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## Bacteria from the “Saline di Tarquinia” marine salterns reveal very atypical growth profiles with regards to salinity and temperature

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### Abstract

Several thousand bacterial colonies were isolated from “Saline di Tarquinia” (Italy) marine salterns; after dereplication, thirty-one strains were obtained, confirming the low culturable bacterial diversity attributed to similar hypersaline environments. Strains were identified by 16S ribosomal DNA sequencing and phylogenetic analysis; most strains belonged to the class Gammaproteobacteria, with minorities from Actinobacteria, Bacilli and Alphaproteobacteria. *Halomonas* and *Salinivibrio* were predominant genera. To profile growth preferences, strains were cultivated at different salinities and temperatures. Non-metric multi-dimensional scaling (nMDS) analysis on growth data at different salinities identified four groups that were significantly correlated with the ecological categories conventionally used to describe halophiles. The majority were moderate halophiles with optimal growth at 40-80‰ salinity. Some were markedly euryhaline and grew from 40-80‰ to 240-280‰.

Very uncommon behaviour was observed for some strains, with a clear optimum growth in the slight to moderate halophilic range, but also the ability to grow without salt. These bacteria could barely be included in the mentioned traditional definitions and we suggest to consider them as “norm-tolerant halophilic”, being somehow in-between the slight halophiles and the halotolerants. As for temperature, nMDS groups were less defined and uncorrelated to ecological categories. Most strains were mesophilic-psychrotolerant or mesophilic, and some psychrotolerants were also recorded. Generally, diffused eurythermism was observed and various strains displayed unusual “flat growth profiles” that showed a broad range of thermal optima. Overall, the majority of strains appeared to be well adapted to the “Saline di Tarquinia” environment and showed great temperature and salinity variations compared to species observed in other salterns.

**Keywords:** Temperature preferences; salinity preferences; halophilic bacteria; marine salterns; Saline di Tarquinia; adaptation.

### Introduction

Transition coastal areas play an important ecological role along seas and oceans. Some characteristics of these biotopes include high productivity, coastal protection, water quality improvement and support of unique habitats for many species (Dungeon *et al.*, 2006; Cimmaruta *et al.*, 2010; Barghini *et al.*, 2014). Marine salterns are peculiar transition areas where chemico-physical parameters can be subjected to intense daily and seasonal variations; a wide salinity gradient is generally present. As demonstrated for a few other unstable extreme environments (Reboleiro Rivas *et al.*, 2013; Andrade *et al.*, 2014), sudden and repeated fluctuations in temperature and water availability subject organisms to high environmental stress and make these sites good prototypical examples of some global-change phenomena, as suggested by Barghini *et al.* (2014). Moreover, they could represent interesting

sources of new microorganisms for use in biotechnology. For example, both microorganisms and their products (i.e., enzymes) could be employed in industrial applications that deal with the bio-treatment of effluents that contain high and sometimes variable amounts of salts. These effluents could represent serious environmental issues and are generated from numerous sources: concentrates from decantation plants, effluents from sewerage treatment plants, brine from natural salty lakes and salt harvesting activities and effluents from pulp/paper and textile industries (Amoozegar *et al.*, 2007; Sabet *et al.*, 2009; Salgaonkar *et al.*, 2013; Sarwar *et al.*, 2015; Quadri *et al.*, 2016).

Generally, marine salterns present a number of shallow pools connected in sequence and show an increasing salinity gradient from approximately 35-300‰ (Anton *et al.*, 2000; Litchfield *et al.*, 2000). These characteristics produce strong environmental heterogeneity that leads

to the establishment of complex biological communities distributed along the gradient. The communities include both highly specialised and marine species (Halse *et al.*, 2002; Cimmaruta *et al.*, 2010; Barghini *et al.*, 2014). The presence of microorganisms in the various ponds is due to active growth of specifically adapted taxa or to external inputs. In any case, at the highest salt concentrations, prokaryotic communities predominate and represent valuable models to study adaptation strategies adopted to cope with a severe life condition in some extreme environments (Salgaonkar *et al.*, 2013; Barghini *et al.*, 2014).

The “Saline di Tarquinia” salterns (ST), covering an area of ca. 135 ha, are located along a low sandy coast on the shore of the North Tyrrhenian Sea and are separated from the sea by a dunes array. The increasing salinity gradient is developed along a series of approximately one hundred shallow interconnected ponds (Angeletti *et al.*, 2016). ST, productive from 1805, were dismissed in 1977 to be transformed into a natural reserve (1980). The conversion to a natural reserve, with mutated management of water balance and a dramatic drop in anthropic activities, changed the entire site structure. In particular, the separation between the various ponds became less sharp and caused significant mixing of waters with diverse salinities. ST can still be considered an extreme environment with wide and abrupt salinity and temperature variations in most of the ponds.

Although rather well investigated concerning its plant and animal ecology and diversity (Frondoni & Iberite 2002; Bellisario *et al.* 2013), ST are practically unknown from the microbiological point of view. The only study dealing with its prokaryote communities, carried out in a very limited number of pools by culture and culture-independent methods, found that there was low bacterial diversity (Barghini *et al.*, 2014). In the present work, bacterial strains were isolated from ST ponds subjected to broad environmental variations during a two-year sampling campaign. In order to investigate their adaptation to stressing conditions and to get materials for future applicative studies, the isolates, identified by 16S ribosomal DNA (rDNA) sequencing and phylogenetic analysis, were cultured under wide gradients of salinity and temperature.

## Materials and Methods

### Sample collection

Water samples were obtained monthly (May 2012 - May 2014) from seven pools that represented different ranges of salinity variation (Fig. 1). For each sampling site (pond), waters were collected at three different points (at the same time) at ca. 20 cm depth using sterile containers connected to a long telescopic shaft and pooled together to obtain an integrated sample (Hervas & Casamayor, 2009; Reboleiro-Rivas *et al.*, 2013). Pool temperature and salinity were measured by a digital thermometer and a refractometer, respectively. After sampling, 1,000

mL of water were vacuum-filtered on sterile membranes (0.22  $\mu\text{m}$ ; Millipore, USA) as reported by Pesciaroli *et al.* (2012). Bacterial cultures were obtained by placing the membranes on Petri dishes that contained Plate Count Agar (PCA; Difco, USA) with different concentrations of marine salt (40, 80, 160 or 240‰; Difco, USA) and then incubated at 10°C and 25°C.

