in the desiccation survival assay (See Table 2). When the Weibull distribution model was applied to the experimental data all the strains of *P. aeruginosa* examined had a concave survival curve with $p < 1$ (See Table 2). The

average value for the scale parameter p was determined to be 0.313.

3.2. Influence of Mucoid Phenotype on Desiccation Survival. The value of δ was greater for all the mucoid strains of *P. aeruginosa* than the corresponding nonmucoid strain. This difference reached statistical significance for the Unique CF ($P = 0.006$) and Paediatric strains ($P = 0.0476$) (See Figure 2).

3.3. Influence of Antibiotic Preincubation on Desiccation Survival on Time to First Decimal Reduction. Preincubation with ceftazidime did not have any significant effect on any of the nonepidemic strains of *P. aeruginosa*. For epidemic strains it significantly increased the time to first decimal reduction for the Paediatric nonmucoid strain and the Seacroft/Liverpool strain (Paediatric non-mucoid $P < 0.0001$; Seacroft/Liverpool $P = 0.0002$) and significantly reduced the time to first decimal reduction Liverpool mucoid strain ($P = 0.0143$). The other epidemic strains were not significantly affected.

All nonepidemic strains had a significant reduction in δ following preincubation with meropenem (Environmental $P = 0.0002$; Unique CF $P < 0.0001$; Unique CF Mucoid $P < 0.0001$). All epidemic strains of *P. aeruginosa* also had a significant reduction in δ following preincubation with meropenem, apart from the Paediatric, Seacroft, and Liverpool strains where the reduction in δ was not statistically significant (Liverpool mucoid $P < 0.0001$; Manchester $P < 0.0001$; Paediatric mucoid $P < 0.0001$; Seacroft/Liverpool $P = 0.0007$).

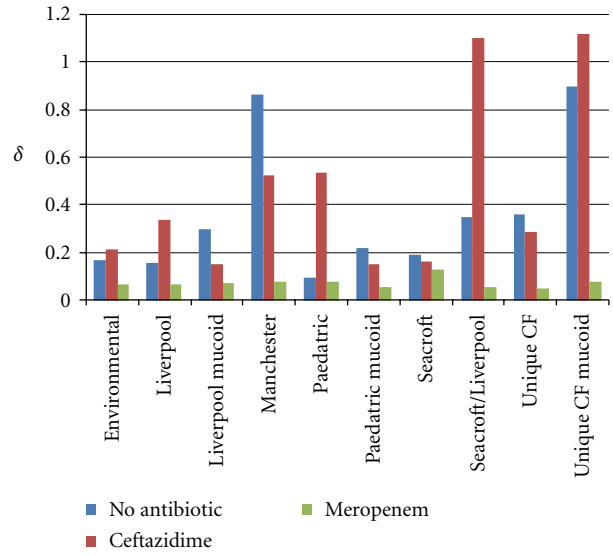

FIGURE 2: Values for δ determined from the Weibull distribution using a fixed value for the scale parameter p with preincubation with no antibiotic (δ), ceftazidime (δ_{cef}), and meropenem (δ_{mero}).

4. Discussion

There have been a number of studies demonstrating *P. aeruginosa* cross-infection between patients with cystic fibrosis [6, 7, 9, 23, 24]. The method of cross-infection is not clear. Dry surface contamination and aerosol dispersion have both been postulated as potential routes of transmission [15, 25]. An important factor that contributes to the loss of viability of bacteria both within aerosols and on dry surfaces is desiccation.

The use of the Weibull model to compare the survival curves of the different strains of *P. aeruginosa* allows for comparison of parameters of the survival curves and eliminates the impact of variations of the initial concentration of bacteria that may influence the time innocula may survive.

We have demonstrated that the mucoid phenotype is important for resistance to desiccation. All three strains available as both mucoid and nonmucoid phenotypes demonstrated greater resistance to desiccation when expressing the mucoid phenotype. This improved resistance to desiccation may be due to the alginate coating reducing the rate of evaporation of water from the bacteria, hence improving the ability of the organism to survive. Panagea et al. demonstrated no difference in survival of the Liverpool epidemic strain of *P. aeruginosa* regardless of the expression of a mucoid or nonmucoid phenotype [15]. Comparing an alginate deficient mutant of PAO1 with wild type, Chang et al. demonstrated that alginate production promoted desiccation resistance which would support the findings presented in the current study [26]. Skaliy and Eagon demonstrated that the *P. aeruginosa* cells in exponential phase of growth were most susceptible to the effects of desiccation compared to those in the stationary phase and that the addition of extracellular slime did not improve the desiccation resistance of exponential growth bacterial cells [27]. This would suggest that the high rate of metabolic activity associated with exponential growth may be more important to desiccation resistance than the presence of extracellular slime.

There was no pattern of improved desiccation resistance between the epidemic strains and nonepidemic strains of *P. aeruginosa*. All three groupings of strains according to the value of δ contained both epidemic and nonepidemic strains of *P. aeruginosa*. These data are contrary to the data presented by Panagea et al [15]. They demonstrated that the Liverpool epidemic strain demonstrated prolonged survival compared to other strains. One explanation for the differences between the previous and current study would be the lack of control of relative humidity in the study of Panagea et al. [15].

A consistent effect of preincubation with meropenem was to reduce desiccation resistance in most of the strains studied. Carbapenems at sub-MIC levels have been demonstrated to decrease outer membrane permeability, improve heat and osmotic stress responses, and increase bacterial susceptibility to neutrophil phagocytosis [12].

Ceftazidime had the lesser effect on the strains studied, causing a significant change in survival in only three of the bacterial strains studied. While cephalosporins have been shown to reduce bacterial adherence to pneumonocytes and polymorphonuclear phagocytosis, they do not appear to affect the hydrophobicity of the bacterial surface [13, 14].

Other β-lactam antibiotics have also been demonstrated to modify the survival characteristics of *P. aeruginosa*. Preincubation with piperacillin-tazobactam has been shown to decrease adhesion, reduce motility, reduce twitching, reduce biofilm formation, and increase the sensitivity to oxidative stress [28].

This study demonstrated that *P. aeruginosa* can survive within a dry environment for prolonged periods of time and that the mucoid phenotype is an important factor promoting survival. It also demonstrates that preincubation with sub-MIC levels may have important effects on the physiology of the bacteria in relation to their resistance to desiccation. Promoting bacterial survival through antibiotic exposure could have important clinical consequences by potentiating the risk of cross infection between people with CF. Further studies should be undertaken looking at the role sub-MIC concentrations of different antibiotics may have in promoting or inhibiting cross-infection with epidemic strains of *P. aeruginosa* in CF units.

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References

[1] G. P. Bodey, R. Bolivar, V. Fainstein, and L. Jadeja, "Infections caused by *Pseudomonas aeruginosa*," *Reviews of Infectious Diseases*, vol. 5, no. 2, pp. 279–313, 1983.

[2] G. Döring, S. Jansen, H. Noll et al., "Distribution and transmission of *Pseudomonas aeruginosa and Burkholderia cepacia* in a hospital ward," *Pediatric Pulmonology*, vol. 21, no. 2, pp. 90–100, 1996.

[3] G. Döring, M. Ulrich, W. Müller et al., "Generation of *Pseudomonas aeruginosa* aerosols during handwashing from contaminated sink drains, transmission to hands of hospital personnel, and its prevention by use of a new heating device," *Zentralblatt fur Hygiene und Umweltmedizin*, vol. 191, no. 5-6, pp. 494–505, 1991.

[4] D. G. Brown and J. Baublis, "Reservoirs of *Pseudomonas aeruginosa* in an intensive care unit for newborn infants: mechanisms of control," *Journal of Pediatrics*, vol. 90, no. 3, pp. 453–457, 1977.

[5] S. Reuter, A. Sigge, H. Wiedeck, and M. Trautmann, "Analysis of transmission pathways of *Pseudomonas aeruginosa* between patients and tap water outlets," *Critical Care Medicine*, vol. 30, no. 10, pp. 2222–2228, 2002.

[6] M. Denton, K. Kerr, L. Mooney et al., "Transmission of colistin-resistant *Pseudomonas aeruginosa* between patients attending a pediatric cystic fibrosis center," *Pediatric Pulmonology*, vol. 34, no. 4, pp. 257–261, 2002.

[7] A. M. Jones, J. R. W. Govan, C. J. Doherty et al., "Spread of a multiresistant strain of *Pseudomonas aeruginosa* in an adult cystic fibrosis clinic," *Lancet*, vol. 358, no. 9281, pp. 557–558, 2001.

[8] S. J. McCallum, J. Corkill, M. Gallagher, M. J. Ledson, C. Anthony Hart, and M. J. Walshaw, "Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by P aeruginosa," *The Lancet*, vol. 358, no. 9281, pp. 558–560, 2001.

[9] D. Armstrong, S. Bell, M. Robinson et al., "Evidence for spread of a clonal strain of *Pseudomonas aeruginosa* among cystic fibrosis clinics," *Journal of Clinical Microbiology*, vol. 41, no. 5, pp. 2266–2267, 2003.

[10] R. E. W. Hancock and D. P. Speert, "Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment," *Drug Resistance Updates*, vol. 3, no. 4, pp. 247–255, 2000.

[11] L. R. Hoffman, D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones, and S. I. Miller, "Aminoglycoside antibiotics induce bacterial biofilm formation," *Nature*, vol. 436, no. 7054, pp. 1171–1175, 2005.

[12] K. Murakami, T. Ono, D. Viducic et al., "Role for rpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance," *FEMS Microbiology Letters*, vol. 242, no. 1, pp. 161–167, 2005.

[13] M. C. Conejo, A. Pascual, A. I. Suarez, and E. J. Perea, "Effect of cefepime in the adherence and phagocytosis of *Pseudomonas aeruginosa*," *Enfermedades Infecciosas y Microbiologia Clinica*, vol. 10, no. 10, pp. 587–591, 1992.

[14] P. Di Martino, J. Rebière-Huët, and C. Hulen, "Effects of antibiotics on adherence of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* to A549 pneumocyte cells," *Chemotherapy*, vol. 46, no. 2, pp. 129–134, 2000.

[15] S. Panagea, C. Winstanley, M. J. Walshaw, M. J. Ledson, and C. A. Hart, "Environmental contamination with an epidemic strain of *Pseudomonas aeruginosa* in a Liverpool cystic fibrosis centre, and study of its survival on dry surfaces," *Journal of Hospital Infection*, vol. 59, no. 2, pp. 102–107, 2005.

[16] A. M. Jones, M. E. Dodd, C. J. Doherty, J. R. W. Govan, and A. K. Webb, "Increased treatment requirements of patients with cystic fibrosis who harbour a highly transmissible strain of *Pseudomonas aeruginosa*," *Thorax*, vol. 57, no. 11, pp. 924–925, 2002.

[17] A. B. Biodisk, "Etest for MIC determination," Tech. Rep., 1997, Technical guide 3B.

[18] K. Cheng, R. L. Smyth, J. R. W. Govan et al., "Spread of β-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic," *Lancet*, vol. 348, no. 9028, pp. 639–642, 1996.

[19] A. Jawad, H. Seifert, A. M. Snelling, J. Heritage, and P. M. Hawkey, "Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates," *Journal of Clinical Microbiology*, vol. 36, no. 7, pp. 1938–1941, 1998.

[20] A. Wexler, "Constant humidity solutions," in *Handbook of Chemistry and Physics*, D. R. Lide, Ed., p. 33, Taylor & Francis, Boca Raton, Fla, USA, 87 edition, 2006.

[21] P. Mafart, O. Couvert, S. Gaillard, and I. Leguerinel, "On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model," *International Journal of Food Microbiology*, vol. 72, no. 1-2, pp. 107–113, 2002.

[22] P. Mafart, O. Couvert, S. Gaillard, and I. Leguerinel, "On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model," *International Journal of Food Microbiology*, vol. 72, no. 1-2, pp. 107–113, 2002.

[23] S. J. McCallum, J. Corkill, M. Gallagher, M. J. Ledson, C. Anthony Hart, and M. J. Walshaw, "Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by P aeruginosa," *The Lancet*, vol. 358, no. 9281, pp. 558–560, 2001.

[24] F. P. Edenborough, H. R. Stone, S. J. Kelly, P. Zadik, C. J. Doherty, and J. R. W. Govan, "Genotyping of *Pseudomonas*

aeruginosa in cystic fibrosis suggests need for segregation," *Journal of Cystic Fibrosis*, vol. 3, no. 1, pp. 37–44, 2004.

[25] A. M. Jones, J. R. W. Govan, C. J. Doherty et al., "Identification of airborne dissemination of epidemic multiresistant strains of *Pseudomonas aeruginosa* at a CF centre during a cross infection outbreak," *Thorax*, vol. 58, no. 6, pp. 525–527, 2003.

[26] W. S. Chang, M. Van De Mortel, L. Nielsen, G. N. De Guzman, X. Li, and L. J. Halverson, "Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions," *Journal of Bacteriology*, vol. 189, no. 22, pp. 8290–8299, 2007.

[27] P. Skaliy and R. G. Eagon, "Effect of physiological age and state on survival of desiccated *Pseudomonas aeruginosa*," *Applied Microbiology*, vol. 24, no. 5, pp. 763–767, 1972.

[28] A. P. Fonseca, C. Extremina, A. F. Fonseca, and J. C. Sousa, "Effect of subinhibitory concentration of piperacillin/tazobactam on *Pseudomonas aeruginosa*," *Journal of Medical Microbiology*, vol. 53, no. 9, pp. 903–910, 2004.

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Research Article

Detection of Bacterial Endospores in Soil by Terbium Fluorescence

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Spore formation is a survival mechanism of microorganisms when facing unfavorable environmental conditions resulting in "dormant" states. We investigated the occurrence of bacterial endospores in soils from various locations including grasslands (pasture, meadow), allotment gardens, and forests, as well as fluvial sediments. Bacterial spores are characterized by their high content of dipicolinic acid (DPA). In the presence of terbium, DPA forms a complex showing a distinctive photoluminescence spectrum. DPA was released from soil by microwaving or autoclaving. The addition of aluminium chloride reduced signal quenching by interfering compounds such as phosphate. The highest spore content (up to 10^9 spores per gram of dry soil) was found in grassland soils. Spore content is related to soil type, to soil depth, and to soil carbon-to-nitrogen ratio. Our study might provide a basis for the detection of "hot spots" of bacterial spores in soil.

1. Introduction

The formation of spores is a survival mechanism of microorganisms when exposed to unfavorable environmental conditions (e.g., heavy metal stress, nutrient limitations) leading to a "dormant" or "resting" growth state [1, 2]. A variety of bacteria identified in diverse habitats including soil is able to form endospores. These physiological groups include aerobic heterotrophs (e.g., *Bacillus, Paenibacillus, Brevibacillus, Geobacillus, Thermoactinomyces,* and *Sporolactobacillus*), anaerobes (*Clostridium*, *Anaerobacter,* and *Desulfotomaculum*), microaerophiles (*Sporolactobacillus*), halophiles (*Sporohalobacter*), and phototrophs (*Heliobacterium*, *Heliophilum*) [3, 4]. Bacterial spores are characterized by a series of unique chemical features which can facilitate their identification in natural environments. Besides the high content of minerals (particularly calcium), spores contain high amounts of dipicolinic acid, DPA [5]. DPA is uniquely found in bacterial spores in amounts of up to 25% of the spore dry weight and depends on the bacterial species [6, 7]. In solution, a complex is formed in the presence of terbium which shows a very strong and distinctive fluorescence spectrum [8]. Originally, DPA was used to detect very low concentrations of terbium (III) [9]. On this basis, methods for the detection of bacterial endospores have been developed [10–13]: by the addition of terbium, the DPA content was determined.

However, terbium-DPA fluorescence might be interfered by a series of compounds, especially when DPA has to be determined in complex samples such as sediments or soils. It has been reported that the presence of phosphorus compounds (especially *ortho*-phosphate) reduced terbium fluorescence by as much as 98% [14]. The addition of aluminium compounds (especially aluminium chloride, $AlCl_3$), however, ameliorated the interference caused by the quenching substances [14]. From a series of organic compounds (benzoate, tryptophan, tyrosine, phenylalanine, glucose, malate, riboflavin, NAD, and tryptone) only the latter two (especially tryptone) reduced fluorescence significantly. Carbohydrates (e.g., starch, dextrine) were reported

not to interfere with the terbium fluorescence [15]. Inorganic compounds such as calcium carbonate, sodium chloride, potassium chloride, ammonium sulphate, ammonium nitrate, and sodium nitrate did not lead to a reduction of the fluorescence, but only dipotassium phosphate did [16].

The aim of this study was to adopt the fluorescence-based method to determine the spore content in soils sampled from various locations. In particular, we were interested in the differentiation between different types of soil such as grasslands (pasture, meadow), allotment gardens, and forests, as well as fluvial sediments, the relationship of soil parameters (carbon-to-nitrogen ratio) on the occurrence of bacterial spores, and the distribution of spores in relation to sampling depth.

2. Materials and Methods

2.1. Bacterial Spores. Different *Bacillus* species (*B. megaterium, B. subtilis*) were cultivated in liquid medium containing (in g/l): glucose (3.6), ammonium chloride (2.5), magnesium sulfate (0.2), calcium chloride (0.07), iron sulfate (0.01), EDTA (0.01), potassium dihydrogen phosphate (0.6), dipotassium hydrogen phosphate (0.9), and yeast extract (1.0). Initial pH was adjusted to 7.0. Erlenmeyer flasks (250 ml) containing 100 ml of growth medium were inoculated and incubated for 10 to 15 days (150 rpm, 30°C). To initiate and stimulate sporulation, bacteria were subsequently transferred to a sporulation medium (identical composition, but without glucose and less ammonium chloride [only 1 g/l]). After additional 30 days of incubation—until vegetative cells were not present anymore after inspection by microscopy—spores were harvested by centrifugation, immediately frozen in liquid nitrogen followed by lyophilization.

2.2. Soil Samples. Samples from different locations were collected using a stainless steel soil corer (15 mm in diameter), which was sterilized before each sampling. Cores with a maximum length of 25 cm were obtained, cut in sections of 5 cm, transferred to sterile screw cap Falcon tubes (20 ml), and stored on dry ice. After return to the laboratory, samples were immediately lyophylized or stored at −80°C until further processing.

Sampling sites were located in the surroundings of Zurich (Switzerland): grassland soil, meadow (municipalities of Männedorf; Uerikon; Stäfa; and Dübendorf), allotment garden (University of Zurich, Irchel campus), pasture (University of Zurich, Irchel campus; municipality of Wädenswil), forest soil (municipality of Stäfa), and aquatic sediments (river Glatt in Dübendorf).

Lyophilized aliquots of approximately 1 g were transferred to an Eppendorf micro test tube (2 ml) and ground (by adding a 6 mm glass bead) in TissueLyser (Retsch, Haan, Germany) for 5 × 1 min. Elemental composition (carbon, hydrogen, nitrogen) of soil was performed with a CHN-932 elemental analyzer (Leco Corp., St. Joseph, Minn, USA). Approximately 10 mg of powdered soil was used for analysis. Composition (in % of dry soil) varied between 2.2 and 15.4, 0.2 and 1.4, and 0.2 and 2.0 for total carbon, total hydrogen, and total nitrogen, respectively. Phosphate in aqueous soil extracts (250 mg soil in 5 ml sodium acetate buffer; 0.2 M, pH 5) was determined using commercially available kits (LCK 348 and 349; Hach Lange AG, Hegnau, Switzerland).

2.3. Release of DPA from Spores. 10 mg of dry spore powder was resuspended in 10 ml sodium acetate buffer (0.2 M, pH 5). Spores were counted under the microscope using a Neubauer counting chamber. Soil samples were thawed and 50 mg were suspended in 0.9 ml sodium acetate buffer and 0.1 ml aluminium chloride ($AlCl_3$, 0.5 M). Optimal volumetric amount and concentration of aluminium chloride was determined in preliminary experiments. Samples were microwaved (Berghof Microwave Digester MWS-1, with built-in *in situ* infrared temperature control) in Teflon TFM screw cap digestion vessels. Temperature and power were set to 140°C and approximately 680 W (80%), respectively. Alternatively, DPA was released from spores by autoclaving the samples in screw cap glass test tubes for 15 minutes at 121°C. The presence of spores after microwaving and autoclaving was determined by microscopy. The identical DPA extraction protocol was applied for soil samples. However, microscopy was not possible due to the presence of mineral particles interfering the observation.

2.4. Fluorescence Measurement. After cooling for 30 minutes, 100 μl of the spore suspensions were mixed with 100 μl terbium chloride solution ($TbCl_3$, 30 μM) in white 96-well microtiter plates (in 8 replicates). Fluorescence was immediately measured using a plate reader (SpectraMax M2, Bucher Biotec, Basel, Switzerland) with the following settings: time-resolved fluorescence (delay 50 μs, interval 1200 μs) at an excitation wavelength of 272 nm, emission wavelength of 545 nm, and 10 endpoint readings per sample at 30°C. The number of spores in the soil samples was determined using standard addition method with spores of *B. subtilis* [17]. Spore content was expressed as equivalents of *B. subtilis*.

3. Results and Discussion

Microwave treatment of spore suspensions and soil samples led to a fast release of DPA (Figure 1). Within two minutes, maximum release was obtained. Increased treatment time did not improve DPA mobilization. Bacterial spore content was related to soil type (Figure 2). Highest spore numbers up to 4×10^8 spores per gram dry soil were found in agriculturally used land (meadow, pasture), less in forest soil. Fluvial sediments showed lowest spore numbers. The interference of different compounds present in soil (e.g., phosphate) might lead to quenching of the fluorescence signal. This drawback has been overcome by the addition of aluminium chloride as already shown for the determination of bacterial spores in aquatic sediments [18]. Concentration of *ortho*-phosphate in soil extracts (22.5 μM) was reduced by the addition of aluminium chloride to concentrations below the detection limit (<1.2 μM). Concomitantly, a decolorization of the extract was observed suggesting the removal of humic acids which have also the potential to form complexes with terbium and quench the fluorescence signal [17]. The method based on terbium fluorescence for

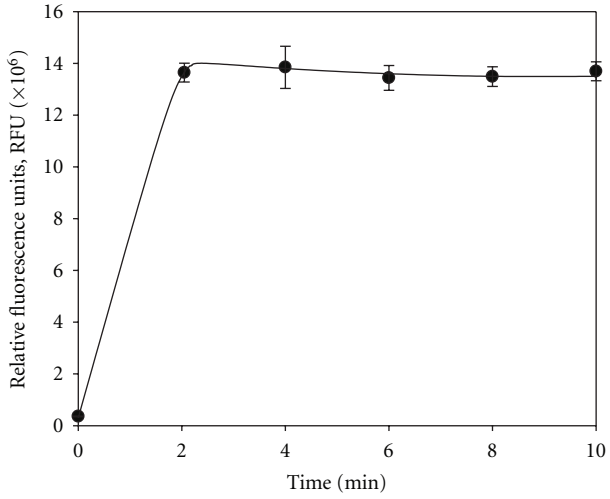

Figure 1: Release of dipicolinic acid (DPA) from a bacterial spore suspension of *B. megaterium* in relation to the duration of the microwave treatment. Points represent mean values of 8 replicates.

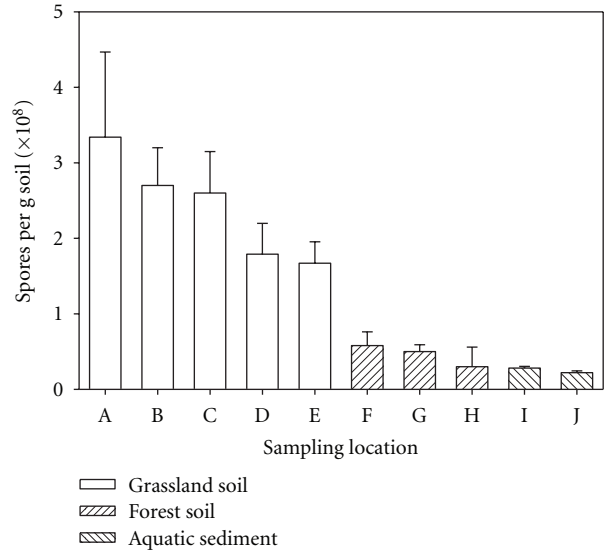

Figure 2: Spore content (expressed as equivalents of *B. subtilis*) of soil from ten different locations. A: grassland soil (municipality of Männedorf, site 1); B: grassland soil (municipality of Männedorf, site 2); C: grassland soil (municipality of Männedorf, site 3); D: grassland soil (municipality of Uerikon); E: grassland soil (municipality of Dübendorf); F: forest soil (municipality of Stäfa, site 1); G: forest soil (municipality of Stäfa, site 2); H: forest soil (municipality of Stäfa, site 3); I: aquatic sediment (river Glatt in Dübendorf, site 1); J: aquatic sediment (river Glatt in Dübendorf, site 2). Bars represent mean values of triplicates. Carbon, hydrogen, and nitrogen content (in % of dry soil) was for grassland soil 6.9 ± 0.6, 0.98 ± 0.07, and 0.54 ± 0.03; for forest soil 4.7 ± 2.3, 0.84 ± 0.29, and 0.32 ± 0.12; for aquatic sediments 6.5 ± 0.4, 0.36 ± 0.09, and 0.11 ± 0.03, respectively.

the detection of total numbers of bacterial endospores in soils is fast and easy.

