Appl Microbiol Biotechnol (2010) 86:1525–1534
DOI 10.1007/s00253-009-2424-7

A preliminary investigation on the growth requirement for monovalent cations, divalent cations and medium ionic strength of marine actinomycete *Salinispora*

Ginger Tsueng · Kin Sing Lam

Received: 20 August 2009 / Revised: 13 December 2009 / Accepted: 24 December 2009 / Published online: 19 January 2010

Abstract In this paper, we report that three species of *Salinispora*, *S. arenicola*, *S. tropica*, and *S. pacifica*, require magnesium and calcium, for growth, with *S. pacifica* having the most stringent growth requirement for these ions. Interaction between these ions in supporting the growth of *Salinispora* was observed. We also demonstrated that the absolute requirement of sodium to support the growth of *Salinispora* has not been established as all three species of *Salinispora* can use either potassium or lithium to replace sodium to support maximum growth. While lithium can replace sodium to support maximum growth of *Salinispora*, it is more toxic to *S. arenicola* than *S. tropica* and *S. pacifica*, inhibiting the growth of *S. arenicola* at 189 mM but without effect on the growth of *S. tropica* and *S. pacifica*. Using both sodium chloride-based and lithium chloride-based media, we showed that *Salinispora* has a growth requirement for divalent ions, magnesium and calcium as well as growth requirement for ionic strength (8.29 to 15.2 mS/cm). *S. arenicola* has a lower growth requirement for ionic strength than *S. tropica* and *S. pacifica*.

Keywords *Salinispora arenicola* · *Salinispora tropica* · *Salinispora pacifica* · Monovalent cations · Divalent cations · Medium ionic strength

G. Tsueng · K. S. Lam (✉)
Nereus Pharmaceuticals, Inc,
10480 Wateridge Circle,
San Diego, CA 92121, USA
e-mail: rlam@marronebio.com

Present Address:
K. S. Lam
Marrone Bio Innovations,
2121 Second Street, Suite B-107,
Davis, CA 95618, USA

Introduction

Since sodium is the most abundant ion present in seawater, it is not surprising that the specific requirement of sodium for growth has been implicated as a primary characteristic of marine microorganisms, although the quantity, specificity, stability, uniqueness, and function of the sodium requirement have been topics of much debate (MacLeod 1965; Pratt 1974; Imhoff 2001). The observation that some marine bacteria possess three types of primary sodium pumps which they use to perform various cellular functions led to the suggestion that the presence of a primary sodium pump is one of the criteria for the definition of marine bacteria (Oh et al. 1991; Unemoto et al. 1992; Kogure 1998; McCarter 2001). However, the requirement of sodium is not unique to marine microorganisms, and several terrestrial prokaryotes and unicellular eukaryotes have been found to require it for growth (Bryant et al. 1959; Sistrom 1960; Larsen 1962; Goldman et al. 1963; Sakazaki et al. 1963; Hunter 1972; Caldwell et al. 1973; Caldwell and Hudson 1974; Chun et al. 2000). A primary sodium pump was also detected in the terrestrial prokaryotes *Bacillus* FTU, *Escherichia coli*, and *Haemophilus influenzae* RD (Avetisyan et al. 1991; Fleischmann et al. 1995; Hayashi et al. 1996; Häse et al. 2001) which have no apparent growth requirement for sodium. Furthermore, marine bacteria that possess a primary sodium pump, but do not require sodium for growth, have been isolated (Oh et al. 1991; Fujiwara-Nagata and Eguchi 2004).

In addition to sodium, marine bacteria have requirements for other ions, such as potassium, magnesium, and calcium for growth (MacLeod 1965, 1968, 1971; Unemoto et al. 1973; Nakamura et al. 1992). Sodium, potassium, magnesium, and calcium all have been shown to play a role in maintaining the integrity of the cells and to provide a

suitable osmotic pressure to support the growth of marine bacteria (Johnson and Harvey 1938; MacLeod and Matula 1961, 1962; MacLeod 1965; Unemoto et al. 1973). Only a limited amount of effort has been spent in the investigation of potassium, magnesium, and calcium on both nutritional growth requirement and osmotic effect on the growth of marine bacteria. Since potassium, magnesium, and calcium are the major cations with important cellular functions for all prokaryotes (Wackett et al. 2004), a thorough examination of the nutritional requirement for these ions, in addition to sodium, to support the growth of marine bacteria is required before we can fully understand the growth requirements for these ions of marine bacteria.