### Strain isolation and culture conditions

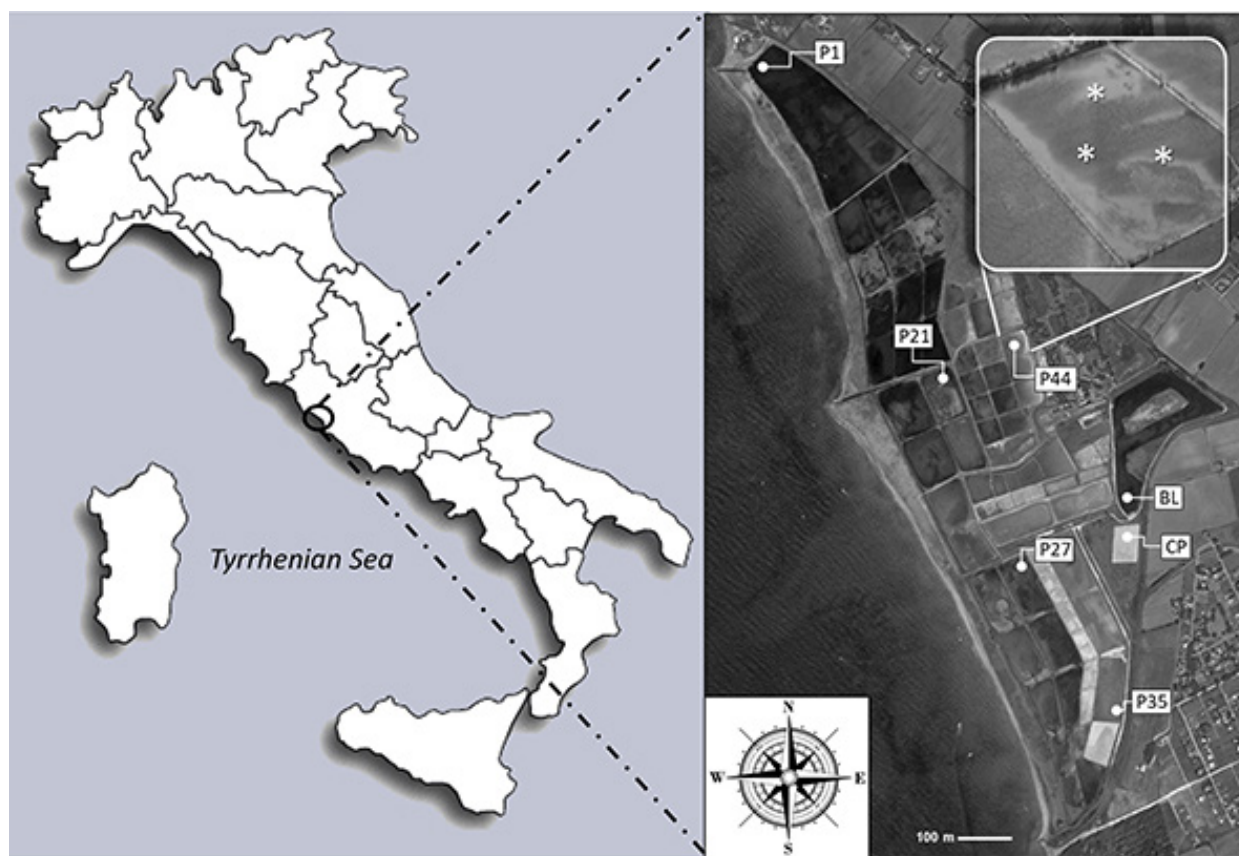
Isolate pure cultures were obtained using the streak plate method. Rejection of duplicates (dereplication) was carried out by preliminary tests that considered some morphological characteristics (shape, colour, morphology, aspect and dimensions), simple biochemical tests (catalase and oxidase production) and Gram reaction; 16S rDNA analysis (cut-off value 100% similarity) was also used in doubtful cases.

Gram staining was carried out using a commercial kit (Merck, Germany). Bacteria were measured on stained specimens using a Leitz Laborlux 11 microscope bearing a calibrated micrometric ocular. Catalase and oxidase tests were performed as reported previously (Kovacs, 1956; Whittenbury, 1964). Dereplication led to the selection of thirty-one strains that were stored in the Culture Collection of Marine Bacteria, DEB, University of Tuscia, maintained at 4°C on agar slants of PCA that contained the necessary concentration of marine salt and routinely subcultured.

### Strain identification

Isolates, grown for 24-48 h on agar plates, were used for genomic DNA extraction by thermal shock as reported by Pesciaroli *et al.* (2012). Amplification of the 16S rDNA sequence was performed in a 25  $\mu\text{L}$  reaction mixture that contained 2x BioMix (BioLine GmbH, Germany), 15-20 ng  $\mu\text{L}^{-1}$  of DNA template and 5 pmol  $\mu\text{L}^{-1}$  of the following universal primers (Sigma-Aldrich, USA): 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1389r (5'-ACGGGCGGTGTGTGTACAAG-3'), as reported by Hongoh *et al.* (2003). Amplifications were carried out as previously reported (Pesciaroli *et al.*, 2012). Amplicons were checked by electrophoresis on agarose gels using the GeneRuler™ 1 kb DNA ladder (Fermentas, Lithuania) and purified using the Nucleospin Extract kit (Macherey-Nagel, Germany). Sequencing was performed by MacroGen sequencing service (MacroGen Inc., Korea). Sequence assembly was done using Chromas software (version 1.5 2009, Technelysium Pty Ltd, Australia).

BLASTn was used to compare ST bacteria sequences to those in the NCBI nucleotide database (Altschul *et al.*, 1997; [https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)). For a preliminary attribution, only database sequences (listed in the BLAST report) with similarity  $\geq 99\%$  were considered. Subsequently, from the BLAST reports, actual identifications were achieved using single trees (maximum-likelihood dendrograms)



**Fig. 1:** Detailed map of the “Saline di Tarquinia” salterns; the pools where strains were isolated are indicated. Abbreviations: P1-P44, pools along the salinity gradient; CP, crystallisation pool; BL, brackish lake. The sampling procedure scheme for each pool is shown in the panel. Pool salinity and temperature range during sampling were as follows: salinity (‰) P1, 20-42; P21, 32-95; P27, 38-174; P35, 40-230; P44, 40-300; CP, 110-360; BL, 4-18; temperature (°C) P1, 7.1-29.4; P21, 6.2-28.5; P27, 6.7-32.0; P35, 7.1-33.2; P44, 6.5-32.2; CP, 9.0-35.9; BL, 5.8-32.0.

that related each ST bacteria sequence with those of the type/reference strains and all other sequences that undoubtedly clustered with them (Timperio *et al.*, 2017). Automatic 16S rDNA sequence alignment and dendrogram construction were carried out using MEGA 7.0 (Kumar *et al.*, 2016). A maximum-likelihood tree is supplied to show relationships among all ST bacteria and their subdivision in classes and orders. Bootstrap tests were done to infer the reliability of branch order, with 1,000 pseudo-replicates.

#### **Determination of growth preferences at different salinity and temperature conditions**

Growth at different salinities was tested on PCA plates (90 mm diameter; Duckworth *et al.*, 2000) at 25°C over the salinity range of 0-320‰ in 40‰ steps. Strain temperature growth preferences were tested on PCA plates over the range of 0-45°C by 5±0.5°C steps at their salinity optima. In both experiments, for each strain, plates were inoculated with triplicated punctiform inocula (diameter 1 mm) and incubated for 30 days in sealed plastic boxes,

humidified by a small beaker of distilled water to prevent evaporation and salt precipitation on plates, as reported by Sabet *et al.* (2009). Growth was measured daily as average colony diameter using imaging analysis (IAS 2000 v.1.0, Delta Sistemi sas).

#### **Statistical analysis**

Data were ordered by non-metric multi-dimensional scaling (nMDS) analysis using a Bray-Curtis matrix of distance and the Kruskal loss function (CAP 4.0, Community Analysis Package, Pisces Conservation Ltd. 2007). Multi-dimensional analyses, carried out separately for salinity and temperature, were made on the normalised colony diameters (maxima) at all the tested salinities (at 25°C) and temperatures (at each strain salinity optima), respectively. The Bray-Curtis index was calculated to evaluate similarity (Greenacre & Primicerio, 2014).

nMDS for molecular data (16S rDNA sequences) was computed on a pairwise distance matrix obtained by estimating the evolutionary divergence between sequences (MEGA 7; Kumar *et al.*, 2016) using the best fit evolu-

tion model implemented in jModeltest software (Posada, 2008): Tamura 3-parameter model (Tamura 1992) with a gamma distribution (shape parameter=0.38). Analysis of similarity (ANOSIM) was performed to evaluate the correspondence among nMDS ordinations and categorical groups designed on the ecological characteristics (salinity: non-halophile, non-halophile halotolerant, slight halophile and moderate halophile; temperature: mesophilic, mesophilic-psychrotolerant and psychrotolerant).