A transect (approximately 100 m in length) through a grass field with different land use management (unused meadow, allotment garden, and pasture) gave spore numbers in the range of 5 to 9×10^8 spores per gram of dry soil (Figure 3). Spore counts were not related to the type of land use: in allotment garden soil, counts were not significantly different from soil samples taken from a pasture ($P = 0.423$; t-test). Regarding the different sampling sites, our results show that grassland soils (meadow, allotment garden, and pasture) contains much more bacterial spores than forest soils and fluvial sediments.

Spore content was related to the carbon-to-nitrogen ratio (Figure 4). At C/N ratios >20 only low spore counts (0.5×10^8 spores per gram of dry soil) were detected as compared to C/N ratios <20. It has been demonstrated in pure cultures of *Bacillus thuringiensis* in a stirred bioreactor that low carbon-to-nitrogen ratios of 4 : 1 resulted in high spore counts [19]. In contrast however, spore formation in *Streptomyces coelicolor* was stimulated under nitrogen-limiting conditions [20]. In particular, C/N ratios between 50 and 100 promoted sporulation, whereas C/N rations <40 did not allow spore formation. Our results showed that in soils with extremely high C/N ratios, spore content was low. The importance of C/N ratio was stressed by Gao and coworkers regarding the sporulation of fungi, although fungal spores do not contain DPA [21]. A carbon-to-nitrogen (C/N) ratio of 20 stimulated spore formation by fungi such as *Penicillium camembertii* [22]. The fungus *Colletotrichum coccodes* produced highest spore counts at a C/N ratio of 5 to 10, whereas at a ratio of 40, spore formation was significantly lower [23]. Similarly, in *Plectosporium tabacinum* optimal spore formation was found when C/N rations were between 5 and 10 [24]. The distribution of spores in marine sediments (determined as DPA) showed only a low correlation with the content of total organic carbon and varied with the sediment type [18].

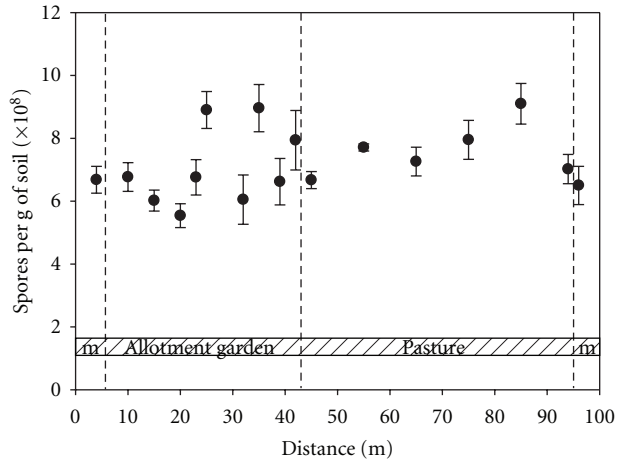

Figure 3: Transect of 100 m through a field showing different land use management: unused meadow (m), allotment garden, and pasture. Bacterial spore content is expressed as equivalents of *B. subtilis*. Data represent mean values of triplicates. Carbon, hydrogen, and nitrogen content (in % of dry soil) was 4.5 ± 0.6, 0.7 ± 0.1, and 0.3 ± 0.1, respectively.

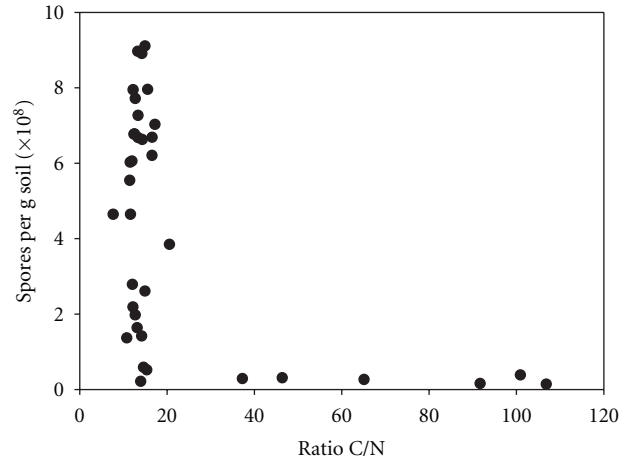

Figure 4: Spore number (expressed as equivalents of *B. subtilis*) as function of soil carbon-to-nitrogen ratio.

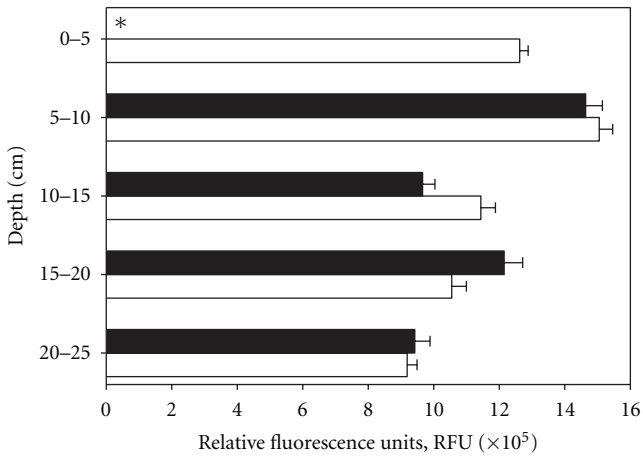

Figure 5: Depth profiles (0 to 25 cm) of bacterial spore content (expressed as equivalents of *B. subtilis*) in soil from a pasture. Comparison between autoclaving (open bars) and microwaving (solid bars) in releasing dipicolinic acid (DPA). Bars represent mean values of triplicate samples. *Sample was lost during filtration step.

Highest numbers have been found in organic-rich black sediments, lowest number in sandy sediments.

It was hypothesized from anthrax outbreaks, that the high numbers of *Bacillus* spores might be related to soils rich in organic matter that is, to a high C/N ratio [25]. These soil environmental conditions are suggested to support the presence and viability of *B. anthracis* spores [26]. However, we could not confirm this hypothesis.

Depth distribution of spores from an area currently used as allotment garden (cultivation of flowers and vegetables) showed highest numbers in a horizon of 5 to 10 cm (Figure 5). The two methods evaluated (microwaving, autoclaving) for the mobilization of DPA from bacterial spores gave similar results. However, microwaving was less time-consuming, whereas autoclaving allowed faster throughput of samples.

4. Conclusions

In summary, microwave treatment of soil samples followed by the measurement of fluorescence after addition of terbium proved to be a fast and easy method to assess the content of bacterial spores. Our study might provide a basis for the detection of "hot spots" of endospores in soil.

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References

[1] W. L. Nicholson, "Roles of *Bacillus* endospores in the environment," *Cellular & Molecular Life Sciences*, vol. 59, no. 3, pp. 410–416, 2002.

[2] M. J. Kennedy, S. L. Reader, and L. M. Swierczynski, "Preservation records of micro-organisms: evidence of the tenacity of life," *Microbiology*, vol. 140, no. 10, pp. 2513–2529, 1994.

[3] A. L. Sonenshein, "Endospore-forming bacteria: an overview," in *Prokaryotic Development*, Y. V. Brun and L. J. Shimkets, Eds., pp. 133–150, ASM Press, Washington, DC, USA, 2000.

[4] D. Fritze, "Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria," *Phytopathology*, vol. 94, no. 11, pp. 1245–1248, 2004.

[5] S. H. Pendukar and P. R. Kulkarni, "Chemical composition of *Bacillus* spores," *Die Nahrung*, vol. 32, no. 10, pp. 1003–1004, 1988.

[6] W. G. Murrell, "The biochemistry of the bacterial endospore," *Advances in Microbial Physiology*, vol. 1, pp. 133–251, 1967.

[7] A. Magge, A. C. Granger, P. G. Wahome et al., "Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*," *Journal of Bacteriology*, vol. 190, no. 14, pp. 4798–4807, 2008.

[8] I. Hemmilä, "Time-resolved fluorometric determination of terbium in aqueous solution," *Analytical Chemistry*, vol. 57, no. 8, pp. 1676–1681, 1985.

[9] T. D. Barela and A. D. Sherry, "A simple, one step fluorometric method for determination of nanomolar concentrations of terbium," *Analytical Biochemistry*, vol. 71, no. 2, pp. 351–357, 1976.

[10] D. L. Rosen, C. Sharpless, and L. B. McGown, "Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence," *Analytical Chemistry*, vol. 69, no. 6, pp. 1082–1085, 1997.

[11] A. A. Hindle and E. A. H. Hall, "Dipicolinic acid (DPA) assay revisited and appraised for spore detection," *Analyst*, vol. 124, no. 11, pp. 1599–1604, 1999.

[12] P. M. Pellegrino, N. F. Fell Jr., and J. B. Gillespie, "Enhanced spore detection using dipicolinate extraction techniques," *Analytica Chimica Acta*, vol. 455, no. 2, pp. 167–177, 2002.

[13] E. D. Lester and A. Ponce, "An anthrax "smoke" detector," *IEEE Engineering in Medicine and Biology Magazine*, vol. 21, no. 5, pp. 38–42, 2002.

[14] N. F. Fell, P. M. Pellegrino, and J. B. Gillespie, "Mitigating phosphate interference in bacterial endospore detection by Tb dipicolinate photoluminescence," *Analytica Chimica Acta*, vol. 426, no. 1, pp. 43–50, 2001.

[15] S. L. Chen, H. C. Zhao, C. Y. Sun, N. Lian, and L. P. Jin, "A study on terbium sensitized chemiluminescence of pipemidic acid and its application," *Analytical Letters*, vol. 35, no. 10, pp. 1705–1714, 2002.

[16] P. M. Pellegrino, N. F. Fell, D. L. Rosen, and J. B. Gillespie, "Bacterial endospore detection using terbium dipicolinate photoluminescence in the presence of chemical and biological materials," *Analytical Chemistry*, vol. 70, no. 9, pp. 1755–1760, 1998.

[17] J. Fichtel, J. Köster, J. Rullkötter, and H. Sass, "Spore dipicolinic acid contents used for estimating the number of endospores in sediments," *FEMS Microbiology Ecology*, vol. 61, no. 3, pp. 522–532, 2007.

[18] J. Fichtel, J. Köster, J. Rullkötter, and H. Sass, "High variations in endospore numbers within tidal flat sediments revealed by quantification of dipicolinic acid," *Geomicrobiology Journal*, vol. 25, no. 7-8, pp. 371–380, 2008.

[19] R. R. Farrera, F. Pérez-Guevara, and M. De La Torre, "Carbon:nitrogen ratio interacts with initial concentration of total solids on insecticidal crystal protein and spore production in *Bacillus thuringiensis* HD-73," *Applied Microbiology & Biotechnology*, vol. 49, no. 6, pp. 758–765, 1998.

[20] A. Karandikar, G. P. Sharpies, and G. Hobbs, "Influence of medium composition on sporulation by *Streptomyces coelicolor* A3(2) grown on defined solid media," *Biotechnology Techniques*, vol. 10, no. 2, pp. 79–82, 1996.

[21] L. Gao, M. H. Sun, X. Z. Liu, and Y. S. Che, "Effects of carbon concentration and carbon to nitrogen ratio on the growth and sporulation of several biocontrol fungi," *Mycological Research*, vol. 111, no. 1, pp. 87–92, 2007.

[22] I. Krasniewski, P. Molimard, G. Feron et al., "Impact of solid medium composition on the conidiation in *Penicillium camemberti*," *Process Biochemistry*, vol. 41, no. 6, pp. 1318–1324, 2006.

[23] X. Yu, S. G. Hallett, J. Sheppard, and A. K. Watson, "Effects of carbon concentration and carbon-to-nitrogen ratio on growth, conidiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccodes*," *Journal of Industrial Microbiology & Biotechnology*, vol. 20, no. 6, pp. 333–838, 1998.

[24] W. Zhang, M. Sulz, and K. L. Bailey, "Growth and spore production of *Plectosporium tabacinum*," *Canadian Journal of Botany*, vol. 79, no. 11, pp. 1297–1306, 2001.

[25] D. C. Dragon, R. P. Rennie, and B. T. Elkin, "Detection of anthrax spores in endemic regions of northern Canada," *Journal of Applied Microbiology*, vol. 91, no. 3, pp. 435–441, 2001.

[26] D. C. Dragon and R. P. Rennie, "The ecology of anthrax spores: tough but not invincible," *Canadian Veterinary Journal*, vol. 36, no. 5, pp. 295–301, 1995.

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Research Article

The Sulfate-Rich and Extreme Saline Sediment of the Ephemeral Tirez Lagoon: A Biotope for Acetoclastic Sulfate-Reducing Bacteria and Hydrogenotrophic Methanogenic Archaea

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Our goal was to examine the composition of methanogenic archaea (MA) and sulfate-reducing (SRP) and sulfur-oxidizing (SOP) prokaryotes in the extreme athalassohaline and particularly sulfate-rich sediment of Tirez Lagoon (Spain). Thus, adenosine-5′-phosphosulfate (APS) reductase α (*aprA*) and methyl coenzyme M reductase α (*mcrA*) gene markers were amplified given that both enzymes are specific for SRP, SOP, and MA, respectively. Anaerobic populations sampled at different depths in flooded and dry seasons from the anoxic sediment were compared qualitatively via denaturing gradient gel electrophoresis (DGGE) fingerprint analysis. Phylogenetic analyses allowed the detection of SRP belonging to Desulfobacteraceae, Desulfohalobiaceae, and Peptococcaceae in ∂-proteobacteria and Firmicutes and SOP belonging to Chromatiales/Thiotrichales clade and Ectothiorhodospiraceae in γ-proteobacteria as well as MA belonging to methylotrophic species in Methanosarcinaceae and one hydrogenotrophic species in Methanomicrobiaceae. We also estimated amino acid composition, GC content, and preferential codon usage for the AprA and McrA sequences from halophiles, nonhalophiles, and Tirez phylotypes. Even though our results cannot be currently conclusive regarding the halotolerant strategies carried out by Tirez phylotypes, we discuss the possibility of a plausible "salt-in" signal in SRP and SOP as well as of a speculative complementary haloadaptation between salt-in and salt-out strategies in MA.

1. Introduction

Molecular oxygen is found only in those biotopes that harbor organisms carrying out oxygenic photosynthesis. In oxygen-deficient systems, the nature of the redox couple and concentrations of electron acceptor/donor determine the succession of dissimilatory metabolisms due to thermodynamic conditions [1]. For a given substrate and under standard conditions, the aerobic dissimilatory metabolisms provide about one order of magnitude more energy than the anaerobic ones, for example, glucose respiration ($\Delta G^{\circ\prime} = -2877$ kJ/mol) versus glucose fermentation ($\Delta G^{\circ\prime} = -197$ kJ/mol) [2]. Therefore, in sedimentary environments oxygen is exhausted at deeper layers and the dissimilatory metabolisms are anaerobic as a result. Anaerobic microorganisms are of interest in extreme environments because environmental parameters such as temperature and salinity regulate the rates of organic matter remineralization [3]. Extreme halophilic microorganisms require at least 15% NaCl and tolerate up to 35% NaCl. Interestingly, the low activity of water and the expense on biosynthesis only select heterotrophs and strict aerobes as extreme halophiles. However, some moderate halophilic and strict anaerobes have been described; one example is the methanogen *Methanohalobium evestigatum,*

which uses methylated compounds (e.g., methylamine and methanol) to generate methane. Methylated substrates yield more energy ($\Delta G^{\circ\prime} = -78.7$ to -191.1 kJ per mol substrate) than H_2/CO_2 ($\Delta G^{\circ\prime} = -34$ kJ/mol substrate) or acetate ($\Delta G^{\circ\prime} = -31$ kJ mol substrate) and allow that methylotrophic methanogens such as *M. evestigatum* can tolerate up to 29.2% NaCl [4]. Differences in bioenergetic yield determine an exclusion of hydrogenotrophic methanogens such as *Methanocalculus halotolerans,* which tolerates a lesser salinity: up to 12% NaCl [5]. A similar pattern has been described for sulfate-reducing prokaryotes: acetoclastic sulfate reducers ($\Delta G^{\circ\prime} = -47.6$ kJ mol substrate), most of them belonging to *Desulfobacteraceae,* cease to tolerate high osmolarity conditions, for example, *Desulfobacter halotolerans* grows up to 13% NaCl [6]; on the other hand, *Desulfohalobiaceae* members have higher salt tolerances (up to 25% NaCl) and grow with H_2/CO_2 ($\Delta G^{\circ\prime} = -152.2$ kJ mol) or lactate ($\Delta G^{\circ\prime} = -160$ kJ/mol).

To define whether extremes of salinity are relevant in composition and persistence of anaerobic ecotypes, the ephemeral systems and spatial gradients constitute appropriate sites of study. Even though there are some studies about microbial communities present along salt gradients, those approaches have been performed on thalassic microbial mats [7]; therefore, they are depleted in sulfate at deep layers, but most of them are also formed only on intertidal zones. Sulfate is the second most abundant electron acceptor on Earth and consequently the dominant electron acceptor for anaerobic metabolism in marine sediments [8]. One interesting ephemeral and sulfate-rich system is Tirez lagoon, or *sabkha,* because it is subjected to flooding/desiccation regimes, located in "La Mancha," an arid region in Spain. Tirez lagoon is athalassic since the ionic composition is far from seawater and it is characterized by a low $Cl : SO_4$ ratio (about 1.18 in flooded season and 0.35 in the dry season), whilst in the Dead Sea this ratio is above 10^3 [9]. This system is maintained at a neutral pH due to a high Mg^{2+} and Ca^{2+} concentration in combination with a low $CO_3{}^{2-}$ content at the saltern and sediment environments. The traces of $CO_3{}^{2-}$ are removed as dolomite ($CaMg(CO_3)_2$) preventing alkalinization [10]. Given this scenario, the primary objective of this study was to characterize the composition of the anaerobic populations in the ephemeral and sulfate rich Tirez Lagoon.

The identification of environmental sulfate reducing prokaryotes (SRP) and sulfur oxidizing prokaryotes (SOP) can be performed by enrichment culturing and molecular ecology fingerprinting; however, a characterization of methanogenic archaea (MA) through isolation techniques is problematic given their slow growth rates [11]. The use of molecular ecology fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) from PCR-amplified genes is informative to assess the temporal and spatial qualitative diversity in natural samples, and it also requires fewer sequencing resources in comparison to clone libraries and/or metagenomic analysis [12]. Instead of the 16S rRNA gene, the use of DGGE from PCR-amplified functional gene markers is profitable to elucidate the composition of the anaerobic pathways of sulfate respiration (SR), sulfur oxidation (SO), and methanogenesis (MT). The 16S rRNA gene-based analysis cannot provide an unambiguous link between gene sequences and its physiological or metabolic role [13].

Whereas the SRP and SOP organisms are phylogenetically and physiologically disperse along the *Bacteria* and *Archaea* domains [14], MA organisms are monophyletic restricted to *Archaea* [15]. In the dissimilatory pathways of sulfate reduction and sulfur oxidation, dissimilatory sulfite reductase (*Dsr*) and adenosine-5′-phosphosulfate (APS) reductase (*Apr*) are considered as key enzymes [14]. In the sulfate-reducing pathway, sulfate has to be activated to APS by ATP-sulfurylase (EC: 2.7.7.4) at the expense of ATP; *Apr* (EC: 1.8.99.2) converts the APS to sulfite and AMP; hereafter, sulfite is reduced to sulfide by *Dsr* (EC: 1.8.7.1). For the sulfur-oxidizing pathway, the reverse direction is operated by homologous and conserved enzymes [16]. The alpha subunits of *Apr* and *Dsr* enzymes are found in all known SRP and most of SOP [17]. Regarding the methanogenesis pathway, the methyl coenzyme-M reductase (*Mcr*) (EC: 2.8.4.1) catalyses the reduction of a methyl group bound to coenzyme-M, with the concomitant release of methane [15]. *McrA* is unique and ubiquitous in all known MA [18]. *McrA* gene fragment provides more information than the 16S rRNA gene; even if the saturation rates are similar between the *McrA* gene fragment and the complete 16S rRNA gene, the number of differences per site in the *McrA* fragment is 2-3 times higher than that in the full-length *16S rrs* [19]. Therefore, assignment of genera with *McrA* sequences offers more conclusive resolution than assignment with 16S rRNA gene sequences. The mutation rates and selective pressures of the *AprA* and *McrA* metabolic gene markers and of the structural 16S rRNA gene are different; however, phylogenetic studies done with partial sequences of *AprA* and *McrA* belonging to the SRP, SOP, and MA lineages have established an agreement with the phylogenetic relationships based on 16S rRNA gene sequences [13, 18]. Therefore, these functional gene markers can provide an estimate of the SR, SO, and MT microbial diversity harbored in sediments of Tirez Lagoon. Indeed, databases have been enriched in sequences of model strains for these two enzymes; as a consequence, the *aprA* and *mcrA* gene markers also provide us information to identify SRP, SOP, and MA selectively in complex microbial communities, for example, [20].

The second aim of this study was to investigate whether the composition and distribution of the encoded amino acids in *aprA* and *mcrA* genes are indicative of haloadaptation to the hypersaline sediment. Diverse lines of evidence report that halophilic microorganisms can bias their amino acid composition to deal with the multimolar salinities of their environment [21, 22]. This adaptative and energetically efficient strategy is characteristic in "salt-in" halophiles, where turgidity is maintained by the intracellular accumulation of K^+ that is usually equilibrated with the presence of extracellular Cl^- [23]. Therefore, an increase of the acidic nature of cytoplasmic proteins, which is offset by an overall decrease in basic amino acids, is needed to maintain an appropriate folding and functionality under osmotic stress [22, 24]. In cytoplasmic proteins, it has been also pointed

out a slight decrease in hydrophobicity as another amino acid haloadaptation [25, 26]. In contrast, "salt-out" halophiles build up concentrations of osmolytes (also named osmoprotectants or compatible solutes) to increase the intracellular osmolarity; thereby maintaining the protein native states in spite of a highest energetic cost to manufacture the organic molecules [27]. Accordingly, only proteins in "salt-out" organisms exposed directly to the hypersaline medium exhibit an excess of acidic amino acids [28]. All eukaryotes, most halophilic bacteria, and the halophilic methanogenic archaea (such as *Methanohalobium evestigatum*) have evolved the "salt-out" strategy [21]. The widely disparate taxonomic position of "salt-in" prokaryotes (Halobacteriales in Archaea, *Salinibacter ruber,* and Halanaerobiales in Bacteria) suggests a convergent evolution of this osmoadaptation strategy [27].

Several studies have also reported that a high genomic CG content (often upwards of 60%) and a GC bias at the codon usage level are common adaptations to hypersaline environments, presumably to avoid UV-induced thymidine dimer formation and accumulation of mutations [21, 23]. For example, the high GC composition (65.9%) of *Halobacterium sp.* NRC1 could reduce the chance of such lesions and its third position GC bias correlates with an overrepresentation of acidic residues (i.e., Asp and Glu) [29]. The unique exception to this general trend has been pointed out so far in another extreme halophile *Haloquadratum walsbyi* shows a remarkably low genomic GC content (47.9%) and a weak GC-bias at the codon usage level [30]. Given that other specific features of nucleotide selection may also be involved in the GC content of organisms, the GC-bias measurements are complementary to the amino acid composition but not decisive in order to infer the "salt-in" strategy [25].

Therefore, the findings of this study try to contribute to the knowledge of diversity and haloadaptation of the SRP and MT thriving at rich sulfate sediment. Additionally, Tirez system is analog to the ocean of Europa, satellite of Jupiter, due to its sulfate abundance and neutral pH [31], and sulfates have been detected on Mars indeed [32]. Thus, this knowledge will provide insight regarding the possible biological limits for life in other analogous places.

2. Materials and Methods

2.1. Study Site and Sampling Procedure. Sediment samples were collected from Tirez lagoon, which has an area of $<1\,km^2$ and it was originated after endorheic inflow under semiarid conditions. The lagoon is located in the southern subplateau of the Iberian region of La Mancha (39° 32′ 42″ N y 03° 21′ O). The salt content fluctuates from 6% (w/v) during winter to 35% (w/v) during spring. In summer, the system becomes an evaporite. Temperature oscillation is about 40°C and −7°C, the mean annual thermal oscillation is 55°C, and the annual mean rainfall is averaged at 400 mm. Water drains through material from the Triassic period; dolomites and Ca-sulfate marls are from the Tertiary period [31].

Samples were collected in February and July 2005 and correspond to the winter and summer seasons, respectively. The winter and summer samplings were done by triplicate in three points at the lagoon; all of them were located in the salt pan or lagoon basin because it is the region covered by salts in summer. The sample cores were obtained from sites separated from each other by several meters. In order to analyze seasonal changes in the lagoon, the summer samples were obtained from the holes-signals leaved by the winter (flooded) sampling. Sediment cores were obtained with a Ring Kit core-sampler for soft soil to a depth of 40 cm. The sampled cores were cooled at −20°C with jelly bags and kept until further processing.