Most of the data available on the growth requirement for ions of marine bacteria were obtained from Gram-negative organisms. Data available in describing the growth requirement for ions of Gram-positive marine bacteria are very limited (Rürgen and Hentzschel 1980) and completely lacking for marine actinomycetes. The exploration of marine actinomycetes as a source for novel drug leads has been the focus of several recent review articles, and the tremendous diversity of marine actinomycetes is undisputable (Lam 2006; Fenical and Jensen 2006; Ward and Bora 2006; Bull and Stach 2007; Jensen and Lauro 2008). A recently described marine actinomycete genus, *Salinispora* (Maldonado et al. 2005), has been shown to be a prolific microorganism for the production of bioactive secondary metabolites (Fenical and Jensen 2006; Oh et al. 2006; Udwary et al. 2007; Oh et al. 2008). Two species of *Salinispora*, *Salinispora arenicola*, and *Salinispora tropica*, have been formally described (Maldonado et al. 2005), while the name of the third species, *Salinispora pacifica*, has been proposed (Oh et al. 2006). One of the unique characteristics of *Salinispora* is the seawater growth requirement, more specifically, a requirement of sodium for growth, reported in its original description as MAR 1 (Mincer et al. 2002) and in the formal taxonomic description (Maldonado et al. 2005). However, the roles of potassium, magnesium, and calcium in supporting the growth of *Salinispora* were not investigated in the above studies.

We recently determined that three strains of *S. tropica*, including the type strain CNB440, do not have an apparent sodium requirement for growth as these three strains grew well in the medium M1 used by Mincer et al (2002) supplemented with salt formulation containing no sodium salt (Tsueng and Lam 2008a, b). The three strains of *S. tropica* require a specific combination of salts to provide a balance of salts and maintain a high enough ionic strength for growth (Tsueng and Lam 2008a, b). In the present study, we expand our investigation on the growth requirement for ions of the other two species of *Salinispora*, *S. arenicola* and *S. pacifica*. We also compare the effects of sodium, potassium, magnesium, and calcium, as well as medium ionic strength on the growth of the three species of *Salinispora*.

Materials and methods

Growth analysis by dry cell weight (DCW), culturing conditions for shake flask, inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) analysis of ions in media and determination of conductivity of media were described in Tsueng et al. (2008) and Tsueng and Lam (2008b). The ICP-DRC-MS analysis and measurement of conductivity were performed on autoclaved media. Growth analysis by DCW was determined in the third growth stage of the cultures to reduce the carryover effect of the undesirable ions from the freeze stocks and the first growth stage of the cultures (Tsueng and Lam 2008b).

Microorganisms

The type strains *S. arenicola* CNH643 and *S. tropica* CNB440 (Maldonado et al. 2005), and the industrial strain *S. tropica* NPS21184 for the manufacturing of proteasome inhibitor NPI-0052 (Tsueng et al. 2008) were deposited with the American Type Culture Collection (ATCC) and assigned the accession numbers ATCC BAA-917^T, ATCC BAA-916^T, and PTA-6685, respectively. *S. arenicola* NPS14034 was isolated from an alga sample collected in Florida. *S. pacifica* NPS14029 was isolated from a sediment sample collected in Hawaii. *S. pacifica* NPS14565 was isolated from a sediment sample collected in Guam. Strains *S. arenicola* NPS14034, *S. pacifica* NPS14565, and *S. pacifica* NPS14029 were deposited with Agricultural Research Service Culture Collection and assigned the accession numbers NRRL B-24795, NRRL B-24796, and NRRL B-24797, respectively. The 16S rRNA sequences of *S. arenicola* CNH643, *S. tropica* CNB440, and *S. tropica* NPS21184 were deposited to GenBank and their accession numbers were reported previously (Maldonado et al. 2005; Tsueng et al. 2008). The 16S rRNA sequences of *S. arenicola* NPS14034, *S. pacifica* NPS14029, and *S. pacifica* NPS14565 were deposited to GenBank with the accession numbers FJ528660, FJ528662, and FJ528657, respectively.

Salt formulations

Salt formulation 1 (SF1), a potassium chloride-based salt formulation, consists of the following per liter of deionized water: 30 g of KCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 0.43 g of $CaCO_3$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 208 μg of $CoCl_2 \cdot 6H_2O$, and 2.6 mg of KF.

Salt formulation 2 (SF2), a sodium chloride-based salt formulation, consists of the following per liter of deionized water: 24 g of NaCl, 0.69 g of KCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 0.43 g of $CaCO_3$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 208 μg of $CoCl_2 \cdot 6H_2O$ and 2.6 mg of KF.