Generalised Procrustes rotation analysis (Bookstein, 1991) was used to check the pairwise correlations based on the distance matrices between the pairs of salinity, temperature and phylogenetic data. Procrustes was used to estimate the significances of the Procrustes statistics to assess similarities between different ordinations. A permutation test with 1,000 bootstrap replicates was then performed to measure the significance of the Procrustes correlation derived from the symmetric Procrustes residual (R version 3.3.1, Package vegan 2.4-1).

One-way analysis of variance (ANOVA) and Tukey posthoc tests were performed to compare strain growth (SYSTAT v. 8.0., SPSS Inc., 1998).

## Results and Discussion

### Strain isolation and identification

The large number of pure cultures obtained at the beginning of the isolation process were subjected to careful dereplication to obtain ninety-eight strains. Some strains, considered as possible duplicates, were initially maintained since they were collected from different pools and could present diverse adaptations to salinity and temperatures (Table 1). Further molecular and physiological characterisation, to improve dereplication, led to the thirty-one isolates studied in this work. In Table 1 the thirty-one ST strains are listed together with information regarding their taxonomical identification, the collection pools and some morphological and biochemical characteristics. The majority (ca. 77%) were Gram negative; all strains were positive for catalase and 68% for oxidase. Most of the isolates (87%) showed a rod shape with a wide range of dimensions (1.0-4.5  $\mu\text{m}$  length; 0.4-1.2  $\mu\text{m}$  width).

The isolates subjected to 16S rDNA sequence analysis were affiliated with the following genera: *Aeromonas*, *Arthrobacter*, *Bacillus*, *Chromohalobacter*, *Erwinia*, *Halomonas*, *Kusheneria*, *Microbacterium*, *Nesterenkonia*, *Pseudomonas*, *Psychrobacter*, *Rhodococcus*, *Ruegeria*, *Salinicola*, *Salinivibrio* and *Yersinia* (BLAST similarity values  $\geq 99\%$ ). Eighteen strains (58%), were identified at the species level with conclusive attributions; the others were identified at the genus level only. Most of strains (74%) belonged to the class Gammaproteobacteria; some Actinobacteria, Bacilli and Alphaproteobacteria were also present (Fig. 2). *Halomonas*, *Salinivibrio* and *Pseudomonas* were the genera found most frequently. A dominance of Gammaproteobacteria is generally reported for

marine salterns (Baati *et al.*, 2008; Sabet *et al.*, 2009). Due to the small number of species found during the sampling campaign, the ST culturable bacterial diversity appeared quite low. This finding confirmed our preliminary work, performed using culture and culture-independent (polymerase chain reaction denaturing gel electrophoresis; PCR-DGGE) methods, that reported the isolation of only twelve species and the presence of just thirty-two different DGGE bands that theoretically corresponded to thirty-two operational taxonomic units (OTUs; Barghini *et al.*, 2014). This result is not surprising since, similar to other extreme environments, bacterial diversity of salterns is generally reported as low in the Mediterranean area and worldwide (Maturrano *et al.*, 2006; Baati *et al.*, 2008; Sabet *et al.*, 2009). Even though the low microbial diversity is widely confirmed in the literature, the available data was generally obtained by very short sampling periods (sometimes involving just a single sample) and could have limited statistical significance. By contrast, the low diversity recorded in our work acquires much more statistical value and ecological importance since samples were obtained during a very long sampling campaign. However, due to the isolation technique used in this work, the information is mainly related to copiotrophic bacteria.

### Salinity preferences for growth

Halophilic microorganisms are classified by function of their growth capability at different salinities. According to current definitions, microorganisms that demand salt for growth are referred to as halophiles. For instance, according to Margesin & Schinner (2001), it is possible to distinguish between slight- (optimal growth up to 30‰ NaCl), moderate- (optimal growth at 30–150‰), extreme- (optimal growth at 250‰) and borderline extreme-halophiles (needing at least 120‰). Ollivier *et al.* (1994) mentioned only three halophile groups: slight (optimal growth at 20-50 ‰ NaCl), moderate (optimal growth at 50–200‰) and extreme (optimal growth at 200-300‰). Non-halophilic microorganisms do not require salt for growth, while those that grow in the presence or absence of salt are defined as non-halophilic halotolerant (Kushner, 1978; Margesin & Schinner, 2001). Considering the salinity ranges for optimal growth, according to the above mentioned definitions (in particular, those of Margesin & Schinner, 2001), ST strains appeared equally distributed between halophiles (52%) and non-halophilic halotolerant (48%). Slight- and moderate-halophiles were 32% and 20% of strains, respectively. It is worth noting that no extreme-halophiles or non-halophiles were found.

nMDS analysis, carried out on growth data, clearly identified four main groups (G1-G4) distributed along the salinity gradient (Fig. 3). However, strain partition among the various groups did not completely correspond to the mentioned categories, since strains that belonged to the same category were distributed in different groups.

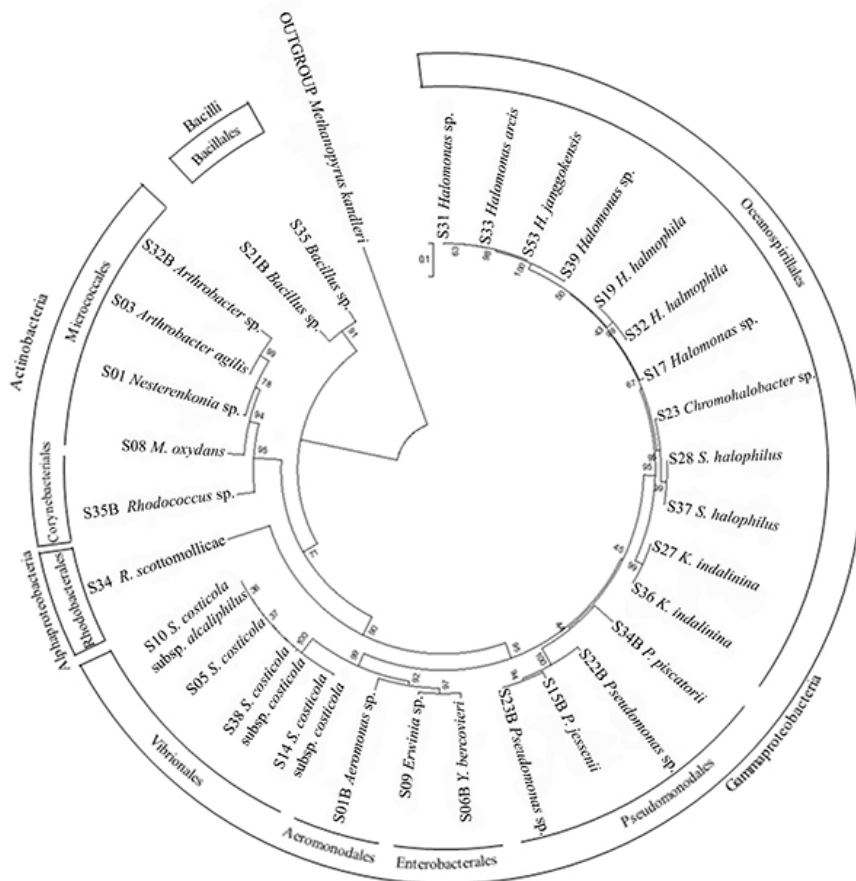
G1 gathered all halophiles (except *Ruegeria scottomollicae*) with a similarity of 72% (Bray-Curtis index).