2.2. Physicochemical Parameters. The sediment cores were sampled in winter and used to perform physicochemical analyses. Eh and pH of the cores were measured with a probe connected to a potentiometer Orion Model 290A + Thermo Orion (Thermo Fisher Scientific). Also, dissolved oxygen and temperature were measured with a Sylant Simplair F-15 oxymeter (Syland Scientific GmbH). In order to determine the interstitial sulfide concentration, the sample cores from surface to 20 cm in depth were sonicated for 5 min (Labsonic B. Braun sonicator). After centrifugation (14,000 rpm 10 min Sorvall RC-5), supernatants were mixed with Zn acetate (2%) and sulfide concentration was determined using the methylene blue method [33]. The core samples used to analyze ion content (Cl^-, SO_4^{2-}, and NH_4^+) and carbon : nitrogen (C : N) ratios were sampled from the surface to 20 cm in depth; samples were dehydrated at 110°C for 12 hrs for ionic chromatography and elemental analysis. Ionic chromatography analysis was completed with 100 mg of pulverized samples diluted in 25 mL of filtered milliQ water, whilst the elemental analysis was performed with dried and pulverized sediment. The sediments were assayed by chromatographic methods with an IC Dionex DX-600 chromatograph and by spectrophotometric methods with a LECO CHNS-932 elemental analyzer at the *Servicio Interdepartamental de Investigación* (UAM).

2.3. Enrichment of SRP. SRP organisms from winter samples were grown in a cysteine-reduced (4.12 mM) medium for sulfate reducers, modified from Raskin et al. [34], and contained glutamic acid (5.2 mM), glycine (0.2 mM), methanol (14 mM), methylamine (27 mM), peptone (250 mg/L), and yeast extract (250 mg/L). The salt content in sulfate-reducing media was 3.5% the Tirez saltern. The inoculation was done with sedimentary slurry from samples collected in February 2005 (winter season). Cell culture growth was monitored through the count of the cell density with 4′,6-diamino-2-phenylindole (DAPI), Molecular Probes (Invitrogen) [35] in a Zeiss Axiovert 200M fluorescent microscope. Sulfide increase was also followed [33] for 12 months of incubation at 30°C. Nonaxenic cultures were subjected to DNA extraction.

2.4. DNA Extraction. Core samples were cut with sterile surgical blades according to depth regions. The three cores with a weight of ∼210 g were mixed with three volumes of PBS 1x at 4°C to reduce microheterogeneities and to wash salts. This mixture was sonicated for 3 min (Labsonic B. Braun sonicator). Integrity of bacterial cells after the

treatment was confirmed by 4′,6-diamino-2-phenylindole (DAPI) 1 μg/mL. Total genomic DNA was extracted from supernatants of washed and centrifuged sediments (500 rpm for 1 min Hettich Mikro 22 R centrifuge to precipitate rocks). In order to collect cells from nonaxenic cultures, 100 mL of the samples were filtered onto 0.22 μm of polycarbonate filters (Millipore). Sediment and soil samples are characterized by the presence of inhibitors such as humic acids and exopolimeric substances, thus we used a specialized DNA extraction kit (*FAST DNA SPIN kit for soil)* (QBiogene, Irving, Calif, USA) which has proved to retrieve a reliable DNA extraction to obtain a broad and intense band patterns, in comparison with variants via phenol DNA extraction [36], and it has been used for analyses of microbial diversity by DGGE in sediments, for example, [20]. Total genomic DNA was purified according to Genomic DNA purification JetQuick kit (Genomed) instructions.

2.5. PCR Amplification. Amplifications were carried out in a Thermal Cycler 2720 (Applied Biosystems). PCR reactions were performed in a mixture of 50 μL containing: 2 μL of template DNA, 1 mM dNTP'S, 0.5 μM of each primer, 3 mM $MgCl_2$, 1x enzyme buffer, and 0.03 U/μL AmpliTaq DNA Polymerase (Roche, Molecular Systems). The *aprA* gene fragment of ~0.4 kb was amplified with the APSfw (TGGCAGATMATGATYMACGGG with a GC clamp) and APSrv (GGGCCGTAACCGTCCTTGAA) primer pairs. The following conditions were implemented: a first denaturing step at 94°C for 3 min, the completion of 35 cycles of 30 s at 94°C, an annealing at 60°C for 55 s and at 72°C for 1 min, and a final extension of 72°C for 7 min [37]. In the *mcrA* gene fragment amplification, at first a 0.76 kb fragment was amplified with the primers ME1 (GCMATGCARATHGGWATGTC) and ME2 (TCATKGCRTAGTTDGGRTAGT). The ME-PCR reaction was carried out with an initial denaturing step at 94°C (5 min), followed by 25 cycles of 1 min at 94°C, an annealing at 57°C for 1 min and at 72°C for 2 min, and a final extension of 72°C during 10 min [38]. ME-PCR product was used as template to amplify an internal 0.47 kb fragment (Figure S4 see in supplementary Material available at doi: 10.1155/2011/753758). Nested PCR was performed with the primers MLf (GGTGGTGTMGGATTCACACARTAYGCWACAGC) and MLr (TTCATTGCRTAGTTWGGRTAGTT) with a GC clamp applying the following conditions: a denaturing step of 5 min at 94°C, 5 cycles at 95°C for 40 s, 55°C for 1 min, 72°C for 90 s (a ramp of 0.1°C/s was included between the annealing to the extension steps), followed by 30 cycles of 95°C for 40 s, 55°C for 1 min, 72°C for 90 s, and an extension of 72°C for 7 min [18]. The GC clamp was equivalent to 40 bp of GC at the 5′ end in order to prevent a complete melting of the DNA fragments. Correct length PCR-DGGE products were visualized on 0.5 μg/mL ethidium-bromide-stained gels at 2% agarose.

2.6. Denaturing Gradient Gel Electrophoresis (DGGE). In order to generate a DGGE pattern, an average of 50–70 μg of DNA from PCR-DGGE products were resolved using a D-Code Universal Mutation Detection System (BioRad Laboratories) in polyacrylamide gels with a horizontal denaturant gradient. All DGGE patterns were achieved under standardized denaturant and electrophoretic conditions: constant temperature of 60°C polyacrylamide composition (acrylamide-N,N′-methylene bisacrylamide, 37 : 1) containing 0–100% of denaturants (7 M urea and 40% formamide deionized with mixed-bed resin), a running time of 4.5 hrs and a constant voltage of 200 V [39]. High resolving band patterns from environmental and culture samples were obtained as follows (denaturant composition is given in percentage): for *aprA* gene fragments, 50–80% from nonaxenic cultures, and 40–70% from environmental samples. In *mcrA* gene fragments from environmental samples, the performed gradients were done by duplicate at 40–70% and 40–60% to increase the resolution of distance among bands. The gels were incubated in ethidium bromide for 20 min and rinsed in distilled water for 30 min. All single bands were excised from the gel with scalpels and eluted in 10 μL of milliQ water to avoid desiccation; hereafter, they were stored overnight at 4°C. DNA was extracted from polyacrylamide by electrophoresis in 2% agarose gels ($\leq$40 mA). The agarose bands were filtered through glass fiber columns at 14,000 rpm for 2 min (Hettich Mikro 22 R centrifuge). 5–10 μL of the precipitate obtained were used as DNA template for the band reamplification of *mcrA* and *aprA* genes under the same PCR conditions however, a minor fraction of the bands were reamplified. The *mcrA* and *aprA* gene PCR products were sequenced using primer pairs APSfw/APSrv and MLf/Mlr in an ABI 377 sequencer (Applied Biosystems). Nucleotide sequences were cleaned and assembled using DNA Baser software (Heracle Software, Germany, http://www.DnaBaser.com/).

2.7. DGGE Band Pattern Analysis. According to the review of Fromin et al. [40], DGGE reproducibility mainly relies on the DNA extraction and/or PCR amplification steps; therefore, the fingerprint analysis (DGGE) was processed once. The reproducibility of DGGE patterns has been tested previously by experimenting differences along the procedure, from sampling to PCR amplification conditions; despite these modifications, the comparison of DGGE patterns was consistent showing changes in band intensities only [41]. Thereby, the pattern of *aprA* DGGE gel from environmental samples was used to define a dendrogram. Then, the bands were qualitatively scored as present/absent and no semiquantitative analyses were performed for band intensity; the band clustering was performed with the maximum likelihood (ML) restriction analysis (RESTML) included in the PHYLIP v.3.67 package [42].

GenBank Accession Numbers. The nucleotide sequence data reported here are available under the GenBank accession numbers: EU722715–EU722732, HM466937–HM466940, HM466943–HM466946 (*aprA* phylotypes), and EU091355–EU091364, HM466948 (*mcrA* phylotypes).

2.8. Phylogenetic Analysis. The translations of the *aprA* and *mcrA* sequences into amino acids were defined using the TRANSLATE tool with a standard code (http://expasy.org/tools/dna.html/). The best frames for all the *aprA*

and *mcrA* fragments were firstly selected by the unstopped amino acid (aa) sequences and, secondly, by matching their best hits with those compiled in the nonredundant database of the GenBank, which were detected through the BLASTP program (*e*-value $\leq 10^{-3}$) [43]. The final inferred aa sequences were compared against the Swiss Prot and GenBank databases in order to obtain their homologous counterparts using the WU-BLAST program [44] with a significant BLASTP *e*-value $\leq 10^{-3}$. From a first approach, we also included *aprA* and *mcrA* sequences from reported environmental samples as seed sequences. A complete list of the sequences included in this study to reconstruct a phylogeny for the *AprA* and *McrA* enzymes can be seen in Supplementary Material (Tables S1 and S2, resp.).

Different filters were used, from the thousands of collected sequences, in order to choose the final candidates involved in the reconstruction of a phylogenetic hypothesis. In this sense, we firstly applied a redundant analysis at 90% identity using the CD-HIT program [45]. From the obtained sequences, a second analysis at 100% identity was done with the CD-HIT program, which excludes redundant phylotypes (subspecies and variants) of the same species, warranting the diversity of sequences only by including strictly different species from the same or different genera. The *AprA* phylotypes aps cw 1 (EU722715) and aps cw 16 (EU722724) showed 100% of identity, as well as the phylotypes aps cw 3 (EU722716) and aps cw 10 (EU722720). Only one sequence was taken as a representative of the identity cluster to reconstruct the phylogeny. In order to support a robust identification in the final phylogeny, we included species from the same genera for those cases in which the homologous counterparts are closely related to the phylotypes obtained in this work. When it was necessary, individual phylogenetic trees for the Tirez phylotypes were done previously in order to improve their identification and to select the counterpart sequences for the final phylogeny. The aa sequences obtained from the previous approaches were then aligned using the CLUSTALX program with default parameters [46]. In order to identify an evolutionary signal from the sequence fragments obtained in this work and their homologous counterparts, we applied a manual and also an automatic approach to edit the alignment.

First, we manually edited the alignment through the use of the BIOEDIT program v.7.0.9 [47] in order to include only the functional domains of the α subunits of *mcr* and *apr* enzymes. The functional description of these domains is fully detailed in Section 4 and in the Supplementary material. The N-terminal domain of the α-subunit of the *AprA* (AprA_alpha_N) harbors the FAD cofactor-binding domain (aa positions: 2–261 and 394–487) and the capping domains (aa positions: 262–393). These functional domains have been characterized from *Archaeoglobus fulgidus* in the reduced state (FAD_{red}-APS, PDB ID: 1JNR) [16] and in the oxidized state (FAD_{ox}-APS, PDB ID: 2FJA) [48] as well as in *Desulfovibrio gigas* (PDB ID: 3GYX) [49]. Thus, a total of 100 aa sequences were included in the *AprA* alignment, where 23 phylotypes are derived from this work. The *AprA* alignment includes two of the nine (the absent sites are Asn-$N^{\alpha 74}$, Tyr-$Y^{\alpha 95}$, Glu-$E^{\alpha 141}$, Val-$V^{\alpha 273}$, Gly-$G^{\alpha 274}$, Leu-$L^{\alpha 278}$, and Arg-$R^{\alpha 317}$) functional active sites of the AprA_alpha_N domain: Arg-$R^{\alpha 265}$ and Trp-$W^{\alpha 234}$, previously reported [48]. See the *AprA* alignment and catalytic sites in Supplementary Material (Figure S2). On the other hand, the C-terminal domain of the α-subunit of the *McrA* enzyme (Mcr_alpha_C) harbors an all-alpha multihelical bundle domain (PFAM domain: PF02249). This functional domain has been characterized in *Methanosarcina barkeri* (PDB ID: 1E6Y, C-terminal domain: 328–460) [50], *Methanothermobacter thermoautotrophicus* (PDB ID: 1MRO, C-terminal domain: 315–440) [51], and *Methanopyrus kandleri* (PDB ID: 1E6V, C-terminal domain: 319–444) [52]. A total of 80 aa sequences, 11 of them derived from this work, were included in the *McrA* alignment. The *McrA* alignment includes five of the seven functional active sites of the Mcr_alpha_C domain (absent sites: Asn-$N^{\alpha 481}$ and Val-$V^{\alpha 482}$; present sites: Phe-$F^{\alpha 330}$, Tyr-$Y^{\alpha 333}$, Phe-$F^{\alpha 443}$, Tyr-$Y^{\alpha 444}$, and Gly-$G^{\alpha 445}$) [51]. See the *McrA* alignment and catalytic sites in the supplementary material (Figure S3).

Finally, we readjusted a final alignment defining the informative sites and conserving the functional active sites of the enzymes previously described, through the use of the software GBLOCKS v.0.91 [53]. Therefore, the final alignments were performed on a region of 137 aas for *AprA* and 139 aas for *MrcA*, from which 122 and 132 positions were involved in the phylogenetic analysis, respectively. In order to reconstruct a phylogenetic tree, a character-based approach for the Apr_alpha_N and Mcr_alpha_C phylogenetic reconstructions was developed using the PROTPARS program in order to construct a maximum parsimony (MP) tree of sequences. *Pyrobaculum aerophilum* and *Archaeoglobus fulgidus* were used as outgroups in the Apr_alpha_N phylogeny; whereas *Methanopyrus kandleri* was used as outgroup in the Mcr_alpha_C phylogeny. A distance approach for the Apr_alpha_N and Mcr_alpha_C phylogenetic reconstructions was also developed using the SEQBOOT program to generate 1000 bootstrapped datasets from the sequences, whereby the pseudoreplicates were used in the PROTDIST program in order to generate a distance matrix through the Jones-Taylor-Thornton (JTT) model of evolution [54]. The evolutionary distances are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution estimated previously shape parameter = 0.9 for *AprA* and = 0.8 for *McrA*. Then, the distance matrix was used in the NEIGHBOR program to construct a neighbor-joining (NJ) tree [55]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates were collapsed with the CONSENSUS program (default parameters). The percentage of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. All these programs belong to the PHYLIP package v.3.68 [42]. No major branching differences were detected between the MP and NJ topologies obtained for both enzymes. The trees were visualized and reannotated using the MEGA and Microsoft Photoshop programs [56].

2.9. Amino Acid Composition Analysis. We calculated the amino acid composition of all AprA and McrA sequences used previously to construct the phylogenies. Using the Perl scripting language, a program was written to read each amino acid sequence (FASTA format) and calculate the frequency for each amino acid. We also calculated two halophilia indicators from the amino acid composition of every sequence: the PAB factor estimates the surplus of polar and acidic amino acid compared to polar and basic ones (i.e., PAB = [Asx + Glx] − [Arg + Lys]) [57], and the AB ratio of the acidic amino acids Glu and Asp to the basic amino acids Lys, His, and Arg (i.e., AB = [Asp + Glu] : [His + Arg + Lys]) [21]. We divided the amino acid profiles from each marker into two different data sets in order to calculate an average and standard deviation of the samples. The first data set is based on salinity adaptation by dividing sequences in Tirez, halophilic, and nonhalophilic species. The second data set is based on the species forming the major taxonomic groups in which the Tirez phylotypes are phylogenetically allocated. For AprA Desulfovibrionales, Desulfobacterales, Peptococcales, and Chromatiales; whilst for McrA, Methanomicrobiales and Methanosarcinales. Therefore, A spreadsheet was created using Microsoft Excel software for data tabulation and graph construction. See Supplementary Material.

2.10. GC Content and Codon Bias Analyses. We performed the corresponding nucleotide alignments for all AprA and McrA sequences used previously to construct the phylogenies. In order to reduce the bias of the GC measurements by the use of sequences with different length and highly divergent regions (i.e., long indels), we manually edited and readjusted the final alignment. Accordingly, highly and long divergent regions (insertions and deletions) were eliminated from the alignment. The final alignments only include the strict codon positions encoding for the functional domains of AprA (375 nucleotide positions, 125 codons) and McrA (399 nucleotide positions, 133 codons) described on the phylogenetic analysis section. Using the Perl scripting language, a program was written to read each nucleotide sequence (FASTA format) and calculate the total nucleotide percentages as well as at the three individual codon positions for each sequence. We divided the GC profiles from each gene marker into the same data sets used to analyze amino acid composition (i.e., salinity adaptation and taxonomic clades) in order to calculate an average and standard deviation of the samples. A correction for the amino acid usage was applied by the calculation of the relative synonymous codon usage (RSCU) values from the nucleotide datasets based on salinity adaptation: Tirez, halophilic, and nonhalophilic species. The RSCU for a particular codon (i) is given by: $\mathrm{RSCU}_i = X_i / \sum\sum X_i/n$, where X_i is the number of times the codon has been used for a given amino acid and n is the number of synonymous codons for that amino acid. RSCU values are the number of times a particular codon is observed, relative to the number of times that the codon would be observed in the absence of any codon usage bias [58]. In the absence of any codon usage bias, the RSCU value would be 1.00. A codon that is used less frequently than expected will have a value of less than 1.00 and *vice versa* for a codon that is used more frequently than expected. Finally, a spreadsheet was created using Microsoft Excel software for data tabulation and graph construction. See supplementary material.

3. Results

3.1. Physicochemical Characterization. Sediment cores from Tirez Lagoon sampled at different depths were subjected to physicochemical analysis. Sulfide showed higher concentrations at the zone of 0–10 cm depth (Figure 1(a)). The occurrence and distribution of sulfide along the depth profile can reflect a biogenic origin by the presence of sulfate-reducing bacteria (SRB) in the hypersaline sediment. The concentration of H_2S coincided with the presence of a black deposit of iron sulfide mainly in winter (Figure 2). Sulfate levels increased with depth, its concentration ranging at 0.2 M, and the highest values were detected at 10–15 cm in depth ($\leq$300 mM), just below the highest concentration zone of sulfide (Figure 1(a)). The complete sediment profile was anoxic and in accordance with a negative redox potential (Figure 1(b)). The redox potential and oxygen levels slightly increased in the deepest zones (15–20 cm in depth). The redox conditions of most part of the sediment core were in the range of −300 and −200 mV, low enough to allow SR and MT activities [59]. The lowest Eh values were reached at 0–10 cm in depth and coincided with the increase in sulfide concentration (Figure 1(b)). Ammonium concentration fluctuated between 1 and 6 μM. Likewise, the highest NH_4^+ concentration (4–6 μM) was observed at 10–15 cm in depth (Figure 1(c)). The $Cl : SO_4$ proportion fluctuated between 0.1 and 0.3, these ratios are lower than the values reported in the saltern [60] and they reflect the athalassic nature of the system. Sulfate concentration in Tirez Lagoon was lower than in the also athalassic Chaka Lake sediment (10^{-1} mM). Even though chloride was undetermined in Chaka Lake sediment, its $Cl : SO_4$ proportion is two times higher than the highest value registered at Tirez Lagoon [61]. Figure 1(d) shows the pH course on sediment depth; it is possible to observe the characteristic neutral pH of the system as well as a slight acidification, probably a consequence of biological volatile fatty acids (VFA) formation and sulfate reduction processes. The C : N ratio determined in the samples showed values characteristic of low photoautotrophic activity at the surface [62] starting at >6 at 0–5 cm depth (Figure 1(d)). Therefore, preferential nitrogen mineralizers should be found at the surface preceding carbon mineralizers at deeper zones. Figure 1(e) describes that divalent cations dominate over monovalents. Finally, the ratio $(Na^+ + K^+)/(Mg^{2+} + Ca^{2+})$ in Tirez is between 1.8 and 0.09, whilst in Salt Lake is >9.0 [9].

3.2. DGGE Patterns from aprA and mcrA Gene Fragments. We applied a denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis through the use of two functional genes: adenosine-5′-phosphosulfate reductase (*Apr*) and the methyl coenzyme-M reductase (*Mcr*), in order to identify ecotypes from the sediments samples and nonaxenic cultures of Tirez Lagoon. *AprA* DGGE profiles are presented in Figure 3 and *McrA* DGGE profile in Figure 4. Thus, we

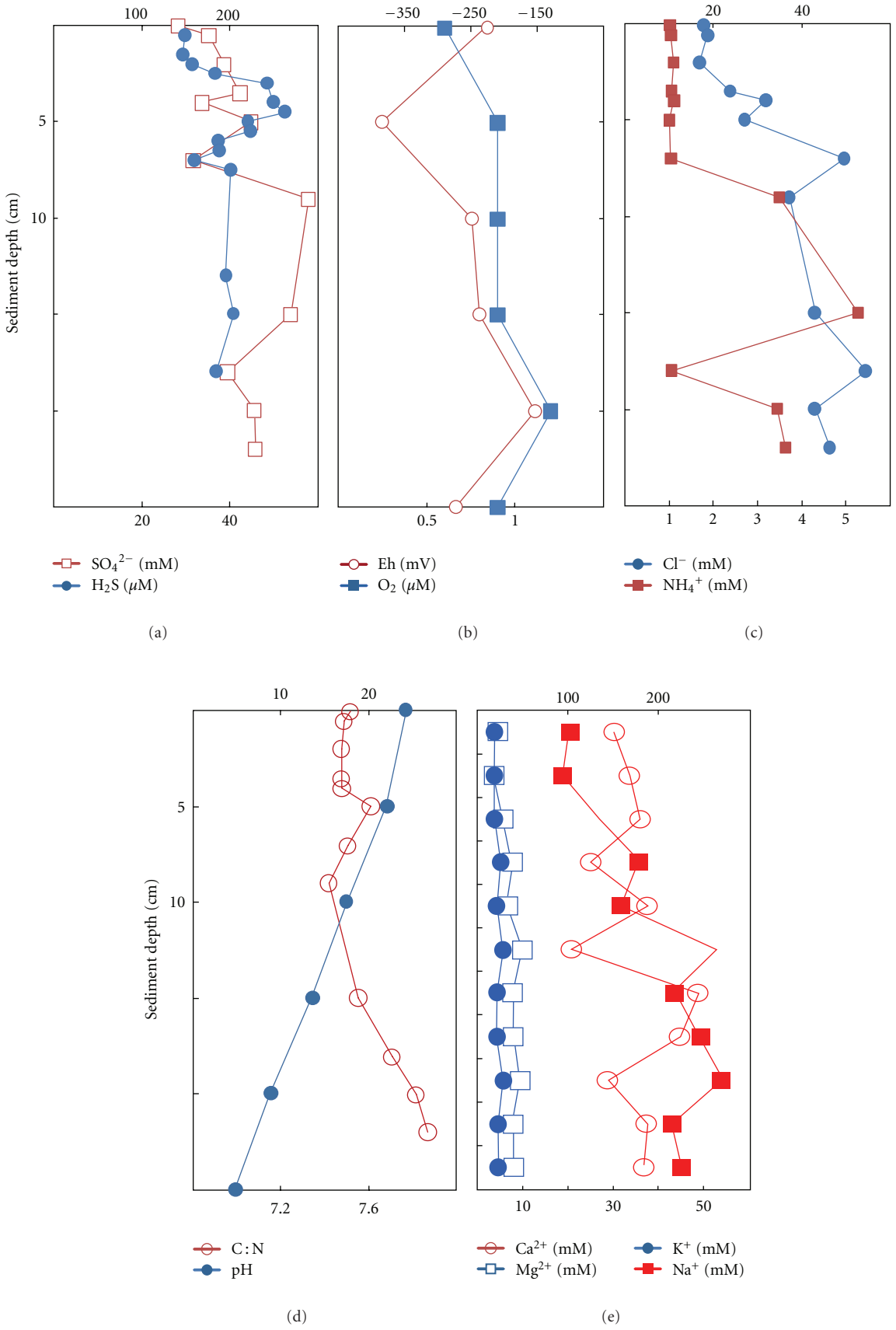
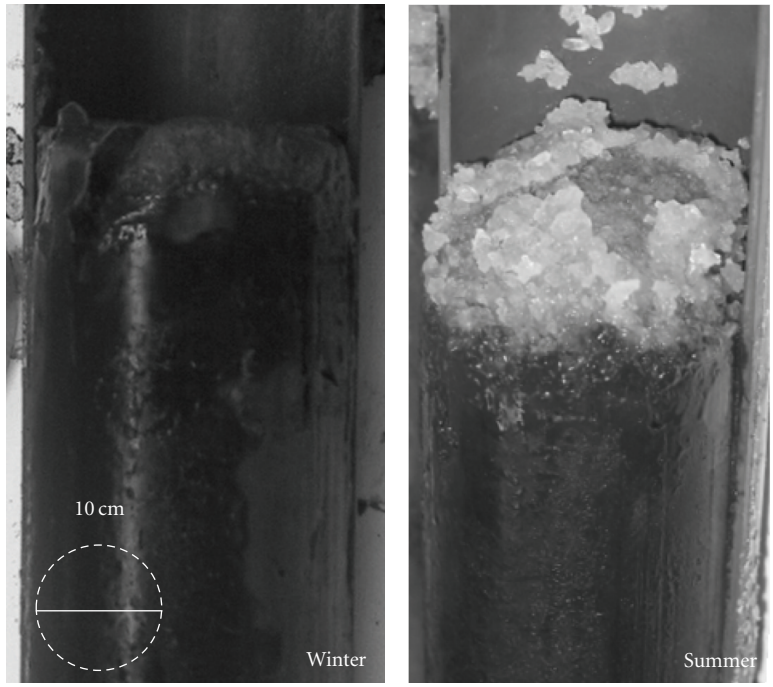


Figure 1: Profiles plotting depth against physicochemical parameters measured in Tirez sediments from winter cores. (a) Sulfide and sulfate; (b) Redox potential (Eh) and oxygen; (c) chloride and ammonium; (d) pH and C : N ratio; (e) magnesium, calcium, potassium, and sodium.