**Table 1.** Taxonomical, morphological and biochemical characteristic of the strains isolated from different ST pools.

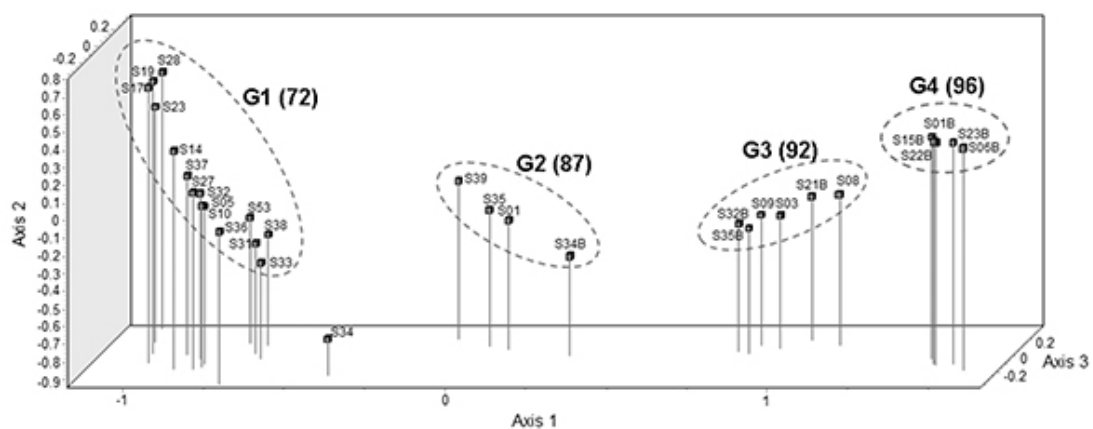
TAXA	Pool							Temp. Range (°C)	Sal. Range (‰)	Colony	Ox.	Accession number
	P01	P21	P27	P35	P44	CP	BL					
<b>Actinobacteria</b>												
<i>Arthrobacter agilis</i>	x	S03	x	x				5-35 (25)	0-80 (0)	P/C/S/C	+	KP715895
<i>Arthrobacter</i> sp.	S32B	x					x	5-30 (25)	0-40 (0)	P/I/S/C	+	KP756687
<i>Microbacterium oxydans</i>	x	S08	x					5-35 (20)	0-80 (0)	Y/C/S/C	-	KP715897
<i>Nesterenkonia</i> sp.			x	S01				0-40 (35)	0-40 (0)	Y/C/R/C	-	KP715894
<i>Rhodococcus</i> sp.	x	x	S35B					0-35 (30)	0-80 (0)	O/C/S/C	+	KP715893
<b>Alphaproteobacteria</b>												
<i>Ruegeria scottmollicae</i>	S34	x	x	x	x			5-35 (20)	40-120 (80)	W/C/S/R	+	KP715909
<b>Bacilli</b>												
<i>Bacillus</i> sp.	x						S35	10-40 (30)	0-160 (0)	P/C/S/R	+	KP715910
<i>Bacillus</i> sp.	S21B	x	x					0-40 (30)	0-80 (0)	P/C/S/R	+	KP756686
<b>Gammaproteobacteria</b>												
<i>Aeromonas</i> sp.	S01B	x						0-40 (35)	0-40 (0)	W/C/S/C	+	KP715888
<i>Chromohalobacter</i> sp.			S23	x	x			10-40 (30)	40-240 (40)	W/C/S/C	-	KP715903
<i>Erwinia</i> sp.	x	x	x	S09				10-35 (30)	0-80 (0)	P/C/S/R	-	KP715898
<i>Halomonas arcis</i>		x	x	S33				5-40 (25)	40-200 (80)	W/C/S/C	+	KP715908
<i>H. halmophila</i>				x	x	S19		5-40 (25)	40-280 (80)	C/C/S/C	+	KP715902
<i>H. halmophila</i>				x	S32	x		20-40 (35)	40-200 (80)	W/C/S/C	+	KP715907
<i>H. janggokensis</i>				x	x	S53		5-40 (30)	40-200 (40)	W/C/S/R	-	KP715922
<i>Halomonas</i> sp.				x	S31	x		5-35 (30)	40-200 (80)	C/C/S/R	-	KP715906
<i>Halomonas</i> sp.	x	x	x	x	x	S39		5-40 (35)	0-160 (40)	C/C/S/C	+	KP715914
<i>Halomonas</i> sp.		x	x	x	S17	x		0-40 (25)	40-280 (40)	W/C/S/C	+	KP715901
<i>Kushneria indalinina</i>		x	x	x	S27			10-35 (30)	40-200 (40)	O/I/S/C	-	KP715904
<i>K. indalinina</i>		S36	x	x				10-35 (30)	40-200 (40)	O/I/R/C	-	KP715911
<i>Pseudomonas jessenii</i>	x	S15B						0-40 (25)	0-40 (0)	Y/C/S/C	+	KP756685
<i>Pseudomonas</i> sp.	S22B							0-35 (30)	0-40 (0)	C/C/S/C	+	KP715890
<i>Pseudomonas</i> sp.	x	S23B						0-40 (30)	0-40 (0)	Y/C/S/C	+	KP715891
<i>Psychrobacter piscatorii</i>	S34B	x	x					0-35 (30)	0-160 (40)	Y/C/S/C	+	KP715892
<i>Salinivibrio costicola</i>			x	x	S05	x		10-40 (30)	40-240 (40)	C/C/S/C	+	KP715896
<i>S. costicola</i> subsp. <i>alcaliphilus</i>			x	x	x	S10		10-40 (30)	40-200 (40)	P/C/R/R	+	KP715899
<i>S. costicola</i> subsp. <i>costicola</i>			x	S14	x	x		10-40 (30-35)	40-240 (40)	C/C/S/C	+	KP715900
<i>S. costicola</i> subsp. <i>costicola</i>			S38	x	x			10-40 (30)	40-160 (80)	C/C/R/C	+	KP715913
<i>Salinicola halophilus</i>				x	x	S28		5-40 (30)	40-240 (80)	Y/C/S/C	-	KP715905
<i>Salinicola halophilus</i>			x	x	S37	x		5-40 (30)	40-200 (80)	C/I/S/C	-	KP715912
<i>Yersinia bercovieri</i>							S06B	0-40 (35)	0-40 (0)	W/I/S/C	+	KP715889

Legend

Pools: X = presence of strains in the various sampled pools; identification code indicated the strain stored in the DEB culture collection of marine micro-organisms (see Fig. 1 for details). Salinity and Temperature range of growth; optima in brackets. Colony, colony characteristics as follows: Colour: C = Cream; O = Orange; P = Pink; W = White; Y = Yellow. Shape: C = Circular; I = Irregular. Texture: S = Smooth; R = Rough. Elevation: C = Convex; R = Raised. Ox. = oxidase.



**Fig. 2:** Phylogenetic dendrogram of ST bacterial cultivable community. The evolutionary history was inferred using the maximum likelihood method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method. The rate variation among sites was modelled with a gamma distribution (shape = 0.38). The analysis involved thirty-two nucleotide sequences. All positions that contained gaps and missing data were eliminated. There was a total of 815 positions in the final dataset. Bootstrap values from 1,000 re-sampled data sets are shown. Strain affiliation to the different classes and orders is also reported.



**Fig. 3:** nMDS analysis, carried out on growth data (maximum colony diameters) at different salinities, for the ST strains. The plot shows strain distribution related to salinity preference. The main groups (G1-G4) are identified by the dashed ellipses, and Bray-Curtis similarity index values are reported in brackets.

nMDS showed that the difference between slight- and moderate-halophiles was very limited and not statistically significant ( $P = 0.37$ ), as confirmed by the ANOSIM test.

Most of strains in this group, beyond their optima, were markedly euryhaline; that is, they were able to grow from 40-80‰ up to 240-280‰ salinity (Fig. 4). Emblematic of this profile was strain S28, *Salinicola halophilus*, that grew from 40-240‰ with a very uncommon growth profile. It showed an optima at 80‰, with slight differences (<20%) in the range of 40-120‰. In addition, over 120‰ and up to 240‰ salt, its growth rate remained particularly high (ca. 75% of the optimum), a finding that indicated a wide and unusual adaptation to salinity variations. By contrast, a common growth profile was recorded for *S. halophilus* S37 in G1, which showed the same optima but reduced euryhalinism (40-200‰) and a typical growth reduction over the optima (Fig. 4B). These two strains could appear as duplicates of the same organism (Fig. 2), but due to slight variations in 16S rDNA sequences (data not shown) and very evident differences in their adaptation to salinity, they must be considered as different strains.

The separation between G3 and G4 evidenced differences within the category of non-halophilic halotolerants and suggests that it could be further subdivided into non-halophilic slight-halotolerants and non-halophilic halotolerants. The non-halophilic halotolerant species grouped in G3 grew optimally with no salt, but their growth at 40‰ salt was still quite high (70-80% of that recorded at 0‰); no growth was recorded above 80‰. By contrast, strains grouped in G4 showed lower halotolerance; they exhibited a dramatic growth reduction (70-90% of growth recorded at 0‰) at only 40‰ salt (Fig. 4A); no growth was recorded over 40‰.

Microorganisms grouped in G2 (S1, *Nesteronkonja* sp., S34B, *Psychrobacter piscatorii*; S35, *Bacillus* sp. and S39, *Halomonas* sp.) should be attributed to halotolerant bacteria since they did not strictly require salt for growth. However, they have intermediate characteristics of halotolerant and halophiles and their attribution is quite difficult. Since their optima was 40‰ salt, considering these strains as “halotolerants” would be incongruous with the concept of “tolerance”: they benefit from the saline environment and just tolerate salt absence (Fig. 4A). These features are more characteristic of halophilic microorganisms; thus, we suggest that it would be more appropriate to classify them as “norm-tolerant halophiles”. The differences between these strains and all the others were confirmed by nMDS that grouped them separately. It is worth noting that the attribution of the nMDS groups to the various ecological categories (G1, halophiles; G2, halophilic norm-tolerants; G3, non-halophilic halotolerants; G4, non-halophilic slightly halotolerants) was statistically significant, as confirmed by ANOSIM analysis ( $P = 0.001$ ).

The statistical elaborations discussed above were performed to correlate strain distribution to the ecological category (salinity). Moreover, an additional nMDS anal-

ysis, on taxonomical distances, was performed on 16S rDNA sequences (data not shown). Subsequently, a generalised Procrustes rotation analysis, performed to check the correlation between the two multivariate ordinations, indicated significant congruence (Protest,  $P = 0.007$ ). Indeed, considering the ST strain taxonomy in relation to salinity groups, 99% of Oceanospirillales and all Vibrionales displayed a halophilic habitus (G1); 80% of Micrococcales were included in G3 (halotolerant) and 80% of Pseudomonadales were included in G4 (non-halophiles). On the contrary, no correlation with taxonomical groups was recorded for G2, since each member belonged to different families.

It is worth noting that the majority of ST strains belonged to species generally found in other marine salterns, and most of them showed broad euryhalinism. This feature appears to be a winning strategy to cope with the very high salinity variations recorded in ST pools, due to its current management, as discussed above.

### Temperature preferences for growth

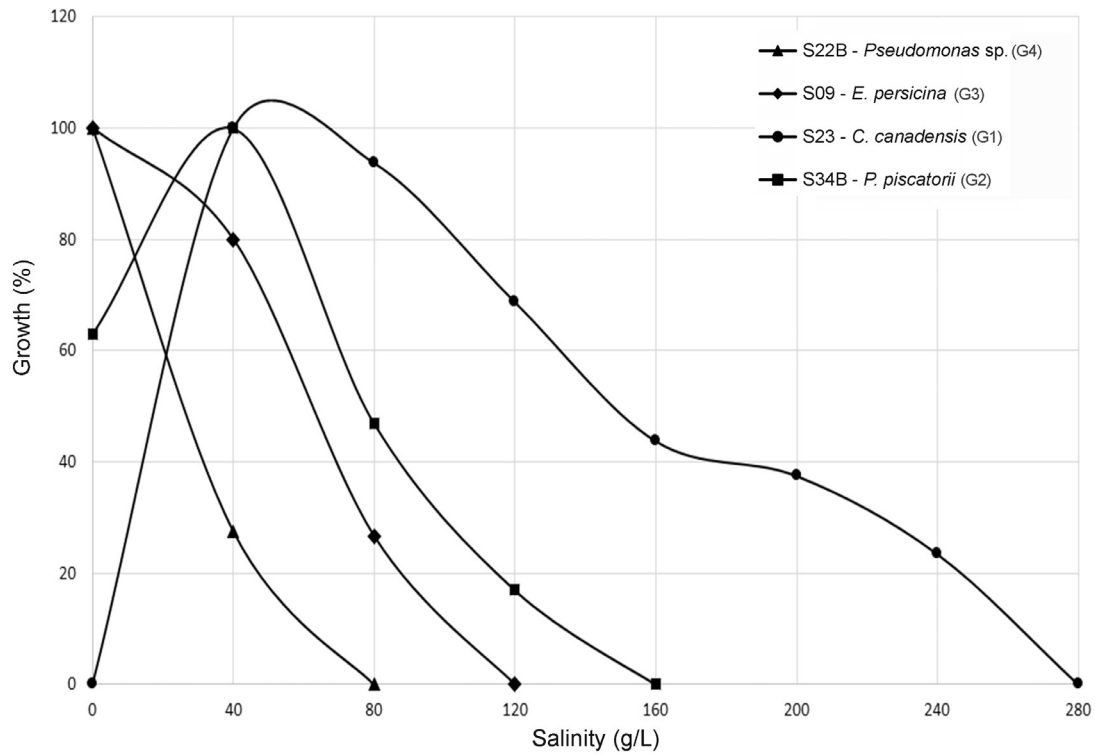
With regards to growth at different temperatures, nMDS analysis revealed a scarcely defined data structure (stress = 0.1); no correlation with temperature ecological categories (ANOSIM,  $P > 0.05$ ) or taxonomical or salinity distributions (Protest,  $P > 0.5$ ) was recorded.