(a) (b)

Figure 2: Cores of sediments of Tirez ephemeral lagoon for analysis collected from winter and summer showing the dark zone in the upper region probably due to metal sulfide precipitation. The evaporite is founded in summer sample.

obtained sequences of diverse phylotypes from DGGE profiles representing the bulk content of three sampling points of the lagoon salt pan. The bands were prefixed as aps and mcr (from the gene marker) and subfixed as cw (from nonaxenic cultures obtained in winter) and ew and es (environmental sediment sampled in winter and summer, resp.).

The *aprA* DGGE pattern from sediment profile (Figure 3(b)) revealed the presence of a more complex banding pattern in comparison with the profile from nonaxenic cultures (Figure 3(a)). At 15–25 cm depth, low yield or no PCR product was obtained (Figure 3(b) lane 5). Given that a considerable number of environmental bands from the *aprA* DGGE profile could not be sequenced or specifically identified, probably due to the presence of residual PCR inhibitors such as humic acids coextracted with genomic DNA [63] that were not purified by the *JetQuick kit* and that comigrate with DNA in the polyacrylamide gel [64] changes in population distribution were estimated through the use of P-analysis with Maximum Likelihood (ML) in Phylip software in order to identify a significant clustering. Bands were taken as species, and patterns were constructed by presence and absence. The clustering pattern is shown in Figure 3(c), and it was more in accordance with a disturbance due to seasonality instead of sediment depth. Additionally, P value showed no significant differences between nodes W and S being $P \leq 0.05$ as significant to reject the hypothesis that two population sets were derived from the same communities.

From previous studies carried out in thalassic communities, where salt gradient is between 8 and 20% (within the range of Tirez), it has been reported that the rate of methanogenesis is below 0.1% of the total sulfate-reduction productivity [65]. Therefore, a lower abundance of MA was expected in Tirez sedimentary community it is inferred from the lower $Cl : SO_4$ ratio. In addition, the population size threshold for DGGE detection is ≤1% [39]. Thereby, we had to perform a nested PCR from the *mcrA* gene in order to improve the detection of the MA community in the sediment samples from Tirez. We firstly obtained a 0.76 kb *mcrA* fragment through the ME primer pair. Because such a length is inadequate to obtain a discernible DGGE pattern [66] and due to low yield in ME amplicons, a small 0.47 kb *mcrA* fragment nested in the ME region was amplified through the ML primer pair (supplementary material, Figure S4). In agreement with Juottonen and collaborators [67], no differences in the diversity of MA organisms were expected from the use of ME and ML PCR products. Different DGGE gradients for the ML-PCR products were tested in order to obtain the best pattern resolution. We detected two distinctive but adjacent bands in all DGGE winter profiles (e.g., mcr-ew1 and mcr-ew2) obtained through several gradients (Figures 4(a) and 4(c), 40–70% and 40–60% gradients, resp.). A pattern of bands in pairs is a result of the low DGGE resolution, where two DNA fragments differ in one or few bases due to the use of ambiguous primers [66]. Though ME-ML primers are ambiguous (see Section 2), the phylotypes were placed in different orders in methanogenic archaea. Thereby, nucleotide sequences have similar electrophoretic mobilities but they represent different sequences and, thus, a double band was ruled out.

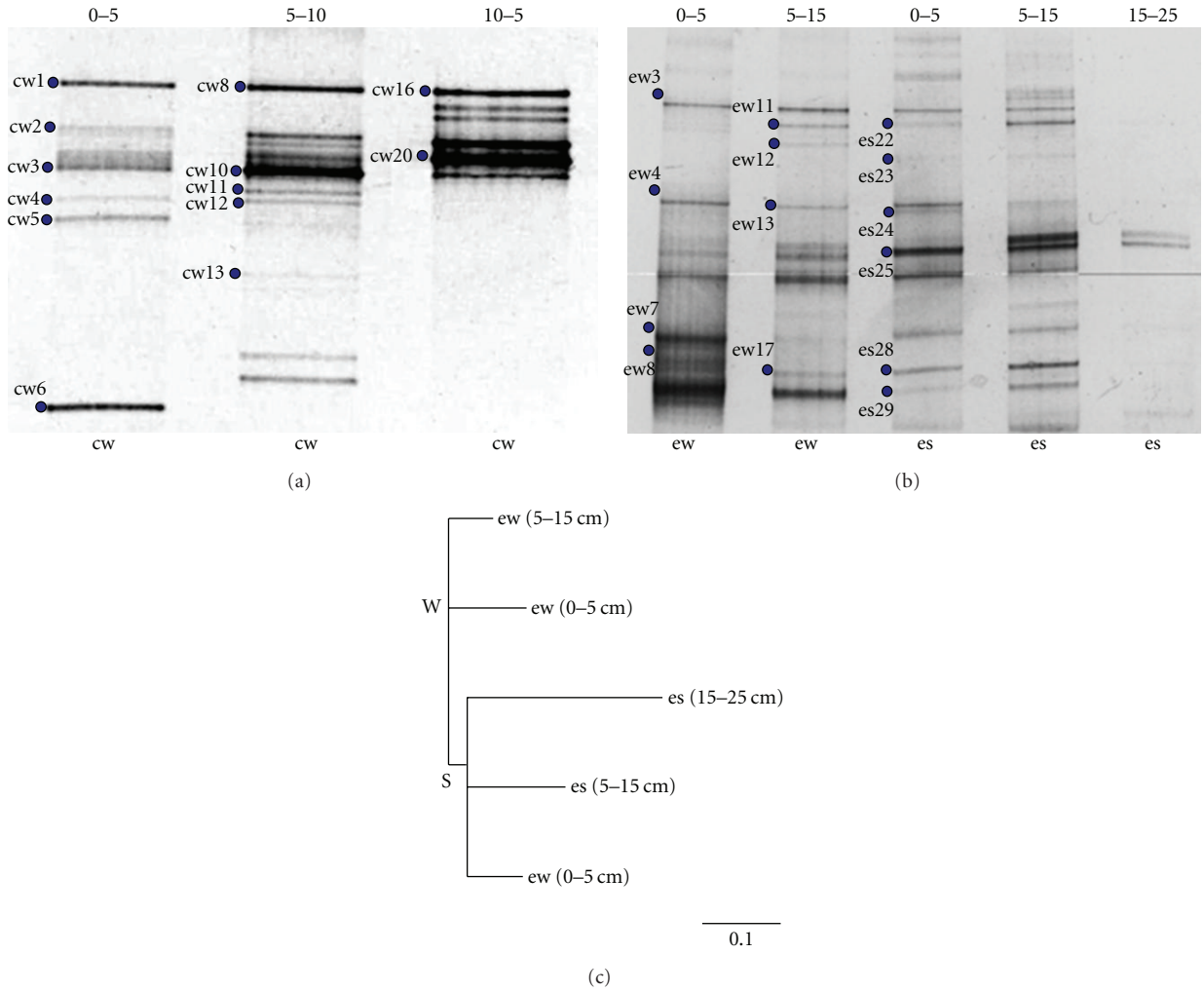


Figure 3: DGGE pattern of PCR-amplified *aprA* gene fragments from nonaxenic SRP cultures (a) and sediment samples (b). (a) series of DGGE patterns obtained from nonaxenic cultures inoculated from winter sediment (cw); (b) Series of DGGE patterns obtained from environmental samples: winter (ew) and summer (es) obtained from different depths (cm). The *aprA* gene fragment from 15–25 cm depth (winter) was not amplified. (c) Maximum Likelihood cluster analysis of the B-pattern DGGE profile the scale bar represents expected numbers of base substitutions.

We also obtained a DGGE pattern from sediment sampled in flooded and dry seasons at different depths (Figure 4(c)). It is interesting to note that the mcr-ew1 band (marked in Figures 4(a) and 4(c)) appeared uniquely associated with flooded season at 0–5 cm in depth (Figure 4(c)).

3.3. Phylogenetic Diversity of Sulfate-Reducing, Sulfate-Oxidizing and Methanogenic Organisms. Phylogenetic reconstructions were done for the inferred amino acid (aa) sequences of *aprA* and *mcrA* gene markers and their homologous counterparts. We decided to analyze aa instead of nucleotides because the latter reduces the inherent variation seen in protein sequences, except for the third codon base. We defined two regions of unambiguously aligned aa, the first one located in the N-terminal domain (137 aas) for the α subunit of *AprA* (AprA_alpha_N), and the second one located in the C-terminal domain (139 aas) of the α subunit of *McrA* (MrcA_alpha_C), both of them containing some of the catalytic sites involved in their metabolic role (supplementary material, Figures S2 and S3, resp.). It is important to note that not only the phylogenetic topologies obtained for the AprA_alpha_N and McrA_alpha_C sequences are robust, as can be seen by the significant bootstrap values in the main clustering branches, but also the internal groups are supported by the expected clustering of the *McrA* and *AprA* crystals previously characterized for (a) the *McrA* in *Methanosarcina barkeri* belonging to Methanosarcinales [50], *Methanothermobacter thermoautotrophicus* from Methanobacteriales [51] and *Methanopyrus kandleri* in Methanopyrales [52]; (b) the *AprA* from *Archaeoglobus fulgidus* in Euryarchaeota [16], and *Desulfovibrio gigas* in Deltaproteobacteria [49].

The phylogenetic analysis of the 25 *AprA* Tirez sequences is presented in Figure 5. This analysis included representative species from diverse SRP and SOP taxonomic groups such

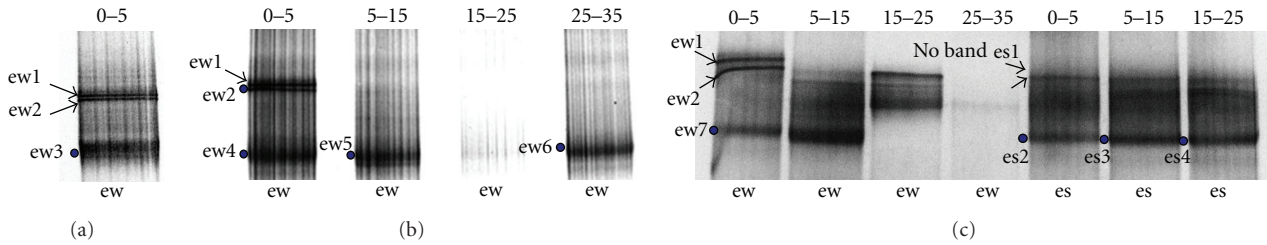


Figure 4: DGGE pattern of PCR-amplified *McrA* gene fragments from environmental sediment samples. (a) winter pattern (0–5 cm depth), (b) winter patterns obtained from different depths, and (c) Winter (ew) and summer (es) patterns from different depths (cm). Bands across several lanes were identified as being in the same genera, and the arrow for ew1 shows its absence in the 0–5 cm depth summer sample (es). Band mcr-es5 is not shown in the figure.

as Euryarchaeota, Crenarchaeota, Firmicutes, β, γ, and ∂-proteobacteria (supplementary material, Table S1). The major fraction (16 phylotypes) was affiliated to the SRP. Some of the environmental *AprA* phylotypes were not resolved at genera level, and the result has been discussed for the next taxonomic rank. Cultured and environmental SRP populations were identified as follows: cultured phylotypes (Desulfohalobiaceae, Peptococcaceae, and Desulfobacteraceae) and environmental phylotyeps (Desulfobacteraceae, and Peptococcaceae). One cluster formed by three phylotypes (aps-cw2, -cw4, and -cw5) was closely related to the halotolerant and alkaliphilic *Desulfonatronovibrio hydrogenovorans*. Interestingly, the summer sediment did not reveal the presence of species in the haloadapted Desulfohalobiaceae. Twelve phylotypes obtained from sediment (summer and winter) and enrichments were related to the acetoclastic and nonhalophilic species *Desulfonema magnum*. Two phylotypes (aps-cw6 -es29) were identified as Peptococcaceae. Whilst the phylotype aps-cw6 was conclusively affiliated to *Desulfotomaculum solfataricum* belonging to Firmicutes, the phylotype aps-es29 was not resolved at genera level; however, aps-es29 was allocated basal to the representative Firmicutes taxa used in this study. Actually, the affiliation of phylotype aps-es29 and other SOP Tirez phylotypes could become particularly uncertain given the well-known horizontal APS reductase (Apr) gene transfer (HGT) events between the SRPs from Firmicutes and δ-proteobacteria as well as between the SOPs from β and γ-proteobacteria, respectively (see Figure 5). Both main Apr-HGT events are identified in this work and are in accordance with previous phylogenetic studies [68].

In four of the environmental SOP phylotypes (aps-ew7, -ew8, and -ew13, aps-es28), the assignment of the *aprA* gene fragment could not be conclusive at species level; thus, a detailed function in Tirez's system remains uncertain. The closest clade for three phylotypes was a group of noncultured microorganisms (endosymbionts) in Hydrogenophilaceae in β-proteobacteria. The phylotype from summer sediment (aps-es28) remained unidentified at species level, and tree topology helped to designate it as γ-proteobacteria. The environmental phylotype aps-ew3 was conclusively affiliated to endosymbionts and close to Thiotrichaceae and Chromatiaceae in γ-proteobacteria. Other three phylotypes were derived from enrichment (aps-cw11, -cw12, and -cw13) and resulted with a short distance with the cultured haloalkaliphilic purple bacteria *Thioalkalivibrio* (Ectothiorhodospiraceae) in γ-proteobacteria.

The phylogenetic reconstruction of the eleven *McrA* sequences obtained from the anoxic Tirez sediments is shown in Figure 6. This analysis included representative MA species within Methanomicrobiales, Methanosarcinales, Methanococcales, and Methanobacteriales (supplementary material, Table S2). The phylogenetic tree allowed the identification of *McrA* phylotypes belonging to the Methanosarcinaceae and Methanomicrobiaceae. Nine phylotypes were proximate to *Methanohalobium evestigatum* often found in high-salt environments. In the same way, phylotype mcr-ew2 was closely related to *Methanolobus zinderi*. Finally, the phylotype mcr-ew1 closely clustered to the hydrogenotrophic and nonosmoadapted species *Methanoplanus petrolearius*.

3.4. Amino Acid Composition, GC content, and Codon Usage Bias in AprA and McrA Phylotypes. The aa composition and GC content in proteins from "salt-in" halotolerant organisms have been related to adaptations to high intracellular concentration in order to favor an osmotic balance within an hypersaline environment [23, 25]. Given that the catalysis of *AprA* and *McrA* enzymes occur in the cytoplasm, we were interested in determine whether Tirez *AprA* and *McrA* sequences show a bias when compared to their halophilic and nonhalophilic homologous counterparts. Thus, we calculated the aa composition from the alignment used to reconstruct the phylogeny in order to estimate the hydrophobic (Gly, Leu, Val, Ile, Phe, Met, Ala, Trp, and Pro), polar (Ser, Thr, Cys, Tyr, Gln, and Asn), basic (His, Arg, and Lys) and acidic (Glu and Asp) contents of the *AprA* and *McrA* enzyme fragments analyzed in this study. Additionally, we used the nucleotide alignment that covers the aa positions selected to reconstruct the phylogeny for each gene marker in order to estimate the general codon bias GC content and the relative synonymous codon usage (RSCU) (see Section 2). For this purpose, we divided the sequence profiles from each gene markers into two data sets the first one is based on salinity adaptation (Tirez, halophilic and nonhalophilic species) and the second one is based on the major taxonomic groups in which the Tirez phylotypes are phylogenetically allocated (*AprA*: Desulfovibrionales, Desulfobacterales, Peptococcales, and Chromatiales; *McrA*: Methanomicrobiales

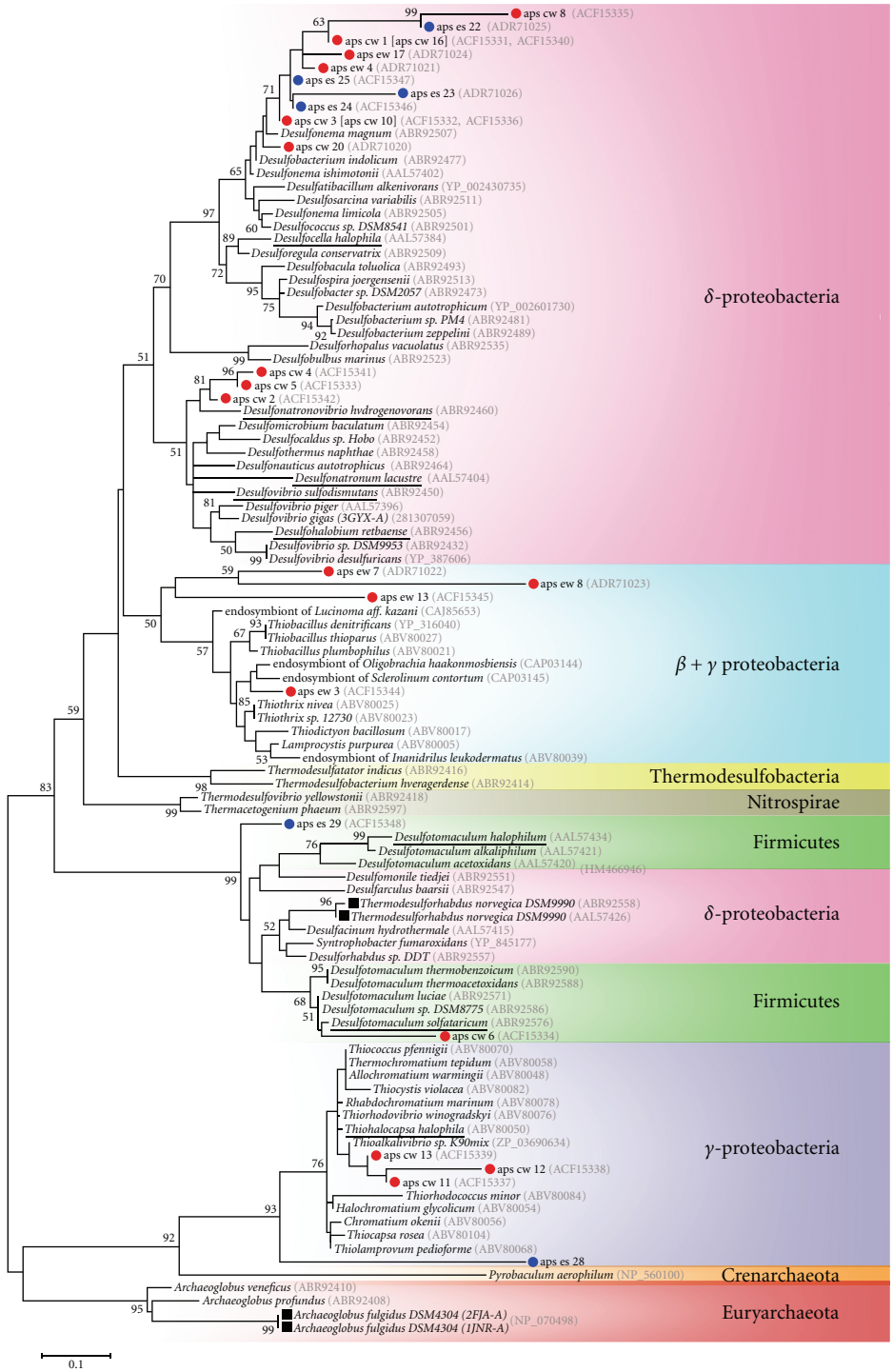


Figure 5: The phylogenetic tree is showing the relationship of *AprA* sequences from environmental samples and nonaxenic cultures from Tirez sediments and *aprA* sequences of characterized SRP and SOP (supplementary material, Table S1). The blue circle identifies the summer phylotypes and red circle is for the winter phylotypes. Halophilic known species are underlined. Same species from different strain with interesting amino acid changes from basic (Lys and K) to polar (Gln, Q and Asn, N) are marked with grey squares (see supplementary Figure S2). The number of redundant phylotypes defined by an identity of 100% is indicated in parenthesis after the accession number. The scale bar represents 0 ± 1 substitutions per aa position. Percentages ≥ 50% of bootstrap are indicated near the nodes. See Section 2 to observe details of the phylogenetic reconstruction done for this tree.

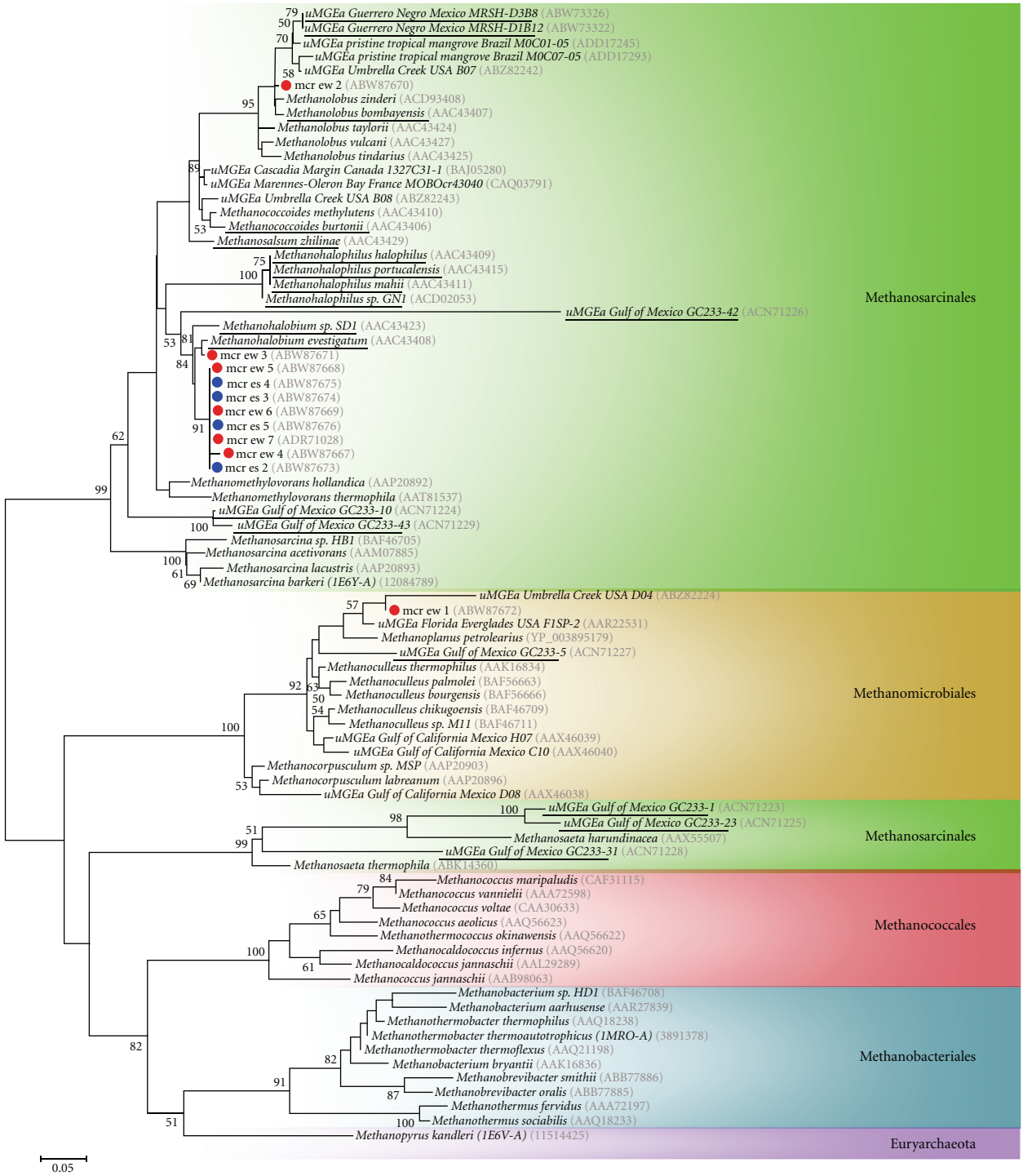


Figure 6: The phylogenetic tree is showing the relationship of *McrA* from Tirez sediments with *McrA* sequences of characterized MA (supplementary material, Table S2). The blue circle identifies the summer phylotypes, and red circle is for the winter phylotypes. Halophilic known species are underlined. The scale bar represents 0 ± 1 substitutions per aa position. Percentages ≥ 50% of bootstrap are indicated near the nodes. See Section 2 to observe details of the phylogenetic reconstruction done for this tree.

and Methanosarcinales) (supplementary material, Tables S1 and S2, resp.).

The degree of excess acidic amino acids and dearth of basic amino acids reflects the prevalence of the "salt-in" strategy and the amount of adaptation necessary to cope with the environmental stress. This can be quantified from two estimations: by calculating the surplus of polar and acidic amino acid compared to polar and basic ones (i.e., PAB = [Asx + Glx] − [Arg + Lys]) [57] and by the ratio of the acidic amino acids Glu and Asp to the basic amino acids Lys,

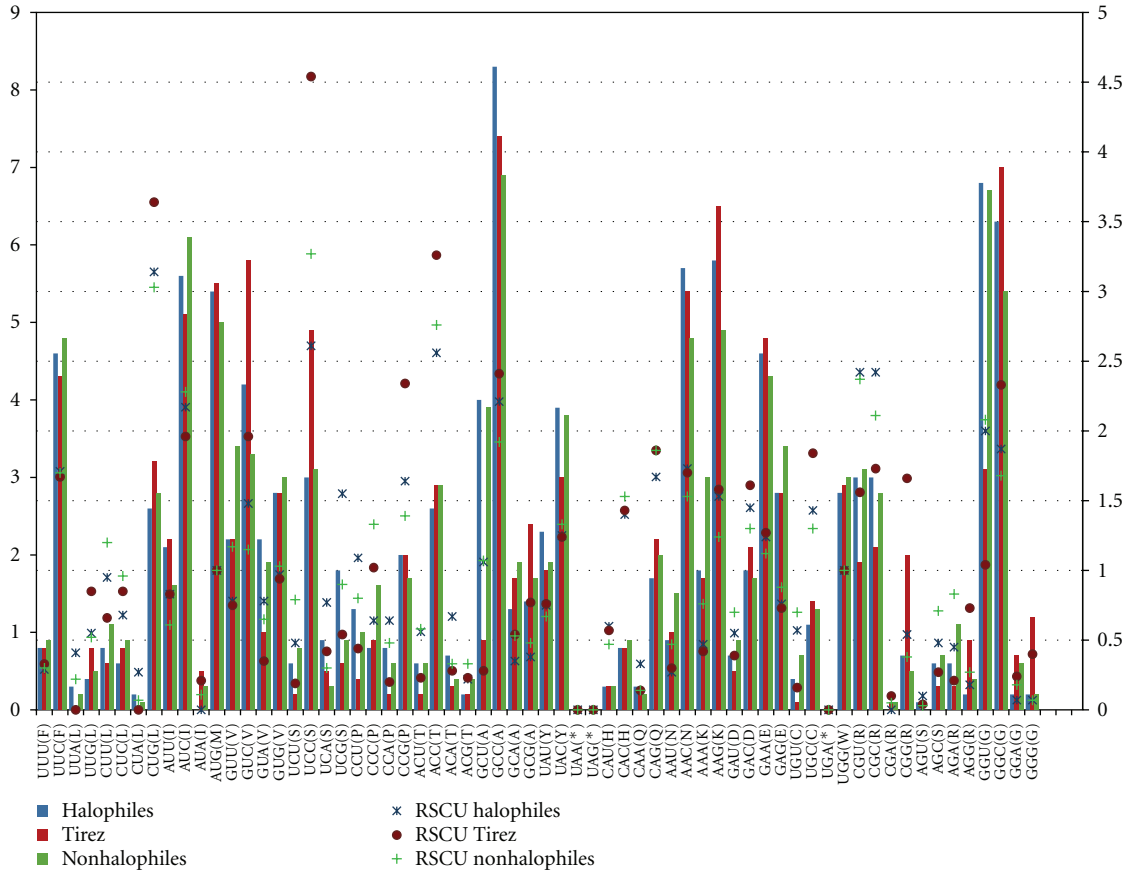


Figure 7: Correspondence analysis of relative synonymous codon usage (RSCU) for *AprA* sequences from halophiles, nonhalophiles, and Tirez phylotypes. The distribution of all codons (including the start and stop codons*) for every amino acid across the three datasets is shown on the *X*-axis. The frequency of each codon (%) is represented with bars on the left *Y*-axis. RSCU values for each codon across the three datasets are represented with differentiated dots on the right *Y*-axis. In the absence of any codon usage bias, the RSCU value would be 1.00. A codon that is used less frequently than expected will have a value of less than 1.00 and *vice versa* for a codon that is used more frequently than expected.

His, and Arg (i.e., AB = [Asp + Glu] : [His + Arg + Lys]) [21]. On average, the amino acid composition measurements (Table 1) indicated that *AprA* Tirez phylotypes (PAB = 2.87, AB = 0.62) were from similar to slightly higher in comparison with halophilic (PAB = 2.70, AB = 0.59) and nonhalophilic sequences (PAB = 2.35, AB = 0.62). However, the observed differences in PAB and AB indicators between Tirez phylotypes and halophilic species are out of proportion to argue a "salt-in" signal in Tirez phylotypes given that *AprA* differences are more than ten times less the difference between *Escherichia coli* and *Halobacterium salinarum* or *Halomonas elongata* and *Halobacterium salinarum* [57].

The total GC content of *AprA* Tirez phylotypes, halophiles, and nonhalophiles organisms is 57.60%, 55.70%, and 55.00%, respectively. The GC content of Tirez phylotypes is higher than the reported for *Escherichia coli* (50.3%) but lower than the extreme halotolerant species from the Dead Sea metagenome (62–67%) and *Halobacterium salinarum* (65.7%) (Table 1). A codon usage in *AprA* Tirez phylotypes is consistent with that expected, when corrected for GC composition (Figure 7). In comparison to halophiles and nonhalophiles, *AprA* Tirez phylotypes show a significant overrepresentation of amino residues with a preferential use for a G or C in the third or first position: Val (GUC), Ser (UCC), Gln (CAG), Lys (AAG), Asn (AAC), Asp (GAC), and Glu (GAA). Even though Arg (CGG, AGG), Ala (GCG), and Cys (UGC) are underrepresented amino acids in *AprA* Tirez phylotypes as well as Leu (CUG) and Gly (GGC, GGG) do not show compositional differences when compared with halophiles and nonhalophiles sequences (supplementary material, Figure S1b), all of them show a significant codon usage with GC bias (RSCU > 1.5) (Figure 7). Accordingly, the first, second, and third codon positions of *AprA* Tirez phylotypes have GC percentages of 54.2%, 42.4%, and 76.0%, respectively, and they agree with the GC content values previously reported in some "salt-in" halophiles (Table 1), with high GC content and a third position GC bias [21, 29]. Similar trends on aa composition and GC content can be seen for the *AprA* clades (Table S3 and Figure S1).

A slighter segregation of the *McrA* Tirez phylotypes from the nonhalophilic species is shown in Table 1. Accordingly, the AB indicator for *McrA* phylotypes was slightly lower (1.50) in comparison with the average of halophilic (1.67) and nonhalophilic species (1.75), whilst an opposite trend

Table 1: The amino acid composition and G + C content of Tirez McrA and AprA sequences, their halophilic and nonhalophilic homologous counterparts, metagenomes, and reference strains.

	Acid Asx + Glx	Acid − Basic[6] (Asx + Glx) − (Arg + Lys)	Acid : Basic[7] (Asp + Glu) : (His + Arg + Lys)	Lys	Asp : Lys	Arg	G + C % in total sequence[8]	G + C % in third codon position[8]
AprA								
Tirez[1]	15.90 ± 2.24	2.87 ± 2.54	0.62 ± 0.13	6.90 ± 1.40	0.35 ± 0.22	6.14 ± 0.90	57.60 ± 4.56	76.00 ± 9.99
Halophilic species[2]	14.90 ± 2.74	2.70 ± 3.02	0.59 ± 0.18	6.20 ± 0.90	0.30 ± 0.09	6.02 ± 0.59	55.70 ± 7.56	66.50 ± 20.70
Nonhalophilic species[2]	15.00 ± 1.95	2.35 ± 2.13	0.62 ± 0.12	6.30 ± 1.10	0.35 ± 0.11	6.34 ± 0.68	55.00 ± 6.94	65.50 ± 19.02
mcrA								
Tirez[1]	22.07± 0.23	16.62 ± 0.24	1.50 ± 0.00	3.38 ± 0.62	2.04 ± 0.37	2.07 ± 0.40	47.20 ± 3.40	46.10 ± 11.70
Halophilic species[3]	21.09 ± 0.52	15.99 ± 0.76	1.67 ± 0.10	3.54 ± 0.29	2.08 ± 1.83	1.56 ± 0.00	51.50 ± 5.20	57.00 ± 12.60
Nonhalophilic species[3,4]	21.75 ± 1.22	16.44 ± 1.63	1.75 ± 0.35	3.65 ± 0.99	2.08 ± 0.80	1.67 ± 0.3	50.30 ±7.20	56.30 ± 21.30
Metagenomic[7]								
Dead Sea	n.d.	n.d.	1.46	n.d.	n.d.	n.d.	62–67	n.d.
Reference strains[5]								
Halobacterium salinarum	31.80	25.36	n.d.	2.34 ± 0.04	n.d.	4.10 ± 0.12	65.7	n.d.
Halomonas elongata	25.98	17.56	n.d.	3.7	n.d.	5.25	n.d.	n.d.
Escherichia coli	26.04	15.85	n.d.	6.03 ± 0.14	n.d.	4.16 ± 0.02	50.3–50.9	n.d.

[1] Average composition from amino acid sequences derived from this study.
[2] Average composition from amino acid sequences listed in supplementary material Table S1.
[3] Average composition from amino acid sequences listed in supplementary material Table S2.
[4] Thermophilic species were not included.
[5] Amino acid composition of the bulk protein content in type species cultures [57].
[6] PAB: amino acid proportions according to [57].
[7] AB: amino proportions according to Rhodes et al. [21].
[8] GC content percentage is calculated as GC% = (G + C/G + C + A + T) ∗ 100.

is shown with the PAB indicator: 16.62 for Tirez, 15.99 for halophiles, and 16.44 for nonhalophiles. In contrast to the *AprA* Tirez phylotypes, the total GC content (47.20%) and the third codon GC bias (46.10%) are significantly lower than the estimated for halophiles and nonhalophilic species (Table 1). The GC content of the first (53.1%) and second (42.4%) codon positions does not change the trend of *McrA* Tirez phylotypes (supplementary material, Figure S3c). Nevertheless, an overrepresentation of amino residues in *McrA* Tirez phylotypes with a preferential codon use (in comparison to nonhalophiles sequences only) can be pointed out for Ile (AUU), Pro (CCA), Ala (GCA), Tyr (UAU), and Asn (AAC, AAU). Even though Lys (AAA), Asp (GAU), Ser (UCC, UCU), and Thr (ACA) are underrepresented amino acids in *McrA* Tirez phylotypes in comparison to halophiles and nonhalophiles sequences (supplementary material, Figure S1b), all of them show a preferential codon usage. Furthermore, it is important to note that the aa composition and GC content trends for *McrA* clade profiles showed a differentiated tendency in contrast to the estimated average from all *McrA* Tirez phylotypes (supplementary material, Table S3). The first, second, and third codon position of Methanomicrobiales present a high GC content values of 52.6% (Tirez 54.1%), 40.0% (Tirez 39.1%), and 73.1% (Tirez 77.4%), respectively. Similarly, the polar and acidic content in Methanomicrobiales (PAB = 17.12 and AB = 1.70) is interestingly higher than the bulk cell protein content reported for *E. coli* (15.85) and close to the haloadaptation threshold of *H. elongata* (17.56) [57].

4. Discussion

4.1. Identification of Anaerobic Prokaryotes in the Sediment by Functional Gene Approach. SRP and MA are the frequent ecotypes responsible of major biogeochemical processes in sedimentary systems. A functional gene PCR-DGGE approach was applied to identify these anaerobic ecotypes. Regarding the sediment profile and community structure along time and depth, the bands identified in the *aprA* DGGE pattern from environmental samples are in agreement with the presence of black sediments below the evaporite layer observed in summer and winter seasons (Figure 2). This mineral precipitation and the sulfide detected in the sediment (Figure 1(a)) are probably attributable to a dissimilatory sulfate reduction where MA were also detected (Figure 3(c)). The use of a nested PCR implies additional amplification cycles, and, thus, it has been used to increase the visualization sensitivity of species present in low numbers by DGGE [69]. Interestingly, our findings via this approach

denote a predominance of the SRP-SOP ecotypes over MA, given that we performed the nested-PCR approach to obtain a positive PCR product of *mcrA* gene fragment, whilst it was not necessary to apply it for the *aprA* gene fragment. Finally, a predominance of SRP-SOP ecotypes in Tirez Lake is in accordance with the high values of sulfate registered on the sediment.

After the clustering analysis of sedimentary populations represented in the *aprA* DGGE pattern, the changes are better explained by a seasonal disturbance in accordance with the ephemeral lagoon. It is suggestible that population resilience is mainly regulated by changes in salinity because the main nodes indicate a partition into dry and flooded patterns (Figure 3(c)); note that salinity fluctuates from 6% (w/v) during winter to 35% (w/v) during spring. However, the strong temperature oscillation can be also associated with salinity over community composition. Additionally, the *P* values (>0.05) indicate that the partition winter/summer is not significant enough to describe well-differentiated communities since flooded node and dry node are more clustered than expected by chance.

Interestingly, most of the SOP, SRP, and MA phylotypes obtained in this work were related to environmental sequences described from alkaliphilic or thalassic hypersaline systems [6, 20]. However, few data is available from athalassic systems [70]. In SRP were detected phylotypes (aps-cw4, -cw-5, and -cw2) from *Desulfonatronovibrio hydrogenovorans,* a lithoheterotrophic, halotolerant (grows in a salinity range of 1–12% NaCl), and alkaliphilic sulfate respirer. Surprisingly, *D. hydrogenovorans* does not grow at pH of 7 and the highest pH of Tirez is below 8.0. Desulfohalobiaceae species are commonly adapted to high osmolarity due to the anabolic metabolism of compatible solute synthesis and dependent on the use of lactate and hydrogen as electron donors [4]. *Desulfohalobium retbaense* is considered the neutrophilic and thalassic counterpart of *D. hydrogenovorans,* but it was not detected in Tirez.

Gram-Positive *Desulfotomaculum solfataricum* (aps-cw6) was detected in enrichments. Another phylotype, aps-es29, is also a member of Peptococcaceae, but it could not be assigned to a specific genus. These phylotypes did not cluster with *Desulfotomaculum halophilum* sequences, which tolerates up to 12% NaCl [71]. However, a previous study reports *Desulfotomaculum* isolates in a salt pan [72]. Sulfate-reducing bacteria in Peptococcaceae perform oxidation from a broad spectrum of electron donors such as lactate [73]. Compatible solutes in Peptococcaceae have not been characterized; however, the theoretical energy yield, for example, in medium supplied with lactate is $\Delta G^{\circ\prime} = -160$ kJ/mol, would give enough energy for the osmoprotectant synthesis or transport as, for example, *Desulfovibrio vulgaris*; *D. vulgaris* is trophically analog to *Desulfotomaculum* species. *D. vulgaris* synthesizes sugars such as trehalose or accumulates amino acids such as glycine betaine and proline as compatible solutes as response to under salt stress. Stress response in *D. vulgaris* is based on genes with homologous in diverse and distant species such as *Bacillus subtilis* [74]; thus, the finding of Peptococcaceae in Tirez, under analog bioenergetic constraints, could be explained in the terms of the "salt-out" strategy (see Section 4).

The presence of *Methanohalobium evestigatum* and *Methanolobus zinderi* in the sulfate-rich and anoxic sediment is easily sustained by functional arguments, even in summer samples, because their metabolism requires methylated substrates; thus, it is noncompetitive with SRP. *M. evestigatum* and *M. zinderi* are theoretically productive in bioenergetic terms [75], enough to exhibit compatible solute synthesis [76]. *Methanolobus zinderi* was isolated from an estuary and grows at the higher rate and tolerates upper levels of divalent cations (Mg^{2+}) in comparison with monovalent Na^{+} [77]. This characteristic is remarkable because *M. zinderi* could be adequate to Tirez given that divalent cation Ca^{2+} dominate over monovalents in the sediment (Figure 1(e)). On the other hand, the increase of ammonium (NH_4^{+} 4–6 μM) at 10–15 cm depth and the decrease of Eh across the sediment profile (Figure 1) suggest the development of strict anaerobic and methylotrophic MA metabolisms [78].

None of the genera detected in both seasons clustered with acetoclastic MA. The absence of acetoclastic MA in hypersaline systems has been widely accepted as a consequence of the low Gibbs free energy dissipated from acetate as substrate [4]. However, acetoclastic MA activity was reported in Napoli mud volcano brines with 4.0 M chloride, where the $Cl : SO_4$ ratio is 200 times higher than the observed in Tirez [79]. In Tirez, the absence of acetoclastic MA is probably explained by substrate outcompetition, because the sulfate-reducing conditions prevail due to the high abundance of sulfate in Tirez and to the putative adaptation of acetoclastic SRP such as *Desulfonema magnum* to the extreme sediment.

The sulfur-oxidizing populations have been frequently described in extreme hypersaline systems. Some of the phylotypes from environmental and enrichment culturing were affiliated to endosymbionts; its potential ecological role in the sediment is supported by the view that the sulfur cycle has been described in marine oligochaetes, where endosymbionts identified as proteobacterial microorganisms participate as sulfur oxidizers [80]. Therefore, it is plausible that the free-living and nonisolated relative populations in Tirez sediment have an analogous metabolic role. Three phylotypes from winter sediment and enrichments were affiliated to the chemoautotrophic genus *Thioalkalivibrio* and sulfur oxidizing endosymbionts in β/γ-proteobacteria clade (Figure 5). These anaerobic ecotypes are expected to be found in the extremely saline sediment as much as the H_2S is present (Figure 1(a)); in turn, H_2S would be oxidized anaerobically by these purple bacteria given that low Eh and partial O_2 pressure were observed in the sampling site (Figure 1). The discrepancy in the finding of *Thioalkalivibrio* is due to its narrow range of optimal pH (9.5–10.0), the fact that species in *Thioalkalivibrio* are true alkaliphilic and are well adapted to athalassic soda lakes, that is, dominated by monovalent cations [81], and considering that other sulfur oxidizing and halophilic SOP species such as *Thiomicrospira halophila* or *Hallochromatium* spp. [82] were not detected and probably better adapted to neutral Tirez saltern. Unfortunately, the SOP Tirez phylotype from summer sediment was not identified at species level.

It has been argued that hypersaline environments are inappropriate for the biological development of anaerobic acetate oxidation as a consequence of the low negative balance of the standard ΔG yielded by this dissimilatory metabolism and due to the high maintenance energy needed for the synthesis/accumulation of compatible solute under high osmotic conditions [4]. However, at high sulfate concentrations, *Desulfonema magnum* populations were unequivocally detected in the evaportitic sediment and winter sediment samples (environmental and derived from enrichment culturing) at 0–15 cm depth under an extreme salinity stress of 35% salts. This acetate-oxidizing Desulfobacteraceae has not been described in hypersaline systems and was the most abundant phylotype identified in Tirez lagoon. *D. magnum* has an optimal salinity about 2.5% NaCl and has been described in marine microbial mats [83]. Previous studies have shown that Desulfobacteraceae are present in thalassic hypersaline basins [70] and athalassic soda lakes [6]. This is a notable finding for the understanding of carbon cycle in extreme hypersaline ecotypes because under extreme conditions there is a decline in organic matter remineralization; thereby, organisms encoding the corresponding *aprA* gene probably face the salinity changes. Halophilic species from Desulfobacterales have not been isolated; *Desulfobacter halotolerans* is member of Desulfobacterales but has an optimum growth with only of 1-2% NaCl [84]. Nevertheless, very little is known about the mechanisms involved in energy conservation that allow acetoclastic SRP organisms to survive in extreme saline conditions. The haloadaptation mechanism "salt-in" osmoadaptation has been suggested for Desulfobacteraceae ecotypes identified in soda lakes to compensate saline stress [6]. Possibly, *Desulfonema,* being an acetoclastic SR, exerts additional energy conserving mechanisms (as in the case of MA and acetogenic bacteria) consisting in extra transference of electrons from membrane complexes dependent on H^+ or Na^+ pumping. Such process is likely to occur in the acetoclastic Desulfobacteraceae *Desulfobacterium autrotrophicum* whose conservation mechanism of chemiosmotic energy is analogous to that in homoacetogenic bacteria [85].

A *mcrA* phylotype from the hydrogenotrophic *Methanoplanus petrolearius* was detected in the surface DGGE profile from winter sediment at 0–5 cm depth (Figure 4), when salt content in the saltern is averaged at 6% w/v. This organism has a maximum tolerance at 5% and an optimal growth at 1–3% NaCl [86]. It is feasible that the *M. petrolearius* salt tolerance determines its absence in summer samples and is correlated with the low energy yielded by the methanogenic pathway based on H_2 and formate as electron donors. MT activity based on these substrates has a low theoretical energy yielded ($\Delta G^{\circ\prime}$). Therefore, it is plausible that *M. petrolearius* is less abundant than methylotrophic MA. *Methanoplanus* clones, which have been reported in thalassic hypersaline sediments but at 2.2 M Cl^- and sulfate below the detection limit [87].

4.2. Halotolerant Strategies in Tirez Lagoon. In order to adjust to lower water activities of the environment and the resulting decrease in cytoplasmic water, microorganisms must accumulate intracellular ions or organic solutes to reestablish the turgor pressure and preserve enzyme activity [27, 88]. "Salt-in" halophiles are adapted to hypersaline environments by a mechanism that involves at least equimolar extracellular and intracellular salt concentrations by a selective influx of potassium ions into the cytoplasm. The "salt-in" strategy favors solubility and is energetically efficient, but unfolds proteins at high concentration [24]. As a consequence, this halotolerant strategy requires that the entire intracellular machinery, that is, proteins, nucleic acids,s and their specific interactions with one another, must be adapted to high salt intracellular levels. The adaptations generally include an increase of the acidic nature of intracellular proteins and/or an increment of genomic CG content and a GC-bias at the codon usage level. Nevertheless, Paul et al. [25] demonstrated common genomic and proteomic trends in halophiles that transcend the boundary of phylogenetic relationship and the genomic GC content of the species. Accordingly, it has been suggested that distantly lineages adopted "salt-in" strategy independently by convergent evolution given its radical nature [27].

All previous studies have estimated average trends of amino acid composition and GC content from selected sequences or enzymes in marine aerobic populations [21, 57] or from completely sequenced genomes obtained from diverse aerobic environments [22, 25]. Even though "salt-in" strategy was recently proposed to explain the finding of the resilience *Desulfobacteraceae* at hypersaline and alkaline lakes [6], this salt-adaptation strategy has been neither reported in species of the SRP-SOP nor in MA; in part, given the absence of complete sequenced genomes and sequenced 16s RNAs from uncultured species. Therefore, we consider it useful to use *AprA* and *McrA* markers to test "salt-in" signals. An intuitive justification would be to expect a naturally biased selection for *AprA* and *McrA* enzymes given their frequent or higher expression levels in the cytoplasm (in comparison to other encoded genes at the genome) in order to cope with their ecological and metabolic role on anaerobic and hypersaline sediments.

Our results cannot be conclusive regarding the halotolerant strategies carried out by Tirez phylotypes, until a large sequence data set can be achieved for these organisms. Nevertheless, the amino acid composition, GC content, and preferential codon usage trends exhibited by the *AprA* marker from Tirez phylotypes suggest a plausible "salt-in" signal when compared to halophiles and non-haphiles. The increase in negatively charged (Asp and Glu) and polar (Ser, Asn, and Gln) residues in *AprA* Tirez phylotypes can be explained by a codon usage with GC bias at the third position. The overrepresentation of these amino acid residues and their preferential codon usage are consistent with reports on "salt-in" adaptation [22, 25]. Similarly, a higher frequency of Val in *AprA* Tirez phylotypes compared to nonhalophiles and halophiles supports the observation of Madern et al. [24] and Paul et al. [25], but disagree with earlier propositions on underrepresentation of all strong hydrophobic residues in halophiles [89]. We also report a slight decrement of the basic residue Arg in *AprA* Tirez phylotypes. The role of

Arg in haloadaptation is quite controversial; its increment in halophilic species can be expected by mutational bias [25] given that five of the six codons have a bias towards GC nucleotides; however, Arg has been also reported in a consistent decrement in specific haloadapted species [29, 57]. Even though the slight increment of Lys observed in *AprA* Tirez phylotypes contradicts all previous propositions on underrepresentation of the most important basic residues in all "salt-in" halophiles [22, 25], it has been recently suggested that dipeptides like Val-Lys significantly contribute to the halostability in proteins [90].