Due to the increased consideration of extreme environments and consequent discovery of unusual microorganisms, traditional classifications have been re-discussed. In a recent study that dealt with the thermal preferences of Arctic bacterial strains, Pesciaroli *et al.* (2012) widely contributed to the debate on this topic and tried to update current definitions. Briefly, psychrotolerant bacteria were defined as those able to grow at 0°C with their optima in the range of 15 - 25°C; mesophilic-psychrotolerant bacteria (those that grow at c.a. 0°C with their optima in the range of 25 - 40°C) and mesophilic bacteria (those with their optima in the range 25 - 40°C, but do not grow below c.a. 10°C). Accordingly, most ST strains (Fig. 5) were mesophiles (ca. 52%), some mesophilic-psychrotolerant bacteria (ca. 23%) were found, while psychrotolerant bacteria comprised 25% of the strains. We observed diffuse eurythermism, a finding that indicates adaptation to the wide temperature variations recorded in this peculiar environment. Moreover, some ST strains (S08, 15B, S17, S19, S33, S34, 34B and S36) showed a very uncommon profile (Fig. 5B) where optimal conditions appeared quite undetermined with no significant growth differences over a broad range of temperatures (15-35°C). Noteworthy, S19, *Halomonas halmophila*, grew from 5 to 45°C and probably far beyond (Fig. 5B). The halophilic strains S28 and S37 of *S. halophilus* that showed very different salinity growth curves displayed identical mesophilic-psychrotolerant profiles.

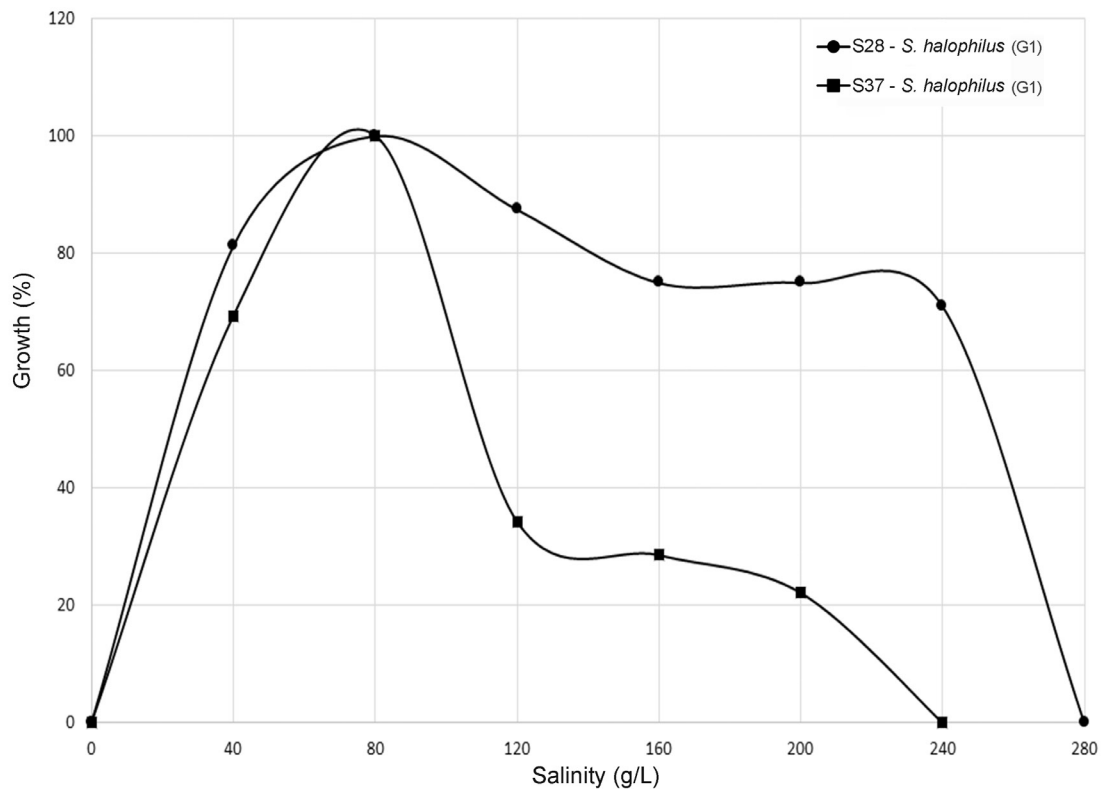
It is worth noting that the growth profiles at different temperatures presented in this study were very similar to those documented for other extreme environments sub-