As described on results, the mcr-ew1 Tirez phylotype allocated in Methanomicrobiales shows an interestingly phylogenetic tendency to use amino acids, not initially biased by GC content or codon usage, that could be involved in a weak-moderate "salt-in" strategy. For example, slight increments of the polar residues Asn, Ser, and Tyr, the negatively charged residues Asp and Glu, and the hydrophobic residues Ala, Ile, and Pro in *McrA* Methanomicrobiales phylotypes are in agreement with salt-in signals previously reported [57]. Charged amino acids prevent charged ions from attaching to proteins and thus they have a significant role in stabilizing proteins against salty conditions and keeping water molecules around these proteins [25, 50]. Similar to *AprA* Tirez phylotypes, we observed a decrement of Arg and an increment of Lys (supplementary material, Table S3). The remaining *McrA* Tirez phylotypes do not exhibit a clear tendency about expected aa composition, GC content, and codon usage bias to carry out "salt-in" haloadaptation. These phylotypes could compensate high salt extracellular concentrations through mechanisms independent of amino acid composition and GC content and that do not compromise the enzymatic activity [91]. The "salt-out" strategy requires the accumulation of specific small-molecular-weight compounds (i.e., compatible solutes or osmolytes) into the cytoplasm. Thereby, "salt-out" signal can be expected on the *McrA* Tirez phylotypes close clustered at *Methanohalobium evestigatum* and *Methanohalobium sp.* species belonging to the Methanosarcinales. This observation is in agreement with the compatible solute characterization described for this clade [92]. It is also well know that *M. evestigatum* uses methylated compounds such as methylamine and methanol to generate methane. These methylated substrates not only provide more energy to *M. evestigatum* than the use of others substrates for anabolic reactions, including the synthesis of compatible solutes, but also allow a tolerance up to 29.2% of NaCl [93]. In "salt-out" strategy, little or no adjustment is required to intracellular macromolecules; in fact, the compatible solutes often act as more general stress protectants as well as just osmoprotectants [27].

Furthermore, halophiles do not live at constant salt concentrations; but in many natural settings they are exposed to changing salinities due to evaporation or rain, and thus also the intracellular conditions change considerably [23]. Accordingly, enzyme activity on "salt-in" halophilic strategy will depend not only on the nature and concentration of the salt, but also on extensive genetic alterations as a prerequisite for adaptation to a saline intracellular environment [24, 27]. Tajima's neutrality test [94] for the *AprA* and *McrA* enzyme fragments (used in this study) shows that both gene markers are evolving under positive selection ($D_{AprA} = 3.13$ and $D_{AprA} = 2.96$) (supplementary material, Table S4). This means that key functional enzymes of anaerobic microorganisms on Tirez lagoon could undergone extensive genetic alterations that, if they help the organism to cope and adapt with a saline intracellular environment, could be clearly differentiated and fast fixed on the populations. Two clear examples of this flexible genetic alterations and selective fixation can be seen on *AprA* sequences of same species but that were obtained from different strains: *Thermodesulforhabdus norvegica* DSM 9990 (EF442952.1) [95]; AF418159.1, [68] and *Archaeoglobus fulgidus* DSM 4304 (PDB: 1JNR-A) [16]; PDB: 2FJA-A, [48], which show interesting amino acid changes from basic (Lys and K) to polar (Gln, Q and Asn, N) residues (see Figure 5 and supplementary Figure S2).

In spite of the considerable diversity in nucleotide content and amino acid composition of the *AprA* and *McrA* enzyme fragments involved in all analyses, it can be seen a crucial conservation of catalytic sites (Arg-$R^{\alpha 265}$-Trp-$W^{\alpha 234}$ in *AprA* and Phe-$F^{\alpha 330}$-Tyr-$Y^{\alpha 333}$-Phe-$F^{\alpha 443}$-Gly-$G^{\alpha 445}$ in *McrA*) as well as of cofactor and nucleotide binding sites in both gene markers (Figures S1 and S2 for *AprA* and *McrA* aa alignments, resp.). As previously reported for *McrA* [50], the same conservative trend holds true for most of the surrounding residues of the *AprA* and *McrA* catalytic sites. Probably, the amino acid conservation and/or the structural localization of these catalytic regions on *AprA* and *McrA* gene markers underestimate the general trend composition of "salt-in" adaptation from moderately to high halotolerant organisms in Tirez lagoon. In fact, it is not possible to figure out at the moment if the diversity, weakness, or absence of amino acid, GC content, and codon usage patterns reported for Tirez phylotypes in this study are a consequence of a minor and biased coverage of their not completely sequenced genomes or if these inconclusive trends are true salt-in signals or a consequence of the use of complementary salt-adaptation strategies in bioenergetically constrained species, given that Tirez phylotypes have a clear anaerobic mode of life on highly saline and sulfate sediments.

Accordingly, we do not discard the presence of mixed types of osmoadaptation in *AprA* and *McrA* Tirez phylotypes, where K^+ accumulates to high levels ("salt-in") along with neutral and negatively charged organic solutes ("salt-out"), as previously reported for many slightly and moderately halophilic methanogens [96]. For example, *Methanohalophilus portucalensis* grows in 2.0 M NaCl and its intracellular concentration of K^+ is 0.76 M, indicating that concentration of intracellular K^+ need not be the same as that of extracellular Na^+. Presumably, *M. portucalensis* uses three zwitterions and other osmolytes to balance osmotic pressure [92, 96]. Likewise, K^+ plays an important role in the response of *Methanococcus thermolithotrophicus* to hyperosmotic (increased NaCl) or hypoosmotic (decreased NaCl) shock. At the beginning of higher NaCl extracellular concentration, *M. thermolithotrophicus* internalizes K^+ until reach a new steady-state intracellular concentration; then, synthesis and accumulation of L-α-glutamate occur. The K^+-α-glutamate pair functions as a temporary osmolyte

whilst the nonmetabolizable zwitterion (Ne-acetyl-b-lysine) is synthesized and accumulated by *M. thermolithotrophicus* exclusively in response to high salt concentrations [96, 97].

4.3. Implications of Anaerobic Diversity for Tirez Biogeochemistry. The characterization of SRP, SOP, and MA diversity in Tirez lagoon contributes to the knowledge of anaerobic diversity of microorganisms in athalassohaline systems and has inferences on the survival and adaptation of life under steep salt gradients. A characterization of the anaerobic diversity in Tirez lagoon is a first step to explain functional issues such as why not all anaerobic dissimilatory pathways occur optimally in extreme biotopes and whether an anaerobic way of life faces higher energetic constraints in hypersaline systems in terms of salt composition [4]. Any quantitative interpretation can be inferred because PCR-DGGE fingerprint is an inconclusive source of information and fluorescence *in situ* hybridization (FISH), parallel experiments designed specifically to quantify ∂-proteobacteria and methanogen populations along the sediment profile (winter and sediment), failed to yield any positive result (data not shown).

The structure and activity of hydrogenotrophic methanogenesis and acetoclastic sulfidogenesis under thalassic hypersaline systems have been extensively studied [98, 99]. But, it should be kept in mind that Tirez sediment is a sulfate-rich system with a peculiar salt composition, considering that in the evaporitic period minerals such as gypsum ($CaSO_4 \cdot 2H_2O$), epsomite ($MgSO_4 \cdot 7H_2O$), and hexahydrite ($MgSO_4 \cdot 6H_2O$) are deposited and dominate over halite (NaCl) [60], and most importantly, the sulfate has a relevant role in anaerobic systems as electron acceptor. Thus, present results might be of importance for the understanding of acetate mineralization as a key process for carbon cycling in extreme environments. In Tirez sulfate-rich sediment, among all the detected phylotypes, *Desulfonema magnum* and *Methanoplanus petrolearius* are the ecotypes of major interest due to energetic constraints; therefore, these ecotypes constitute a probable signal of haloadaptation in anaerobic populations.

Although it was possible to characterize several anaerobic prokaryotes involved in distinctive metabolic lineages across the Tirez sediment, DGGE and phylogenetic analyses revealed a poor SRP, SOP, and MA phylotype composition; probably underestimated in comparison with other extreme systems [100]. Nevertheless, extant conditions in Tirez, as well as in other hypersaline environments, enable the persistence of low energetic anaerobic metabolic capabilities such as the *Halanaerobiales* fermenting bacteria (manuscript in prep.), which use a well-adapted fermentation of organic compounds to produce CO_2/H_2 and volatile fatty acids (VFA) such as acetate by the use of the "salt-in" strategy [101].

Typically, the carbon cycle in halophilic communities implicates low rates of carbon mineralization to CO_2 which explains the accumulation of acetate at salt saturation levels [102]. In addition to H_2 and acetate, methylated compounds as fermentation products of compatible solutes can be mineralized by MA [78, 103]. The perspective for the nitrogen cycle is different in Tirez, its completion is predictable given that it shares the characteristics of other hypersaline systems, where methylotrophic MA contribute to nitrogen mineralization [103]. About the sulfur cycle, the sulfate-reducing microorganisms were identified in the sulfate-rich sediment and represent probable suppliers of sulfide for sulfur-oxidizing populations. This understanding is useful to infer possible biological processes in analogous systems such as Europa because the ocean present in the satellite is rich in sulfates and divalent cations and probably it is also in anoxic state [31, 104].

5. Conclusion

Extensive phylogenetic and physiological characterizations of thalassic and alkaline anaerobic biotopes have been reported. Phylogenetic studies have been traditionally determined by physiological characterization of marine species, and the records of anaerobic phylotypes in hypersaline systems are dominated by thalassic species. Tirez lagoon has *sabkha* properties thus, it is a brine of interest to analyze strong spectra in salinity. Also, Tirez lagoon is characterized by a low chloride/sulfate ratio; this is remarkable considering that sulfate serves as terminal electron acceptor in the marine systems; however, few biological descriptions have been made when this anion is abundant under hypersaline conditions. Using the PCR-DGGE fingerprint technique for the functional adenosine-5′-phosphosulfate (*aprA*) and the methyl coenzyme M reductase (*mcrA*) gene markers, we have confirmed the occurrence of hydrogenotrophic methanogenic and acetoclastic sulfate-reducing organisms in Tirez sediment. Despite the steep osmotic change along the year in the lagoon, changes in composition of PCR-DGGE dendrogram reflected weak differences on winter-summer community structure.

The persistence of Desulfobacteraceae phylotypes in summer sediment as well as the finding of Methanomicrobiales at the hypersaline and sulfate-rich sediment is remarkable (hydrogenotrophic MA are outcompeted by SRP in high concentrations of sulfate). Probably, these ecotypes are energetically constrained and, unfortunately, our findings on amino acid and nucleotide compositions cannot be currently conclusive regarding the halotolerant strategies carried out by Tirez phylotypes until a large sequence data set can be achieved for these uncultured, anaerobic and bioenergetically constrained organisms. Nevertheless, it looks like *AprA* gene marker could be a useful "salt-in" indicator for different environmental (e.g., marine versus sedimentary) samples, not only because its amino acid overrepresentation and codon usage bias well correlate with those found in halophiles but also because *AprA* gene marker could exhibit a preferential use of amino acid (e.g., Val and Lys) on sediments in contrast to those found in marine and aerobic environments. Similarly, *McrA* gene marker shows an unexpected amino acid and nucleotide composition with nonclear "salt-in" signals exhibited. However, we speculate that the diverse and not conclusive salt-in signals in these ecotypes (perhaps due to the absence of complete sequenced *McrA* genes) could reflect that whereas protective osmolytes

"salt-out" can be produced by MA Tirez populations in response to salt stress, probably also a weak "salt-in" strategy may contribute to adaptation of osmotic stress on sedimentary MA Tirez populations.

An extended understanding for acetoclastic sulfate reducing activity under high osmolarity conditions is needed in order to elucidate mechanisms that are involved in the biological carbon mineralization. On the long term, the findings of this work will provide valuable information to determine habitable conditions of Europa, the most interesting moon of Jupiter for the Astrobiology field, as an anoxic and hypersaline environment.

Authors' Contribution

L. Montoya, I. Lozada-Chávez, I. Marín and R. Amils conceived the study. L. Montoya, I. Marín, R. Amils and N. Rodriguez were involved in the fieldwork. L. Montoya performed the experimental work. I. Lozada-Chávez performed the sequence analysis. L. Montoya and I. Lozada-Chávez performed the analysis and interpretation of data, and wrote the paper. All authors read, improved, and approved the final paper.

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References

[1] R. Cord-Ruwisch, H. J. Seitz, and R. Conrad, "The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor," *Archives of Microbiology*, vol. 149, no. 4, pp. 350–357, 1988.

[2] D. C. Catling, C. R. Glein, K. J. Zahnle, and C. P. McKay, "Why O_2 is required by complex life on habitable planets and the concept of planetary "Oxygenation time"," *Astrobiology*, vol. 5, no. 3, pp. 415–438, 2005.

[3] K. Tabuchi, H. Kojima, and M. Fukui, "Seasonal changes in organic matter mineralization in a sublittoral sediment and temperature-driven decoupling of key processes," *Microbial Ecology*, vol. 60, no. 3, pp. 551–560, 2010.

[4] A. Oren, "Thermodynamic limits to microbial life at high salt concentrations," *Environmental Microbiology*, vol. 13, no. 8, pp. 1908–1923, 2011.

[5] B. Ollivier, M. L. Fardeau, J. L. Cayol et al., "Methanocalculus halotolerans gen. nov., sp. nov., isolated from an oil-producing well," *International Journal of Systematic Bacteriology*, vol. 48, no. 3, pp. 821–828, 1998.

[6] M. Foti, D. Y. Sorokin, B. Lomans et al., "Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes," *Applied and Environmental Microbiology*, vol. 73, no. 7, pp. 2093–2100, 2007.

[7] J. G. Dillon, S. Miller, B. Bebout, M. Hullar, N. Pinel, and D. A. Stahl, "Spatial and temporal variability in a stratified hypersaline microbial mat community," *FEMS Microbiology Ecology*, vol. 68, no. 1, pp. 46–58, 2009.

[8] A. Teske and K. B. Sørensen, "Uncultured archaea in deep marine subsurface sediments: have we caught them all?" *ISME Journal*, vol. 2, no. 1, pp. 3–18, 2008.

[9] D. J. Kushner, "The Halobacteriaceae," in *The Bacteria: A Treatise on Structure and Function*, C. R. Woese and R. S. Wolfe, Eds., pp. 171–214, Academic Press, 1985.

[10] J. S. Kargel, J. Z. Kaye, J. W. Head et al., "Europa's crust and ocean: origin, composition, and the prospects for life," *Icarus*, vol. 148, no. 1, pp. 226–265, 2000.

[11] D. L. Valentine, "Adaptations to energy stress dictate the ecology and evolution of the Archaea," *Nature Reviews Microbiology*, vol. 5, no. 4, pp. 316–323, 2007.

[12] A. Nocker, M. Burr, and A. K. Camper, "Genotypic microbial community profiling: a critical technical review," *Microbial Ecology*, vol. 54, no. 2, pp. 276–289, 2007.

[13] B. Meyer and J. Kuever, "Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using aprA as functional marker gene," *Applied and Environmental Microbiology*, vol. 73, no. 23, pp. 7664–7679, 2007.

[14] B. Meyer and J. Kuever, "Homology modeling of dissimilatory APS reductases (AprBA) of sulfur-oxidizing and sulfate-reducing prokaryotes," *PLoS One*, vol. 3, no. 1, Article ID e1514, 2008.

[15] R. K. Thauer, A. K. Kaster, H. Seedorf, W. Buckel, and R. Hedderich, "Methanogenic archaea: ecologically relevant differences in energy conservation," *Nature Reviews Microbiology*, vol. 6, no. 8, pp. 579–591, 2008.

[16] G. Fritz, A. Roth, A. Schiffer et al., "Structure of adenylylsulfate reductase from the hyperthermophilic Archaeoglobus fulgidus at 1.6-Å resolution," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 4, pp. 1836–1841, 2002.

[17] W. M. Hipp, A. S. Pott, N. Thum-Schmitz, I. Faath, C. Dahl, and H. G. Trüper, "Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes," *Microbiology*, vol. 143, no. 9, pp. 2891–2902, 1997.

[18] P. E. Luton, J. M. Wayne, R. J. Sharp, and P. W. Riley, "The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill," *Microbiology*, vol. 148, no. 11, pp. 3521–3530, 2002.

[19] E. Springer, M. S. Sachs, C. R. Woese, and D. R. Boone, "Partial gene sequences for the A subunit of methylcoenzyme M reductase (mcrI) as a phylogenetic tool for the family Methanosarcinaceae," *International Journal of Systematic Bacteriology*, vol. 45, no. 3, pp. 554–559, 1995.

[20] R. Wilms, H. Sass, B. Köpke, H. Cypionka, and B. Engelen, "Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea," *FEMS Microbiology Ecology*, vol. 59, no. 3, pp. 611–621, 2007.

[21] M. E. Rhodes, S. T. Fitz-Gibbon, A. Oren, and C. H. House, "Amino acid signatures of salinity on an environmental scale with a focus on the Dead Sea," *Environmental Microbiology*, vol. 12, no. 9, pp. 2613–2623, 2010.

[22] S. Fukuchi, K. Yoshimune, M. Wakayama, M. Moriguchi, and K. Nishikawa, "Unique amino acid composition of proteins in halophilic bacteria," *Journal of Molecular Biology*, vol. 327, no. 2, pp. 347–357, 2003.

[23] J. Soppa, "From genomes to function: haloarchaea as model organisms," *Microbiology*, vol. 152, no. 3, pp. 585–590, 2006.

[24] D. Madern, C. Ebel, and G. Zaccai, "Halophilic adaptation of enzymes," *Extremophiles*, vol. 4, no. 2, pp. 91–98, 2000.

[25] S. Paul, S. K. Bag, S. Das, E. T. Harvill, and C. Dutta, "Molecular signature of hypersaline adaptation: insights from genome and proteome composition of halophilic prokaryotes," *Genome Biology*, vol. 9, no. 4, article R70, 2008.

[26] G. W. Hutcheon, N. Vasisht, and A. Bolhuis, "Characterisation of a highly stable α-amylase from the halophilic archaeon *Haloarcula hispanica*," *Extremophiles*, vol. 9, no. 6, pp. 487–495, 2005.

[27] H. Santos and M. S. da Costa, "Compatible solutes of organisms that live in hot saline environments," *Environmental Microbiology*, vol. 4, no. 9, pp. 501–509, 2002.

[28] A. Oren, F. Larimer, P. Richardson, A. Lapidus, and L. N. Csonka, "How to be moderately halophilic with broad salt tolerance: clues from the genome of Chromohalobacter salexigens," *Extremophiles*, vol. 9, no. 4, pp. 275–279, 2005.

[29] S. P. Kennedy, W. V. Ng, S. L. Salzberg, L. Hood, and S. DasSarma, "Understanding the adaptation of Halobacterium species NRC-1 to its extreme environment through computational analysis of its genome sequence," *Genome Research*, vol. 11, no. 10, pp. 1641–1650, 2001.

[30] H. Bolhuis, P. Palm, A. Wende et al., "The genome of the square archaeon Haloquadratum walsbyi: life at the limits of water activity," *BMC Genomics*, vol. 7, article 169, 2006.

[31] O. Prieto-Ballesteros, N. Rodríguez, J. S. Kargel, C. G. Kessler, R. Amils, and D. F. Remolar, "Tírez lake as a terrestrial analog of Europa," *Astrobiology*, vol. 3, no. 4, pp. 863–877, 2003.

[32] D. T. Vaniman, D. L. Bish, S. J. Chipera, C. I. Fialips, J. W. Carey, and W. G. Feldman, "Magnesium sulphate salts and the history of water on Mars," *Nature*, vol. 431, no. 7009, pp. 663–665, 2004.

[33] J. D. Cline, "Spectrophotometric determination of hydrogen sulfide in natural waters," *Limnology and Oceanography*, vol. 14, pp. 454–458, 1969.

[34] L. Raskin, B. E. Rittmann, and D. A. Stahl, "Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms," *Applied and Environmental Microbiology*, vol. 62, no. 10, pp. 3847–3857, 1996.

[35] R. L. Kepner and J. R. Pratt, "Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present," *Microbiological Reviews*, vol. 58, no. 4, pp. 603–615, 1994.

[36] Á. Aguilera, V. Souza-Egipsy, E. González-Toril, O. Rendueles, and R. Amils, "Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs," *International Microbiology*, vol. 13, no. 1, pp. 21–32, 2010.

[37] B. Deplancke, K. R. Hristova, H. A. Oakley et al., "Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract," *Applied and Environmental Microbiology*, vol. 66, no. 5, pp. 2166–2174, 2000.

[38] B. A. Hales, C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders, "Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis," *Applied and Environmental Microbiology*, vol. 62, no. 2, pp. 668–675, 1996.

[39] G. Muyzer, E. C. De Waal, and A. G. Uitterlinden, "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA," *Applied and Environmental Microbiology*, vol. 59, no. 3, pp. 695–700, 1993.

[40] N. Fromin, J. Hamelin, S. Tarnawski et al., "Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns," *Environmental Microbiology*, vol. 4, no. 11, pp. 634–643, 2002.

[41] E. O. Casamayor, R. Massana, S. Benlloch et al., "Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern," *Environmental Microbiology*, vol. 4, no. 6, pp. 338–348, 2002.

[42] J. Felsentein, *PHYLIP (3.68 edn)*, Department of Genetics, University of Washington, 1993, Distributed by the author.

[43] S. F. Altschul, T. L. Madden, A. A. Schäffer et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.

[44] R. Lopez, V. Silventoinen, S. Robinson, A. Kibria, and W. Gish, "WU-Blast2 server at the European Bioinformatics Institute," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3795–3798, 2003.

[45] W. Li, L. Jaroszewski, and A. Godzik, "Tolerating some redundancy significantly speeds up clustering of large protein databases," *Bioinformatics*, vol. 18, no. 1, pp. 77–82, 2002.

[46] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, "The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools," *Nucleic Acids Research*, vol. 25, no. 24, pp. 4876–4882, 1997.

[47] T. Hall, *BIOEDIT. (7.0.5. edn)*, Ibis Therapeutics, Division of Isis Pharmaceuticals, 1997, Biological sequence alignment editor, Distributed by the author.

[48] A. Schiffer, G. Fritz, P. M. H. Kroneck, and U. Ermler, "Reaction mechanism of the iron-sulfur flavoenzyme adenosine-5′-phosphosulfate reductase based on the structural characterization of different enzymatic states," *Biochemistry*, vol. 45, no. 9, pp. 2960–2967, 2006.

[49] Y. L. Chiang, Y. C. Hsieh, J. Y. Fang et al., "Crystal structure of adenylylsulfate reductase from Desulfovibrio gigas suggests a potential self-regulation mechanism involving the C terminus of the β-subunit," *Journal of Bacteriology*, vol. 191, no. 24, pp. 7597–7608, 2009.

[50] W. Grabarse, F. Mahlert, S. Shima, R. K. Thauer, and U. Ermler, "Comparison of three methyl-coenzyme M reductases from phylogenetically distant organisms: unusual amino acid modification, conservation and adaptation," *Journal of Molecular Biology*, vol. 303, no. 2, pp. 329–344, 2000.

[51] U. Ermler, W. Grabarse, S. Shima, M. Goubeaud, and R. K. Thauer, "Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation," *Science*, vol. 278, no. 5342, pp. 1457–1462, 1997.

[52] S. Rospert, J. Breitung, K. Ma et al., "Methyl-coenzyme M reductase and other enzymes involved in methanogenesis from CO_2 and H_2 in the extreme thermophile Methanopyrus kandleri," *Archives of Microbiology*, vol. 156, no. 1, pp. 49–55, 1991.

[53] J. Castresana, "Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis," *Molecular Biology and Evolution*, vol. 17, no. 4, pp. 540–552, 2000.

[54] D. T. Jones, W. R. Taylor, and J. M. Thornton, "The rapid generation of mutation data matrices from protein sequences,"

Computer Applications in the Biosciences, vol. 8, no. 3, pp. 275–282, 1992.

[55] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Molecular Biology and Evolution*, vol. 4, no. 4, pp. 406–425, 1987.

[56] K. Tamura, J. Dudley, M. Nei, and S. Kumar, "MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0," *Molecular Biology and Evolution*, vol. 24, no. 8, pp. 1596–1599, 2007.

[57] A. Oren and L. Mana, "Amino acid composition of bulk protein and salt relationships of selected enzymes of *Salinibacter ruber*, an extremely halophilic bacterium," *Extremophiles*, vol. 6, no. 3, pp. 217–223, 2002.

[58] P. M. Sharp, T. M. F. Tuohy, and K. R. Mosurski, "Codon usage in yeast: cluster analysis clearly differentiates highly and lowly expressed genes," *Nucleic Acids Research*, vol. 14, no. 13, pp. 5125–5143, 1986.

[59] K. R. Sowers and J. E. M. Watts, "The study of strictly anaerobic microorganisms," in *Extremophiles*, F. A. Rainey and A. Oren, Eds., pp. 739–764, Elsevier-Academic Press, New York, NY, USA, 2006.

[60] J. A. de la Peña, "Las lagunas de la Mancha: un ejemplo de ambiente continental," in *Memorias de la Real Academia de Ciencias Exactas, Físicas y Naturales*, pp. 79–93, 1987.

[61] H. Jiang, H. Dong, G. Zhang, B. Yu, L. R. Chapman, and M. W. Fields, "Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China," *Applied and Environmental Microbiology*, vol. 72, no. 6, pp. 3832–3845, 2006.

[62] V. N. Bashkin, *Modern Biogeochemistry*, Kluwer Academic Publishers, 2003.

[63] M. A. Schneegurt, S. Y. Dore, and C. F. Kulpa, "Direct extraction of DNA from soils for studies in microbial ecology," *Current Issues in Molecular Biology*, vol. 5, no. 1, pp. 1–8, 2003.

[64] M. R. Frost and J. A. Guggenheim, "Prevention of depurination during elution facilitates the reamplification of DNA from differential display gels," *Nucleic Acids Research*, vol. 27, no. 15, p. e6, 1999.

[65] K. B. Sørensen, D. E. Canfield, and A. Oren, "Salinity responses of benthic microbial communities in a solar saltern (Eilat, Israel)," *Applied and Environmental Microbiology*, vol. 70, no. 3, pp. 1608–1616, 2004.

[66] G. Muyzer and K. Smalla, "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology," *Antonie van Leeuwenhoek*, vol. 73, no. 1, pp. 127–141, 1998.

[67] H. Juottonen, P. E. Galand, and K. Yrjälä, "Detection of methanogenic Archaea in peat: comparison of PCR primers targeting the mcrA gene," *Research in Microbiology*, vol. 157, no. 10, pp. 914–921, 2006.

[68] M. W. Friedrich, "Phylogenetic analysis reveals multiple lateral transfers of adenosine-5′-phosphosulfate reductase genes among sulfate-reducing microorganisms," *Journal of Bacteriology*, vol. 184, no. 1, pp. 278–289, 2002.

[69] S. A. Dar, J. G. Kuenen, and G. Muyzer, "Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities," *Applied and Environmental Microbiology*, vol. 71, no. 5, pp. 2325–2330, 2005.

[70] S. Borin, L. Brusetti, F. Mapelli et al., "Sulfur cycling and methanogenesis primarily drive microbial colonization of the highly sulfidic Urania deep hypersaline basin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 23, pp. 9151–9156, 2009.

[71] C. Tardy-Jacquenod, M. Magot, B. K. C. Patel, R. Matheron, and P. Caumette, "Desulfotomaculum halophilum sp. nov., a halophilic sulfate-reducing bacterium isolated from oil production facilities," *International Journal of Systematic Bacteriology*, vol. 48, no. 2, pp. 333–338, 1998.

[72] S. Kerkar, *Studies on bacteria of the dissimilatory reductive processes of the sulphur cycle from the salt pans of Goa*, M.S. thesis, Marine Sciences, Goa University, 2004.

[73] E. V. Belyakova and E. P. Rozanova, "Newly discovered properties of spore-forming sulfate-reducing bacteria, *Desulfotomaculum* strains 435 and 781," *Microbiology*, vol. 73, no. 2, pp. 237–239, 2004.

[74] Z. He, A. Zhou, E. Baidoo et al., "Global transcriptional, physiological and metabolite analyses of the responses of *Desulfovibrio vulgaris* Hildenborough to salt adaptation," *Applied and Environmental Microbiology*, vol. 76, no. 5, pp. 1574–1586, 2010.

[75] A. Oren, "Bioenergetic aspects of halophilism," *Microbiology and Molecular Biology Reviews*, vol. 63, no. 2, pp. 334–348, 1999.

[76] R. S. Oremland and S. Polcin, "Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments," *Applied and Environmental Microbiology*, vol. 44, pp. 1270–1276, 1982.

[77] S. N. Doerfert, M. Reichlen, P. Iyer, M. Wang, and J. G. Ferry, "*Methanolobus zinderi* sp. nov., a methylotrophic methanogen isolated from a deep subsurface coal seam," *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 5, pp. 1064–1069, 2009.

[78] A. Oren, "Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments," *Antonie van Leeuwenhoek*, vol. 58, no. 4, pp. 291–298, 1990.

[79] C. S. Lazar et al., "Methanogenic diversity and activity in hypersaline sediments of the centre of the Napoli mud volcano, Eastern Mediterranean Sea," *Environ Microbiol*, 2011.

[80] B. Meyer and J. Kuever, "Phylogenetic diversity and spatial distribution of the microbial community associated with the Caribbean deep-water sponge Polymastia cf. corticata by 16S rRNA, aprA, and amoA gene analysis," *Microbial Ecology*, vol. 56, no. 2, pp. 306–321, 2008.

[81] D. Y. Sorokin, H. Banciu, M. van Loosdrecht, and J. G. Kuenen, "Growth physiology and competitive interaction of obligately chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria from soda lakes," *Extremophiles*, vol. 7, no. 3, pp. 195–203, 2003.

[82] D. Y. Sorokin, T. P. Tourova, A. M. Lysenko, and G. Muyzer, "Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats," *Microbiology*, vol. 152, no. 10, pp. 3013–3023, 2006.

[83] M. Fukui, A. Teske, B. Assmus, G. Muyzer, and F. Widdel, "Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (*genus Desulfonema*)," *Archives of Microbiology*, vol. 172, no. 4, pp. 193–203, 1999.

[84] K. K. Brandt and K. Ingvorsen, "Desulfobacter halotolerans sp. nov., a halotolerant acetate-oxidizing sulfate-reducing bacterium isolated from sediments of Great Salt Lake, Utah," *Systematic and Applied Microbiology*, vol. 20, no. 3, pp. 366–373, 1997.

[85] A. W. Strittmatter, H. Liesegang, R. Rabus et al., "Genome sequence of *Desulfobacterium autotrophicum* HRM2, a marine sulfate reducer oxidizing organic carbon completely

to carbon dioxide," *Environmental Microbiology*, vol. 11, no. 5, pp. 1038–1055, 2009.

[86] B. Ollivier, J. L. Cayol, B. K. C. Patel, M. Magot, M. L. Fardeau, and J. L. Garcia, "*Methanoplanus petrolearius* sp. nov., a novel methanogenic bacterium from an oil-producing well," *FEMS Microbiology Letters*, vol. 147, no. 1, pp. 51–56, 1997.

[87] P. J. Waldron, S. T. Petsch, A. M. Martini, and K. Nüslein, "Salinity constraints on subsurface archaeal diversity and methanogenesis in sedimentary rock rich in organic matter," *Applied and Environmental Microbiology*, vol. 73, no. 13, pp. 4171–4179, 2007.

[88] A. D. Brown, Ed., *Microbial Water Stress Physiology: Principles and Perspectives*, John Wiley & Sons.

[89] K. L. Britton, P. J. Baker, M. Fisher et al., "Analysis of protein solvent interactions in glucose dehydrogenase from the extreme halophile *Haloferax mediterranei*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 13, pp. 4846–4851, 2006.

[90] E. Ebrahimie, M. Ebrahimi, N. R. Sarvestani, and M. Ebrahimi, "Protein attributes contribute to halo-stability, bioinformatics approach," *Saline Systems*, vol. 7, no. 1, article 1, 2011.

[91] A. D. Brown, "Microbial water stress," *Bacteriological Reviews*, vol. 40, no. 4, pp. 803–846, 1976.

[92] M. C. Lai, K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus, "Distribution of compatible solutes in the halophilic methanogenic archaebacteria," *Journal of Bacteriology*, vol. 173, no. 17, pp. 5352–5358, 1991.

[93] J. L. Garcia, B. K. C. Patel, and B. Ollivier, "Taxonomic, phylogenetic, and ecological diversity of methanogenic *Archaea*," *Anaerobe*, vol. 6, no. 4, pp. 205–226, 2000.

[94] F. Tajima, "Statistical method for testing the neutral mutation hypothesis by DNA polymorphism," *Genetics*, vol. 123, no. 3, pp. 585–595, 1989.

[95] B. Meyer and J. Kuevert, "Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5′-phosphosulfate (APS) reductase from sulfate-reducing prokaryotes—origin and evolution of the dissimilatory sulfate-reduction pathway," *Microbiology*, vol. 153, no. 7, pp. 2026–2044, 2007.

[96] D. D. Martin, R. A. Ciulla, and M. F. Roberts, "Osmoadaptation in archaea," *Applied and Environmental Microbiology*, vol. 65, no. 5, pp. 1815–1825, 1999.

[97] D. D. Martin, R. A. Ciulla, P. M. Robinson, and M. F. Roberts, "Switching osmolyte strategies: response of *Methanococcus thermolithotrophicus* to changes in external NaCl," *Biochimica et Biophysica Acta*, vol. 1524, no. 1, pp. 1–10, 2000.

[98] K. U. Kjeldsen, A. Loy, T. F. Jakobsen, T. R. Thomsen, M. Wagner, and K. Ingvorsen, "Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah)," *FEMS Microbiology Ecology*, vol. 60, no. 2, pp. 287–298, 2007.

[99] E. G. Potter, B. M. Bebout, and C. A. Kelley, "Isotopic composition of methane and inferred methanogenic substrates along a salinity gradient in a hypersaline microbial mat system," *Astrobiology*, vol. 9, no. 4, pp. 383–390, 2009.

[100] J. B. H. Martiny, B. J. M. Bohannan, J. H. Brown et al., "Microbial biogeography: putting microorganisms on the map," *Nature Reviews Microbiology*, vol. 4, no. 2, pp. 102–112, 2006.

[101] E. N. Detkova and M. A. Pusheva, "Energy metabolism in halophilic and alkalophilic bacteria," *Mikrobiologiia*, vol. 75, no. 1, pp. 5–17, 2006.

[102] B. Ollivier, P. Caumette, J. L. Garcia, and R. A. Mah, "Anaerobic bacteria from hypersaline environments," *Microbiological Reviews*, vol. 58, no. 1, pp. 27–38, 1994.

[103] D. T. Welsh, "Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate," *FEMS Microbiology Reviews*, vol. 24, no. 3, pp. 263–290, 2000.

[104] K. P. Hand, R. W. Carlson, and C. F. Chyba, "Energy, chemical disequilibrium, and geological constraints on Europa," *Astrobiology*, vol. 7, no. 6, pp. 1006–1022, 2007.

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Review Article

Stress Responses of *Shewanella*

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The shewanellae are ubiquitous in aquatic and sedimentary systems that are chemically stratified on a permanent or seasonal basis. In addition to their ability to utilize a diverse array of terminal electron acceptors, the microorganisms have evolved both common and unique responding mechanisms to cope with various stresses. This paper focuses on the response and adaptive mechanism of the shewanellae, largely based on transcriptional data.

1. Introduction

Stress is an inevitable part of the life of all organisms. This is especially true about microorganisms, which reside and thrive in almost all environments on earth, including some considered extremely harsh [1]. Common environmental factors that affect the activities of microorganisms include temperature, pH, water availability, nutrient limitation, presence of various chemicals, osmolarity, pressure, and radiation [2]. Consequently, for every microorganism the ability to adapt rapidly to changes in environments is essential for its survival and prosperity. Regulation that modulates the microbial adaptation to environmental disturbances is rather complex. The most important and efficient control occurs at the level of transcription. Many single stress-induced regulatory circuits have been identified, which enable cells to cope with specific stresses. However, given that microbial cells live in a dynamic environment where multiple factors fluctuate constantly at the same time, stress responses are generally carried out by a regulatory network composed of a series of individual circuits which are highly connected [3].

Most of our understanding of microbial stress response mechanisms has come from the study of model microorganisms, particularly *Escherichia coli* and *Bacillus subtilis*. Extensive physiological and genetic analyses of the stress response systems in these two bacteria have helped us to elucidate the complexity of the process, function of critical proteins, and regulation [4]. While model organisms will continue to provide insights into the fundamental properties of the stress response systems, efforts should be extended to other microorganisms, especially those that are of scientific, environmental, and economic importance.

As one of representatives, the family of *Shewanellaceae* (order Alteromonadales, class γ-proteobacteria) is emerging in recent years. The genus *Shewanella* consists of rod-shaped, Gram-negative, aerobic or facultatively anaerobic, polarly flagellated, readily cultivated γ-proteobacteria [5–8]. While many *Shewanella* isolates remain uncharacterized, 52 species have been recognized to date [9]. Shewanellae are renowned for its ability to use a diverse range of electron acceptors for anaerobic respiration, including fumarate, nitrate, nitrite, thiosulfate, elemental sulfur, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), Fe(III), Mn(III) and (IV), Cr(VI), U(VI), As(V), V(V), and others [10, 11]. As a result of this property, Shewanellae have drawn much attention in the fields of bioremediation, biogeochemical circulation of minerals, and bioelectricity [12, 13]. In addition, Shewanellae have now served as the model for ecological and evolutionary studies at the whole genome level because of its diverse habitats and the availability of up to 26 genome sequences [14, 15].

A number of *Shewanella* strains are currently under physiological investigation [11]. However, stress responses have focused nearly exclusively on *Shewanella oneidensis*, the first genome of the shewanellae to be sequenced [16]. The availability of the genome sequence allowed development of high-throughput technologies such as microarrays and proteomics tools, with which an array of assays has been

carried out to decipher the ability of *S. oneidensis* to respond to and survive external stresses. While impacts of most of common environmental factors have been examined, oxidative stress imposed by H_2O_2 is surprisingly untouched. In this paper, we consider all insights into the stress response mechanisms revealed thus far in *S. oneidensis* and broaden our discussion to other sequenced species if necessary.

2. Stress Responses to Temperature Fluctuation

Variation in growth temperature is a common stress encountered in nature. Stress response to sudden fluctuation in growth temperature, has become a model system for studying the impact of environmental stresses on biological systems. The hallmark of this adaptive cellular response is the induction of a limited set of proteins, called Heat shock proteins (Hsps) or Cold shock proteins (Csps). In general, Hsps play important roles in protein folding, degradation, assembly of protein complexes, and transport of proteins across membranes whereas Csps function as RNA chaperons to regulate ribosomal translation, rate of mRNA degradation and termination of transcription [17–19].

Using whole-genome DNA microarrays, temporal gene expression profiles of *S. oneidensis* MR-1 in response to temperature variations have been investigated [20, 21]. Expression profiles indicate that temperature fluctuation has a pleiotropic effect on the bacterial transcriptomes. Both heat and cold shock responses appear to share a couple of common features, including that approximately 15% of the total genes are significantly affected ($P < 0.05$) over a 25-min period, that the global changes in mRNAs are rapid and transient, and that a similar set of proteins are induced to manage energy production and protein damage. For instance, most of genes encoding enzymes in the Entner-Doudoroff pathway and the pentose cycle are highly induced upon a temperature alteration.

In the case of heat shock response, two lines of evidence suggest that *S. oneidensis* copes with the situation with mechanism similar to that employed by *E. coli*. First, the majority of the genes that showed homology to known Hsps in *E. coli* such as DnaK, DnaJ, GroEL, GroES, GrpE, HtpG, and Lon/La proteases were highly induced. Second, the identified σ^{32} consensus sequences (CTTGAAA-13/15bp-CCCCAT) of both bacteria for heat shock gene promoters are virtually the same (Figure 1), indicating that the induction of most Hsps owns to a rapid and transient increase in the intracellular concentration of an alternative σ factor, σ^{32} encoded by *rpoH*. Nevertheless, novel findings are not scarce. After numerous attempts, we failed to remove *rpoH* from the genome, implicating that σ^{32} is essential in *S. oneidensis* (unpublished result). Additionally, some hypothetical proteins (i.e., SO2017) are under the control of σ^{32}, suggesting that *S. oneidensis* recruits new proteins to overcome increased temperature (Table 1).

Unlike *E. coli*, most *Shewanella* strains are psychrotolerant. In terms of the canonical Csps *S. oneidensis* possesses three (of which two (SO1648 and SO2787) are cold inducible) whereas *E. coli* has nine (of which four are cold inducible) [19]. Both SO1648 and SO2787 are important in growth at low temperatures evidenced in the mutational analysis [21]. The *S. oneidensis* genome carries two more genes encoding Csd(cold shock domain)-containing proteins (SO0733, 203 aa; SO1732, 224 aa) whose C-terminal lacks sequence similarity to any known proteins. Intriguingly, such a structure has been found only in eukaryotes, with the exception of *Mycobacterium* [24]. Neither SO0733 nor SO1732 is found to be induced upon a decrease in temperature or influences growth at low temperature, indicating that these Csd-containing proteins may not be involved in cold stress response.

S. piezotolerans WP3 is another *Shewanella* that has been studied in respect of response to low temperatures. Strikingly, none of its Csps are cold inducible, suggesting that these proteins may not play an indispensible role in the process [25]. Instead, the organism utilizes other strategies to overcome temperature downshifts. These include increased production of EPA (eicosapentaenoic acid) and BCFA (branched-chain fatty acid) [26], induced expression of RNA helicase DeaD which may facilitate transcription, morphological changes in cell membrane, and elevated assembly of lateral flagella (The organism possesses both polar and lateral flagella.) [25]. In addition, a novel filamentous phage (SW1) is found to be significantly induced at low temperature but the significance of this event in the cold adaptation of *S. piezotolerans* WP3 is unknown [27].

3. Stress Responses to Acidic and Alkaline pH

Microorganisms live in a volatile environment where extracellular pH changes frequently. To minimize the acid- or alkaline-induced damage, various adaptive strategies have evolved [28, 29]. Studies on *E. coli* have revealed that bacterial cells activate outward H^+ pumps such as K^+/proton antiporters in response to acute cytoplasmic acidification and sodium proton antiporters, which bring in 2 H^+ for each Na^+ extruded, to adapt to alkaline pH in the presence of Na^+. To survive upon prolonged acid stress exposure, cells rely on the arginine and glutamate decarboxylase/antiporter systems, which are thought to counteract external acidification through the consumption of intracellular protons and the generation of alkaline amines. Additional acid tolerance responses include regulation of proton permeability by induction of membrane proteins and lipid modification enzyme. In the case of alkaline stress, amino acid metabolic enzymes such as tryptophan deaminase (TnaA) and *o*-acetylserine sulfhydrylase A (CysK) are induced to reverse alkalinization by metabolizing amino acids to produce acidic products.

The response of *S. oneidensis* to acid and alkaline stresses intersects with other stresses evidenced by elevated expression of RpoS, a central regulator of stationary-phase gene expression [30]. It is reasonable to speculate that *S. oneidensis* cells upon altered pH mimic those at the stationary phase. In respect of response to acidic pH, the mechanism of *S. oneidensis* is fundamentally different from that of *E. coli*. The most important and effective player of *E. coli* in mediating acid resistance is the glutamate-dependent (Gad) system, which is missing in all sequenced *Shewanellae* [31].

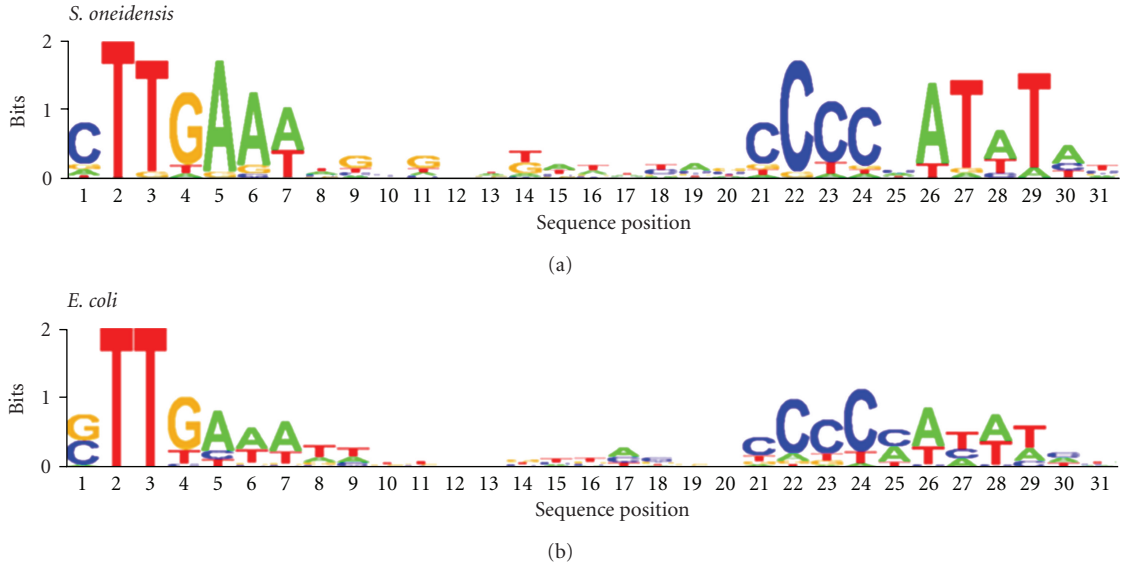

Figure 1: Comparison of consensus σ^{32}-recognition sequences of *E. coli* and *S. oneidensis*. The *E. coli* and *S. oneidensis* sequences used were from the published reports by Nonaka et al. [22] and Gao et al. [20], respectively. The sequences were initially aligned by clustalx and the sequence logo was prepared using public software at http://www.bioinf.ebc.ee/EP/EP/SEQLOGO/.

Additionally, none of genes encoding H^+ ex-pumps are found to be induced. Instead, proteins showing substantial induction are rather diverse, including those functioning in cell envelope structure (e.g., *csg* genes), glycogen biosynthesis (*glg* operon), fatty acid metabolism (*fadBA*), glutamate synthesis (*gltBD*), phosphate transport (*so1724* and *pstB-1*), and regulation (e.g., *rpoS* and *phoU*). This observation indicates that the molecular effects of acute acidic pH are profound and multifarious. Upon alkaline pH, as in *E. coli* Na^+/H^+ antiporter systems (NhaA) are particularly important in maintaining a pH-homeostatic mechanism, thus enabling *S. oneidensis* to survive and adapt to external alkaline conditions.

4. Stress Responses to Osmolarity

The bacterial response to hypertonic stress includes a range of mechanisms. The most important one is regulation of aquaporins in the outer membrane for water intake by the stationary-phase sigma factor, RpoS [32]. It is common that upon the stress condition K^+ uptake is activated and K^+ ions are maintained at high levels. Additionally, cells accumulate neutral, polar, small molecules, such as glycine betaine (GB), proline, trehalose, or ectoine [33]. These compatible solutes serve as osmoprotectants and are synthesized and/or imported into the cell. Many *Shewanella* species are marine microorganisms and therefore are naturally tolerant to relatively high levels of salt. Although some like *S. oneidensis*, are obtained from freshwater environments, they are able to grow in the presence of up to 0.6 M NaCl [34].

The primary response of *S. oneidensis* to hyperosmotic conditions is similar to *E. coli*. Genes encoding K^+ uptake proteins, Na^+ efflux system components, and glutamate synthesis are found to be highly induced. Nonetheless, some novel mechanisms are observed. Genes encoding proteins involved in accumulation of compatible osmolytes are either missing in the genome or transcriptionally unaffected when encountered stress. Interestingly, genes encoding TCA cycle are particularly active, probably producing much needed ATP for ion transport. This may also explain that *S. oneidensis* shows reduced motility and chemotaxis responding capability under the stress given that the assembly of flagella is extremely energy consuming [34].

5. Stress Responses to Radiation

Radiation is potentially lethal and mutagenic to all organisms. Although DNA is the major chromophore in general, effects of radiation are in fact pleiotropic [35, 36]. *S. oneidensis*, one of the most radiation-sensitive organisms known so far, is approximately 1 order of magnitude more susceptible to all wavelengths of solar UV, UV, and ionizing radiation than *E. coli* [35, 37–40]. This is strikingly because the organism similar to *E. coli* possesses the complete set of genes for photo-reactivation, and nucleotide excision repair, and SOS response, primary mechanisms that protect cells from DNA damages and radiation-induced oxidative stress [16, 41, 42]. All of these *S. oneidensis* genes appear to be functional and crucial in the cellular response to radiation, supported by significant upregulation in transcriptional analyses. It is interesting to note that *Shewanella* strains vary significantly in their susceptibility to radiation although compared to *E. coli* they are still much less resistant. The general trend is that the more radiation exposure is in the habitat where the organisms are isolated the less sensitive they are [37]. For instance, *S. oneidensis* MR-1 from lake sediment and *S. putrefaciens* 200 from a crude oil pipeline are more sensitive to radiation than *S. algae* from the surface of a red alga and *S. oneidensis* MR-4 from the surface of the Black Sea [33].

Table 1: Genes predicted to be under the direct control of σ^{32} in *S. oneidensis*.