A

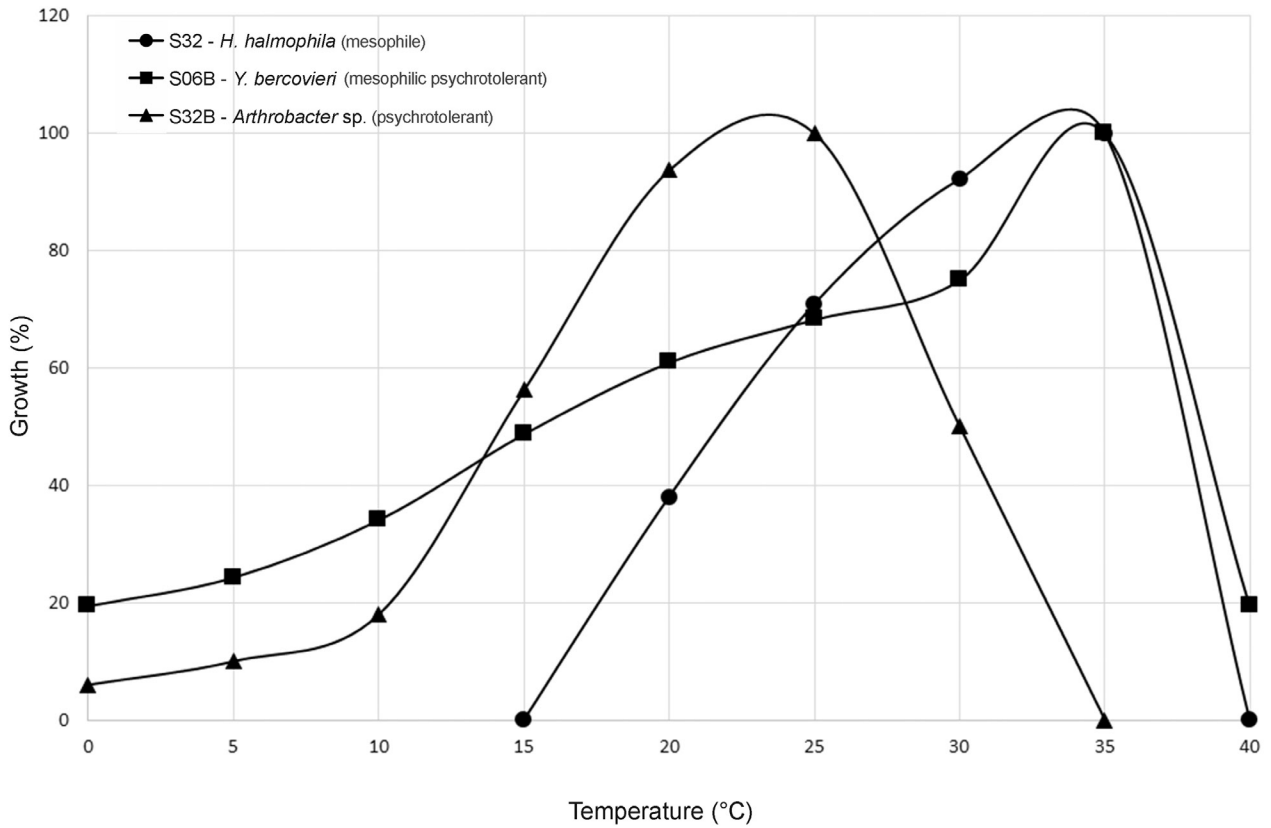


B

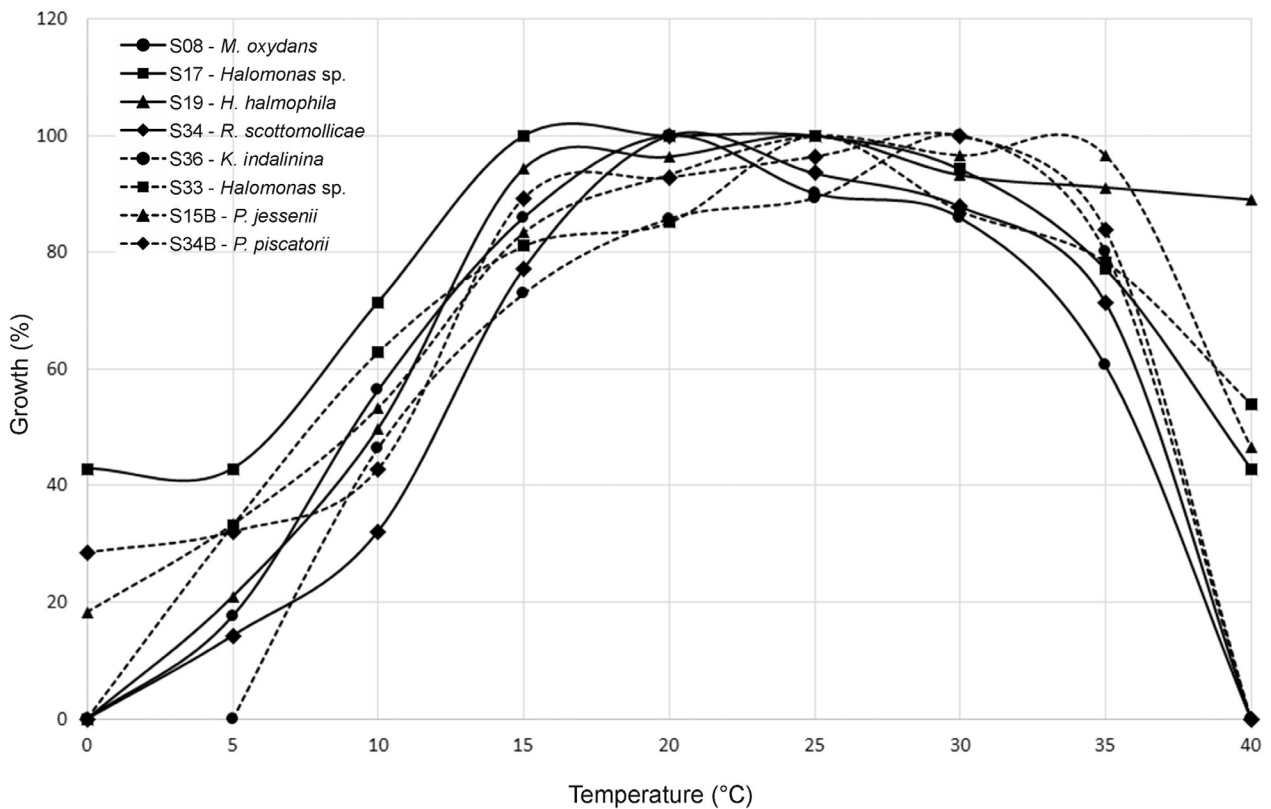


**Fig. 4:** Growth profiles of some ST strains at different salinities (0-320‰). To obtain normalised values, growth is reported as percentage of the maximum colony diameter. A: profiles of prototypical strains that belong to different categories of salinity preferences. B: profiles of strains S28 and S37 of *Salinicola halophilus*. Growth was measured by image analysis as the average increase of colony diameter. Data are the mean of three replicates; standard deviation was less than 10%.

A



B



**Fig. 5:** Growth profiles of some ST strains at different temperatures in the range 0-40°C. To obtain normalised values, growth is reported as percentage of the maximum colony diameter. A: profiles of prototypical strains that belong to different categories of temperature preferences. B: uncommon profiles of various strains that showed broad eurythermism. Growth was measured by image analysis as the average increase of colony diameter. Data are mean of three replicates; standard deviation was less than 10%.

mitted to intense temperature variations. Pesciaroli and co-workers (2012) reported analogous trends for various bacteria isolated from an Arctic sub-extreme environment characterised by very wide thermal excursions; thus, the uncommon growth profiles discussed above appear to be related to an adaptive strategy that would be associated with frequent and intense thermal excursions rather than to a specific environment.

Fig. 6 shows strain distribution considering both their preference for salinity and temperature. Halophiles and non-halophilic halotolerants were found in all temperature categories, while mesophilic psychrotolerant strains were found in all salinity categories. Mesophilic strains that exhibited halophilic behaviour were the most numerous group.

Comparison of our results with those of others scientists is not easy. Most of the available papers deal with a single strain or a limited group of strains. In addition, the sample sites investigated by other authors are

generally located in regions with very different environmental features and, consequently, strains that belong to the same species but isolated from different sites could show different adaptations.

Table 2 compares some ST strains with others that belong to same species but were isolated in different climatic areas regarding their adaptation to salinity and temperature. In general, the minimal amount of salt required for ST strains seems to be higher than that recorded for other strains. In particular, the ability of *Halomonas* spp. to grow with very limited amounts of NaCl (0-5‰) (Bouchotroch *et al.*, 2001; Duckworth *et al.*, 2000; Xu *et al.*, 2007) was not confirmed by our results. Indeed, we found that all ST *Halomonas* strains required a minimum of c.a. 40‰ salt and none was able to grow without salt, as reported by Xu *et al.* (2007) for *H. arcis*. The same considerations could be made for the various ST species of *Salinivibrio costicola*; their growth always required salt and no growth was recorded at 0‰ NaCl, as previ-