Locus	Gene	Product	Start	End	Sequence	Weight
SO2016	*htpG*	heat shock protein HtpG	−84	−55	CTTGAAAAGTGGATTTGCAGCCCCATTTTA	20.3
SO4162	*hslV*	ATP-dependent protease HslV	−83	−54	CTTGAATTCTGGCTATCCATCCCCATATTT	20.1
SO1126	*dnaK*	chaperone protein DnaK	−78	−48	CTTGAAAAAAAATGCGTCCGGCCCCATATCT	18.6
SO0406	*trxA*	thioredoxin 1	−80	−51	CTTGAAAAGCTATTTTTCAGCCCCAATATA	18.4
SO1524	*grpE*	heat shock protein GrpE	−74	−45	CTTGAAACGTCAAAATTGATCCCCATAATA	18.2
SO2593		conserved hypothetical protein	−262	−232	CTTGAAATGGGGAGTTTAACTCCCCATTTTT	17.9
SO3577	*clpB*	clpB protein	−77	−48	CTTGAATTTGGTTAAATAGCCCCCATCTTT	16.8
SO0452	*trxC*	thioredoxin 2	−60	−31	CTTTAAATTCGCCGCAGCGCCCCCATATCT	15.7
SO2017		conserved hypothetical protein	−106	−76	CTTGAGTTGAGACGCAAGTGCCCCGATTTAC	14.4
SO1796	*lon*	ATP-dependent protease La	−68	−39	ATTGAAAGGGCATAAACCGCCCCAATATAC	14
SO2277	*ibpA*	16 kDa heat shock protein A	−167	−138	CTTGAAATCCGTTTTCCTATCCTTATATCT	13.5
SO0703	*groES*	chaperonin GroES	−123	−93	CTTGGATCTGGCGGGGGTGAACCCCATATCA	13.3
SO4492		conserved hypothetical protein	−76	−48	GTTGAAAAGAATTGATTTGCCCCAAGATA	12.8
SO1794	*clpP*	ATP-dependent Clp protease, proteolytic subunit	−83	−55	CTTGACTTGATTAGCAGTTCGCCATTTAT	12.8
SO1163		conserved hypothetical protein	−60	−31	CTTGAATCGGGTATAATCGCCACCATATAG	12.7
SO3863	*modA*	molybdenum ABC transporter, periplasmic molybdenum-binding protein	−206	−177	CTTGAGTAAATGTTATTGTCCCCGATCAAT	12.3
SO1196		ribosomal RNA large subunit methyltransferase J	−65	−36	GTTGAAAAACCGCTATTCTACCCTTATATA	12.2
SO2723	*rrmJ*	HIT family protein	−47	−17	ATTGAATTGCTAGTATACTATCCCAATTAAC	11.8
SO1213		hydrolase, TatD family	−240	−211	GTTTAAAGGCGGTGATTCACCGCCTTTTTT	11.8
SO2705	*topA*	DNA topoisomerase I	−77	−49	CTTGAAACTCTCAGTGCAACCCTCTATAT	11.1
SO3501		conserved hypothetical protein	−297	−268	CATGAATTTGGCAACGGCACCGCCATTTTC	11
SO2728	*htpX*	peptidase HtpX	−101	−71	GTAGAAAAACTCTTATCTTTACCCCTTGAAT	10.6
SO1473	*smpB*	SsrA-binding protein	−69	−39	GTTGAAATAGCTCAAATAAACCCTTATATCC	10.3
SO0698	*fsxA*	fxsA protein	−64	−34	CTTGAATTAAGACCGGATTGCCCCCATTTAG	10.3
SO3402		hypothetical protein	−396	−367	ATTGAAAAGGGCCTTTATGGCCCTTTTTCG	10.2
SO1937	*fur*	ferric uptake regulation protein	−164	−135	CTTGAATTGCCGCAATTTATTGCAATTTCA	10.2
SO2706	*astB*	succinylarginine dihydrolase	−40	−11	TTTGAATAAATAATAACCTTCCCTATCACA	9.7
SO0868		hypothetical protein	−93	−63	GTTTAAATGGGGAGAAAACAACTCCATTTTA	9.4
SO3961	*rpoN*	RNA polymerase sigma-54 factor	−83	−53	CTTGAATTTGGCAGCGCAAAGCGCCATCAGT	9.4
SO0930		Transketolase	−161	−133	CTTGAATAGTTCATCCTTAAGCCATTTTT	9.3
SO3528		hypothetical protein	−195	−167	AATGAAAAGAGGCTTTTAGCCTCTTTTTT	9.3
SO1580		TonB-dependent heme receptor	−57	−28	CTTTGATGCCTATAATGCCGCCCTATTTTT	9.3
SO2314	*tkt*	ISSo1, transposase OrfA	−227	−197	GTTAAAATGACAAGCATGGAGCGCAATATCT	9.2
SO1903		hypothetical protein	−71	−42	TTTGGGATTATTTAATTCCCCCCCATTTAT	9.2
SO1097		conserved hypothetical protein	−63	−33	CATGAAATCTGCGATAATCAGCGCCTTATTT	9.2
SO0595		hypothetical protein	−327	−298	CTTGATTAGAGCCACGTCGCTCCAATTTTT	9.2
SO4719		conserved hypothetical protein	−44	−16	CTAGGCATTTGAGTTGGAACCCTATTTTT	9.1
SO4287	*motA*	chemotaxis motA protein	−127	−99	CTTGAATTTAGTAGATTTTCCTTATAATG	9.1
SO3113	*tgt*	queuine tRNA-ribosyltransferase	−96	−67	GTTGAACCTTTTAGATCTGTCCCTATCTCT	9

Genome screening with σ^{32} weight matrix is performed using RSAT at http://rsat.ulb.ac.be/rsat/RSAT_home.cgi [23]. Genes with a weight score over 9 are shown.

It has been suggested that the hypersensitivity to radiation may be in part due to the activation of prophage [38–40]. Radiation has been used as a standard approach to induce prophage in a variety of bacteria [43, 44]. In *S. oneidensis*, upon radiation the majority of LambdaSo, MuSo1, and MuSo2 genes are induced and phage particles have been found in the cultures, indicating that a great number of cells are lysed by lytic phages. It has also been implicated that a large number of iron-containing proteins may be partially accountable for the susceptibility. Compared to *E. coli* which hosts only five to seven cytochrome *c* proteins, *S. oneidensis* contains 41 such proteins, some of which are electron transport proteins and essential in respiration [45, 46]. Damages on these proteins by reactive oxygen species (ROS) generated in cells upon radiation would likely cause two detrimental results [47]. First, damaged proteins *per se* may be dysfunctional, directly reducing ability to survive or thrive. Second, damaged proteins release irons into cultures, which further induce ROS production [48]. This second wave of ROS may be more fatal because it comes at the onset of recovery of seriously damaged cells. Furthermore, the finding that the intracellular Mn/Fe concentration ratios correlate well with resistance to radiation may explain the hypersensitivity of *S. oneidensis*, which has the lowest ratio among bacteria tested so far [35, 49].

6. Stress Responses to Heave Metals

Many of metal elements are required for microbial growth mostly as cofactors in metabolic pathways. However, they exert deleterious effects under conditions of elevated concentration [50]. *Shewanellae* have attracted much attention because of their ability to reduce metal ions including chromium, cobalt, iron, manganese, technetium, uranium, and vanadium, some of which are not needed and highly toxic for most organisms [10, 51, 52]. At the low level these metal ions are taken as electron acceptors by cells and mildly induced some stress-associated genes [53]. However, at the high concentration some of them elicited a distinctively different pattern [54–60]. The cellular resistance mechanisms displayed by microorganisms are diverse and include biosorption, diminished intracellular accumulation through either direct obstruction of the ion uptake system or active chromate efflux, precipitation, and reduction of metals to less toxic form. Multiple regulatory circuits are found to work together to cope with the stress response of *S. oneidensis* to heavy metal compounds. The major ones include those modulating oxidative stress protection, detoxification, protein stress protection, iron acquisition, and DNA repair [50].

The molecular response of *S. oneidensis* to heavy metal shock elicits a distinctively different transcriptional profile compared with metal reduction [53–60]. This observation is consistent with that metal reduction and toxicity resistance mechanisms are to be unlinked cellular processes [61]. Responses of *S. oneidensis* to acute stresses imposed by a variety of heavy metals share a common strategy: survive first and then exert both general and specific stress responses. As a result, *S. oneidensis* up-regulates its resistance-nodulation-cell division (RND) protein family genes that facilitate cation export and thus confer heavy metal resistance. Once the first line of defense is initiated, cells employ both general and specific stress responses that are inseparable from each other to recover from the crisis. Alternative sigma factors including RpoS, RpoH, RpoE, along with stress-response-related genes are induced, leading to induction of a variety of detoxification, resistance, and transport functions. Such coordinated expression of stress response and detoxification mechanisms in *S. oneidensis* may offer an advantage to thrive in anoxic metal-reducing conditions in aquatic sediment and submerged soil systems where substantial amounts of heavy metals can be generated.

Two specific responding mechanisms are particularly worth noting. The first is that genes/proteins involved in iron transport are transcriptionally active and implicated to play an important role in the process. Although induction of siderophore biosynthetic and iron transport genes may not be a direct consequence of intracellular iron limitation, several lines of evidence suggest that it is more likely to be indirect by interfering with the Fur (ferric uptake regulator) protein, which eventually results in derepression of the iron regulon. Several reports have demonstrated that Co^{2+}, Mn^{2+}, or other divalent cations interact with the Fur-binding sites [62, 63]. Moreover, iron-chelating siderophores from other microorganisms have been shown to be able to bind other metals, such as thorium, uranium, vanadium, and plutonium [64, 65]. By increasing siderophore production, cells can reduce toxicity of heavy metals by sequestration. The other is that sulfur transport and assimilation is promoted. While the underlying mechanism is currently unknown, an explanation is offered. In *S. oneidensis*, reactive oxygen species (ROS) produced in cells by heavy metal stresses can damage iron-containing proteins. As cysteine residues in these proteins are essential to their functions, an extra mount of cysteine is needed for protection. To this end, cells elevate transportation of inorganic sulfate which is reduced and incorporated into bioorganic compounds via assimilatory sulfate reduction, which is the major route of cysteine biosynthesis in most microorganisms [66].

7. Concluding Remarks

As a potential strategy for the reductive immobilization or detoxification of environmental contaminants, *in situ* bioremediation has received much interest and attention in last 20 years and are becoming more prevalent today. As its intrinsic feature, the application puts its work force, mostly bacteria, "*in situ*" facing the unpredictability of individual microbial processes and constant fluctuations in environments. Thanks to the availability of the *S. oneidensis* genome sequence, stress responses of the microorganism have been extensively investigated, generating a handful of insights into mechanisms adopted to cope with detrimental conditions. Nonetheless, adaptive mechanisms of *Shewanella* to environmental stresses are still a large playing field for three reasons. First, a number of common stressful agents, especially reactive oxygen species, are not visited. Second, the complex components and regulation in the bacterial stress responses discussed in this paper are mostly based on

transcriptional profiling and thus experimental validation is urgently warranted. Last, but definitely not the least, the genus is composed of members which are not only isolated from extremely diverse habitats but also lack unifying phenotypic features, prompting exploration to be extended to other ecological groups of the Shewanellae.

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References

[1] C. Gerday and N. Glansdorff, *Physiology and Biochemistry of Extremophiles*, American Society for Microbiology, Washington, DC, USA, 2007.

[2] G. Storz and R. Hengge-Aronis, *Bacterial Stress Responses*, American Society for Microbiology, Washington, DC, USA, 2000.

[3] E. Ron, "Bacterial stress response," in *The Prokaryotes*, M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt, Eds., Springer, Singapore, 2006.

[4] S. Fields and M. Johnston, "Whither model organism research?" *Science*, vol. 307, no. 5717, pp. 1885–1886, 2005.

[5] M. T. MacDonell and R. R. Colwell, "Phylogeny of the *Vibrionaceae*, and recommendation for 2 new genera, *Listonella* and *Shewanella*," *Systematic and Applied Microbiology*, vol. 6, no. 2, pp. 171–182, 1985.

[6] G. Gauthier, M. Gauthier, and R. Christen, "Phylogenetic analysis of the genera *Alteromonas, Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations," *International Journal of Systematic Bacteriology*, vol. 45, no. 4, pp. 755–761, 1995.

[7] E. P. Ivanova, S. Flavier, and R. Christen, "Phylogenetic relationships among marine *Alteromonas*-like *proteobacteria*: emended description of the family *Alteromonadaceae* and proposal of *Pseudoalteromonadaceae* fam. nov., *Colwelliaceae* fam. nov., *Shewanellaceae* fam. nov., *Moritellaceae* fam. nov., *Ferrimonadaceae* fam. nov., *Idiomarinaceae* fam. nov. and *Psychromonadaceae* fam. nov.," *International Journal of Systematic and Evolutionary Microbiology*, vol. 54, no. 5, pp. 1773–1788, 2004.

[8] H. Gao, A. Obraztova, N. Stewart et al., "*Shewanella loihica* sp. nov., isolated from iron-rich microbial mats in the Pacific Ocean," *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 8, pp. 1911–1916, 2006.

[9] P. Verma, P. K. Pandey, A. K. Gupta et al., "*Shewanella indica* sp. nov.,isolated from the sediment of Arabian Sea," *International Journal of Systematic and Evolutionary Microbiology*. In press.

[10] K. H. Nealson and D. Saffarini, "Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation," *Annual Review of Microbiology*, vol. 48, no. 1, pp. 311–343, 1994.

[11] J. K. Fredrickson, M. F. Romine, A. S. Beliaev et al., "Towards environmental systems biology of *Shewanella*," *Nature Reviews Microbiology*, vol. 6, no. 8, pp. 592–603, 2008.

[12] J. K. Fredrickson and J. M. Zachara, "Electron transfer at the microbe-mineral interface: a grand challenge in biogeochemistry," *Geobiology*, vol. 6, no. 3, pp. 245–253, 2008.

[13] D. R. Lovley, "The microbe electric: conversion of organic matter to electricity," *Current Opinion in Biotechnology*, vol. 19, no. 6, pp. 564–571, 2008.

[14] K. T. Konstantinidis, M. H. Serres, M. F. Romine et al., "Comparative systems biology across an evolutionary gradient within the *Shewanella* genus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15909–15914, 2009.

[15] P. J. L. Simpson, D. J. Richardson, and R. Codd, "The periplasmic nitrate reductase in *Shewanella*: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB," *Microbiology*, vol. 156, no. 2, pp. 302–312, 2010.

[16] J. F. Heidelberg, I. T. Paulsen, K. E. Nelson et al., "Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*," *Nature Biotechnology*, vol. 20, no. 11, pp. 1118–1123, 2002.

[17] S. Lindquist and E. A. Craig, "The heat-shock proteins," *Annual Review of Genetics*, vol. 22, pp. 631–677, 1988.

[18] T. Yura and K. Nakahigashi, "Regulation of the heat-shock response," *Current Opinion in Microbiology*, vol. 2, no. 2, pp. 153–158, 1999.

[19] M. H. Weber and M. A. Marahiel, "Bacterial cold shock responses," *Science Progress*, vol. 86, no. 1-2, pp. 9–75, 2003.

[20] H. Gao, Y. Wang, X. Liu et al., "Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*," *Journal of Bacteriology*, vol. 186, no. 22, pp. 7796–7803, 2004.

[21] H. Gao, Z. K. Yang, L. Wu, D. K. Thompson, and J. Zhou, "Global transcriptome analysis of the cold shock response of *Shewanella oneidensis* MR-1 and mutational analysis of its classical cold shock proteins," *Journal of Bacteriology*, vol. 188, no. 12, pp. 4560–4569, 2006.

[22] G. Nonaka, M. Blankschien, C. Herman, C. A. Gross, and V. A. Rhodius, "Regulon and promoter analysis of the *E. coli* heat-shock factor, sigma32, reveals a multifaceted cellular response to heat stress," *Genes and Development*, vol. 20, no. 13, pp. 1776–1789, 2006.

[23] J. V. Turatsinze, M. Thomas-Chollier, M. Defrance, and J. van Helden, "Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules," *Nature Protocols*, vol. 3, no. 10, pp. 1578–1588, 2008.

[24] S. Phadtare and M. Inouye, "Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of *Escherichia coli*," *Journal of Bacteriology*, vol. 186, no. 20, pp. 7007–7014, 2004.

[25] S. Li, X. Xiao, P. Sun, and F. Wang, "Screening of genes regulated by cold shock in *Shewanella piezotolerans* WP3 and time course expression of cold-regulated genes," *Archives of Microbiology*, vol. 189, no. 6, pp. 549–556, 2008.

[26] F. Wang, X. Xiao, H. Y. Ou, and Y. Gai, "Role and regulation of fatty acid biosynthesis in the response of *Shewanella piezotolerans* WP3 to different temperatures and pressures," *Journal of Bacteriology*, vol. 191, no. 8, pp. 2574–2584, 2009.

[27] F. Wang, Q. Li, and X. Xiao, "A novel filamentous phage from the deep-sea bacterium *Shewanella piezotolerans* WP3 is induced at low temperature," *Journal of Bacteriology*, vol. 189, no. 19, pp. 7151–7153, 2007.

[28] J. W. Foster, "Microbial responses to acid stress," in *Bacterial Stress Responses*, G. Storz and R. Hengge-Aronis, Eds., American Society for Microbiology, Washington, DC, USA, 2000.

[29] H. Saito and H. Kobayashi, "Bacterial responses to alkaline stress," *Science Progress*, vol. 86, no. 4, pp. 271–282, 2003.

[30] A. B. Leaphart, D. K. Thompson, K. Huang et al., "Transcriptome profiling of *Shewanella oneidensis* gene expression following exposure to acidic and alkaline pH," *Journal of Bacteriology*, vol. 188, no. 4, pp. 1633–1642, 2006.

[31] J. W. Foster, "*Escherichia coli* acid resistance: tales of an amateur acidophile," *Nature Reviews Microbiology*, vol. 2, no. 11, pp. 898–907, 2004.

[32] L. N. Csonka, "Physiological and genetic responses of bacteria to osmotic stress," *Microbiological Reviews*, vol. 53, no. 1, pp. 121–147, 1989.

[33] M. Jebbar, L. Sohn-Bosser, E. Bremer, T. Bernard, and C. Blanco, "Ectoine-induced proteins in *Sinorhizobium meliloti* include an ectoine ABC-type transporter involved in osmoprotection and ectoine catabolism," *Journal of Bacteriology*, vol. 187, no. 4, pp. 1293–1304, 2005.

[34] Y. Liu, W. Gao, Y. Wang et al., "Transcriptome analysis of *Shewanella oneidensis* MR-1 in response to elevated salt conditions," *Journal of Bacteriology*, vol. 187, no. 7, pp. 2501–2507, 2005.

[35] D. Ghosal, M. V. Omelchenko, E. K. Gaidamakova et al., "How radiation kills cells: survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress," *FEMS Microbiology Reviews*, vol. 29, no. 2, pp. 361–375, 2005.

[36] M. J. Daly, E. K. Gaidamakova, V. Y. Matrosova et al., "Protein oxidation implicated as the primary determinant of bacterial radioresistance," *PLoS Biology*, vol. 5, no. 4, article e92, 2007.

[37] X. Qiu, G. W. Sundin, B. Chai, and J. M. Tiedje, "Survival of *Shewanella oneidensis* MR-1 after UV radiation exposure," *Applied and Environmental Microbiology*, vol. 70, no. 11, pp. 6435–6443, 2004.

[38] X. Qiu, G. W. Sundin, L. Wu, J. Zhou, and J. M. Tiedje, "Comparative analysis of differentially expressed genes in *Shewanella oneidensis* MR-1 following exposure to UVC, UVB, and UVA radiation," *Journal of Bacteriology*, vol. 187, no. 10, pp. 3556–3564, 2005.

[39] X. Qiu, J. M. Tiedje, and G. W. Sundin, "Genome-wide examination of the natural solar radiation response in *Shewanella oneidensis* MR-1," *Photochemistry and Photobiology*, vol. 81, no. 6, pp. 1559–1568, 2005.

[40] X. Qiu, M. J. Daly, A. Vasilenko et al., "Transcriptome analysis applied to survival of *Shewanella oneidensis* MR-1 exposed to ionizing radiation," *Journal of Bacteriology*, vol. 188, no. 3, pp. 1199–1204, 2006.

[41] E. C. Friedberg, G. C. Walker, and W. Siede, *DNA Repair and Mutagenesis*, American Society for Microbiology, Washington, DC, USA, 1995.

[42] C. Janion, "Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*," *International Journal of Biological Sciences*, vol. 4, no. 6, pp. 338–344, 2008.

[43] I. Lamont, A. M. Brumby, and J. B. Egan, "UV induction of coliphage 186: prophage induction as an SOS function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 14, pp. 5492–5496, 1989.

[44] Y. Liu, Q. Zhang, C. Fang, S. Zhu, Y. Tang, and S. Huang, "Effect of glutathione on UV induction of prophage lambda," *Archives of Microbiology*, vol. 183, no. 6, pp. 444–449, 2005.

[45] H. Gao, S. Barua, Y. Liang et al., "Impacts of *Shewanella oneidensis* c-type cytochromes on aerobic and anaerobic respiration," *Microbial Biotechnology*, vol. 3, no. 4, pp. 455–466, 2010.

[46] F. R. Blattner, G. Plunkett III, C. A. Bloch et al., "The complete genome sequence of *Escherichia coli* K-12," *Science*, vol. 277, no. 5331, pp. 1453–1462, 1997.

[47] B. Py and F. Barras, "Building Feg-S proteins: bacterial strategies," *Nature Reviews Microbiology*, vol. 8, no. 6, pp. 436–446, 2010.

[48] J. A. Imlay, "Cellular defenses against superoxide and hydrogen peroxide," *Annual Review of Biochemistry*, vol. 77, pp. 755–776, 2008.

[49] M. J. Daly, E. K. Gaidamakova, V. Y. Matrosova et al., "Accumulation of Mn(II) in *Deinicoccus radiodurans* facilitates gamma-radiation resistance," *Science*, vol. 306, no. 5698, pp. 1025–1028, 2004.

[50] D. H. Nies, "Efflux-mediated heavy metal resistance in prokaryotes," *FEMS Microbiology Reviews*, vol. 27, no. 2-3, pp. 313–339, 2003.

[51] C. Liu, Y. A. Gorby, J. M. Zachara, J. K. Fredrickson, and C. F. Brown, "Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria," *Biotechnology and Bioengineering*, vol. 80, no. 6, pp. 637–649, 2002.

[52] W. Carpentier, K. Sandra, I. De Smet, A. Brigé, and L. De Smet, "Microbial reduction and precipitation of vanadium by *Shewanella oneidensis*," *Applied and Environmental Microbiology*, vol. 69, no. 6, pp. 3636–3639, 2003.

[53] A. S. Beliaev, D. M. Klingeman, J. A. Klappenbach et al., "Global transcriptome analysis of *Shewanella oneidensis* MR-1 exposed to different terminal electron acceptors," *Journal of Bacteriology*, vol. 187, no. 20, pp. 7138–7145, 2005.

[54] C. W. Saltikov, A. Cifuentes, K. Venkateswaran, and D. K. Newman, "The ars detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3," *Applied and Environmental Microbiology*, vol. 69, no. 5, pp. 2800–2809, 2003.

[55] R. Bencheikh-Latmani, S. M. Williams, L. Haucke et al., "Global transcriptional profiling of *Shewanella oneidensis* MR-1 during Cr(VI) and U(VI) reduction," *Applied and Environmental Microbiology*, vol. 71, no. 11, pp. 7453–7460, 2005.

[56] S. D. Brown, M. Martin, S. Deshpande et al., "Cellular response of *Shewanella oneidensis* to strontium stress," *Applied and Environmental Microbiology*, vol. 72, no. 1, pp. 890–900, 2006.

[57] S. D. Brown, M. R. Thompson, N. C. VerBerkmoes et al., "Molecular dynamics of the *Shewanella oneidensis* response to chromate stress," *Molecular and Cellular Proteomics*, vol. 5, no. 6, pp. 1054–1071, 2006.

[58] K. Chourey, M. R. Thompson, J. Morrell-Falvey et al., "Global molecular and morphological effects of 24-hour chromium(VI) exposure on *Shewanella oneidensis* MR-1," *Applied and Environmental Microbiology*, vol. 72, no. 9, pp. 6331–6344, 2006.

[59] A.-C. M. Toes, M. H. Daleke, J. G. Kuenen, and G. Muyzer, "Expression of *copA* and *cusA* in *Shewanella* during copper stress," *Microbiology*, vol. 154, no. 9, pp. 2709–2718, 2008.

[60] I. Mugerfeld, B. A. Law, G. S. Wickham, and D. K. Thompson, "A putative azoreductase gene is involved in the *Shewanella oneidensis* response to heavy metal stress," *Applied Microbiology and Biotechnology*, vol. 82, no. 6, pp. 1131–1141, 2009.

[61] C. Cervantes, J. Campos-Garcia, S. Devars et al., "Interactions of chromium with microorganisms and plants," *FEMS Microbiology Reviews*, vol. 25, no. 3, pp. 335–347, 2001.

[62] T. Funahashi, C. Fujiwara, M. Okada et al., "Characterization of *Vibrio parahaemolyticus* manganese-resistant mutants in reference to the function of the ferric uptake regulatory

protein," *Microbiology and Immunology*, vol. 44, no. 12, pp. 963–970, 2000.

[63] K. Hantke, "Iron and metal regulation in bacteria," *Current Opinion in Microbiology*, vol. 4, no. 2, pp. 172–177, 2001.

[64] C. Baysse, D. De Vos, Y. Naudet et al., "Vanadium interferes with siderophore-mediated iron uptake in *Pseudomonas aeruginosa*," *Microbiology*, vol. 146, no. 10, pp. 2425–2434, 2000.

[65] S. G. John, C. E. Ruggiero, L. E. Hersman, C. S. Tung, and M. P. Neu, "Siderophore mediated plutonium accumulation by *Microbacterium flavescens* (JG-9)," *Environmental Science and Technology*, vol. 35, no. 14, pp. 2942–2948, 2001.

[66] M. A. Kertesz, "Riding the sulfur cycle—metabolism of sulfonates and sulfate esters in gram-negative bacteria," *FEMS Microbiology Reviews*, vol. 24, no. 2, pp. 135–175, 2000.