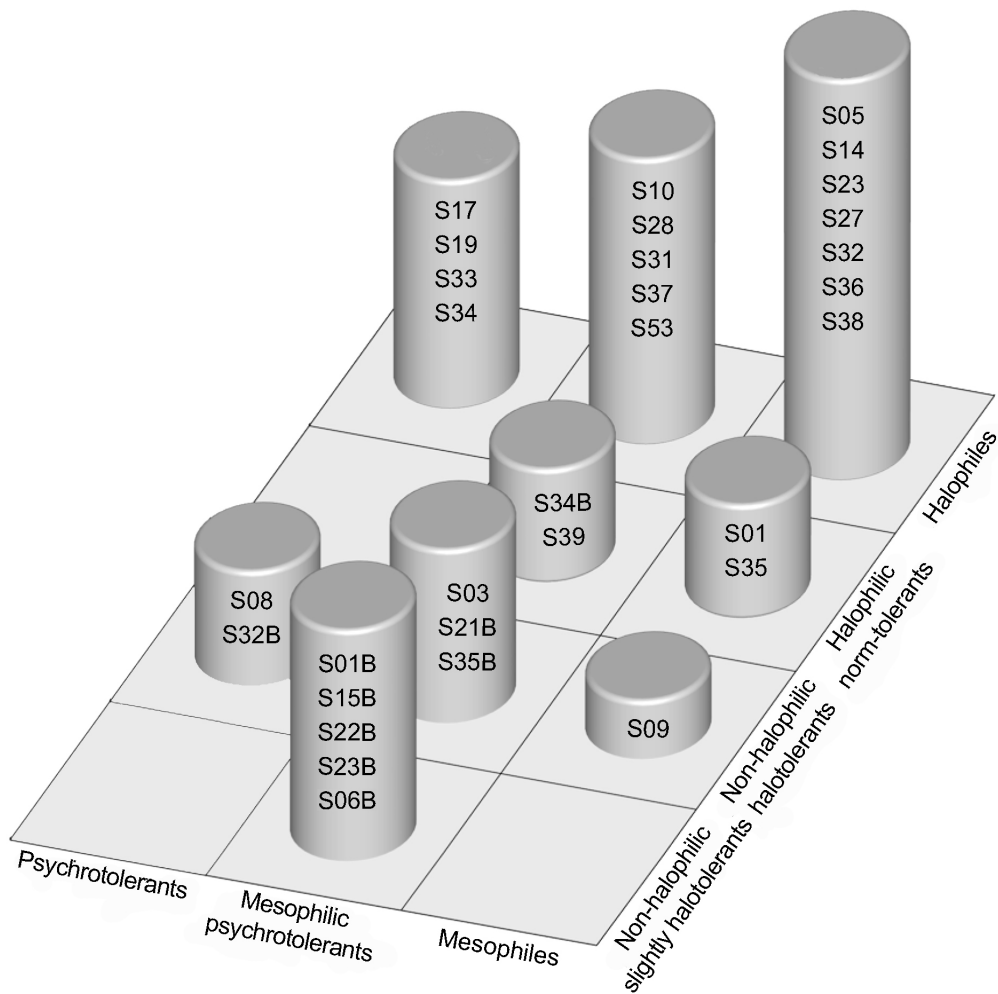


Fig. 6: Distribution of ST strain growth preferences with regards to salinity and temperature categories.

**Table 2.** Comparison between some ST strains and other similar species regarding their adaptation to temperature and salinity variations.

Strain	Taxa	Temperature (°C)		Salinity (‰)		Reference
		ST strain	Literature	ST strain	Literature	
S34	<i>Ruegeria scottomollicae</i>	5-35 (20)	4-40 (ND)	40-120 (80)	10-150 (ND)	Vandecandelaere, 2008
S19	<i>Halomonas halmophila</i>	5-40 (25)	15-45 (37)	40-280 (80)	5-300 (75)	Duckworth et al., 2000; Bouchotroch et al., 2001
S32	<i>H. halmophila</i>	20-40 (35)	15-45 (37)	40-200 (80)	5-300 (75)	
S33	<i>H. arcis</i>	5-40 (25)	4-48 (30)	40-200 (80)	0-200 (10-50)	Xu et al., 2007
S53	<i>H. janggokensis</i>	5-40 (30)	5-45 (25-30)	40-200 (40)	10-200 (100-150)	Kim et al., 2007
S27	<i>Kushneria indalinina</i>	10-35 (30)	15-40 (ND)	40-200 (40)	30-250 (75-100)	Cabrera et al., 2007
S36	<i>K. indalinina</i>	10-35 (30)	15-40 (ND)	40-200 (40)	30-250 (75-100)	
S34B	<i>Psychrobacter piscatorii</i>	0-35 (30)	0-30(25)	0-160 (40)	0-150 (ND)	Yumoto et al., 2010
S05	<i>Salinivibrio costicola</i>	10-40 (30)	5-50(ND)	40-240 (40)	0-200 (ND)	Huang et al., 2000
S10	<i>S. costicola</i> subsp. <i>alcaliphilus</i>	10-40 (30)	10-40 (30)	40-200 (40)	20-250 (ND)	Romano et al., 2006
S14	<i>S. costicola</i> subsp. <i>costicola</i>	10-40 (30-35)	5-50 (ND)	40-240 (40)	0-200 (ND)	Huang et al., 2000
S38	<i>S. costicola</i> subsp. <i>costicola</i>	10-40 (30)	5-45 (37)	40-160 (80)	5-200 (100)	
S28	<i>Salinicola halophilus</i>	5-40 (30)	15-45 (35)	40-240 (80)	30-250 (70-100)	De la Haba et al., 2010
S37	<i>S. halophilus</i>	5-40 (30)	15-45 (35)	40-200 (80)	30-250 (70-100)	

Legend: Values in column separated by “-” indicate the range for growth, while optima are reported in brackets. ND, not determined.

ously reported (Huang *et al.*, 2000; Romano *et al.*, 2005).

With regards to temperature adaptation, for some species the differences between our work and others were even more evident. Various ST strains showed a more manifest psychrophilic attitude than could be strictly related to the sampling site and its climate characteristics. The “Saline di Tarquinia” salterns are located in the North Tyrrhenian Sea (Central Italy) further north of various other Mediterranean marine salterns. Sometimes in the winter, very cold periods could be recorded, including a few days with ice formation and snow. Various strains showed lower optima than those reported in the literature and their growth range was shifted downward. For example, the ST strains of *S. halophilus* showed growth in the range of 5-40°C with an optima at 30°C. The strain studied by De la Haba *et al.* (2010), isolated from a solar saltern in southern Spain (Cabo de Gata, Almeria, c.a. 600 km south of ST) (Aguilera *et al.*, 2007), had optimal growth at 35°C and a growth range between 15-45°C. Strain S19 of *H. halmophila* (growth range 5-40°C; optima at 25°C) revealed a clear psychrotolerant behaviour in

contrast with the mesophilic characteristics of the strain studied by Duckworth *et al.* (2000) that was isolated from an African soda lake (optima at 37°C and growth from 20-45°C).

## Conclusions

This study represents the first attempt to supply a physiological characterisation of the “Saline di Tarquinia” bacteria during a wide sampling campaign. The results could have both ecological value and a potential biotechnology interest. Microorganisms that display very broad euryhalinism and/or eurythermism could be studied as prototypical examples of adaptation to some global change phenomena since various environments are subjected to alternate and opposite stress conditions (i.e., temperature and water availability). In addition, they could be employed in biotechnological processes, where new strains and/or new microbial products are required. Since adaptation to salinity and temperature variations is related to the production of adapted enzymes, activ-

ity over a broad range of conditions could indicate the microorganisms and their enzymes for use in industrial applications that deal with the bio-treatment of effluents that contain high and sometimes variable amount of salts that are generated by many different sources and cause environmental problems. Thus, even if studies in this sense must be carried out, the ST bacteria could represent a fascinating fount of new biodiversity. It is worth noting that in this work, regarding the salinity preferences of the investigated strains, we observed what has been recently stated for other bacteria in relation to their adaptation to temperature: classical definitions of ecological categories are unsatisfying to describe the microbial biodiversity and its strategies to cope with the environmental stress. This is particularly true for those microorganisms adapted to extreme environments.

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