Salt formulation 3 (SF3), a lithium chloride-based salt formulation, consists of the following per liter of deionized water: 5 g of LiCl, 0.69 g of KCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 0.43 g of $CaCO_3$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 208 μg of $CoCl_2 \cdot 6H_2O$ and 2.6 mg of KF.

Salt formulation 4 (SF4) consists of the following per liter of deionized water: 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 0.43 g of $CaCO_3$, 21.5 mg of H_3BO_3, and 15.5 mg of $SrCl_2$.

The composition and the preparation of the Sieburth salt formulation was described by Sieburth (1979). The Sieburth salt formulation consists of the following per liter of deionized water: 21.4 g of NaCl, 0.605 g of KCl, 9.68 g of $MgCl_2 \cdot 6H_2O$, 1.02 g of $CaCl_2$, 3.47 g of Na_2SO_4, 0.17 g of $NaHCO_3$, and 0.14 mg of Na_2HPO_4.

K-Sieburth salt formulation is a modified salt formulation of Sieburth salt formulation by replacing the sodium salts with equimolar potassium salts. The K-Sieburth salt formulation consists of the following per liter of deionized water: 27.9 g of KCl, 9.68 g of $MgCl_2 \cdot 6H_2O$, 1.02 g of $CaCl_2$, 4.26 g of K_2SO_4, 0.20 g of $KHCO_3$, and 0.17 mg of K_2HPO_4.

Salt formulation NaCl consists of 24 g of NaCl per liter of deionized water. Salt formulation KCl consists of 30 g of KCl per liter of deionized water. Salt formulation LiCl consists of 5 g of LiCl per liter of deionized water.

Growth media

Medium M1 consists of the following ingredients per liter of deionized water or salt formulation: 10 g of starch (USB), 2 g of peptone (USB), and 4 g of yeast extract (USB). Medium M1 has the same composition as medium A1 reported in our previous studies (Tsueng and Lam 2008a, b). Medium SHY consists of the following ingredients per liter of deionized water or salt formulation: 10 g of starch (USB), 4 g of Hy Soy (Kerry Biosciences), and 4 g of yeast extract (USB). Agar media have the same composition as the liquid media with addition of 17 g/l of agar (Difco) as a solidifying agent.

Agar cultures

Sterile inoculation loops were used to transfer 20 μl of cultures to agar plates (60×15 mm) containing 10 ml of agar media. After inoculation, the edges of the agar plates were wrapped with parafilm to reduce evaporation. The agar plates were incubated at 28 °C for 2 to 8 weeks to observe growth.

Results

Salinispora strains used in this study

The type strains *S. arenicola* CNH643 and *S. tropica* CNB440 (Maldonado et al. 2005), the industrial strain *S. tropica* NPS21184 for the manufacturing of proteasome inhibitor NPI-0052 (Tsueng et al. 2008), and three strains from Nereus Culture Collection, *S. arenicola* NPS14034, *S. pacifica* NPS14029, and *S. pacifica* NPS14565, were selected for this study. The phylogenic analysis of these six strains, based on their close to complete 16S rRNA sequences, is shown in Fig. 1. Since there is no type strain of *S. pacifica* currently available, the sequences of three reference strains of *S. pacifica* obtained from GenBank were also included in the phylogenic analysis. Phylogenic analysis shows that *S. arenicola* NPS14034 and *S. tropica* NPS21184 match closely to their corresponding type strains *S. arenicola* CNH643 and *S. tropica* CNB440, respectively. The two *S. pacifica* strains NPS14029 and NPS14565 also match closely to the three reference strains.

Growth of *S. tropica*, *S. arenicola*, and *S. pacifica* in M1 liquid medium containing either sodium-, potassium-, or lithium-based salt formulation

We reported in our earlier study that three strains of *S. tropica*, including the type strain CNB440, do not require seawater for growth but require a specific combination of salts and a high enough ionic strength for growth (Tsueng and Lam 2008b). In the present study, we examined the growth yield of two strains each of *S. arenicola*, *S. tropica,* and *S. pacifica* in submerged cultures grown in starch-yeast extract-peptone (M1) media supplemented with either sodium-, potassium-, or lithium-based salt formulations. All six strains grew well with similar growth yields as determined by DCW (4.46 to 6.63 mg/ml) in all three media M1.SF1 (potassium chloride-based), M1.SF2 (sodium chloride-based) and M1.SF3 (lithium chloride-based) (Table 1).

Growth of *S. tropica*, *S. arenicola*, and *S. pacifica* on M1 agar media containing different salt formulations

Since the same M1-based medium was used in both the study of Mincer et al. (2002) and the current study, the discrepancy in the observation of the seawater growth requirement of the *Salinispora* strains between the two

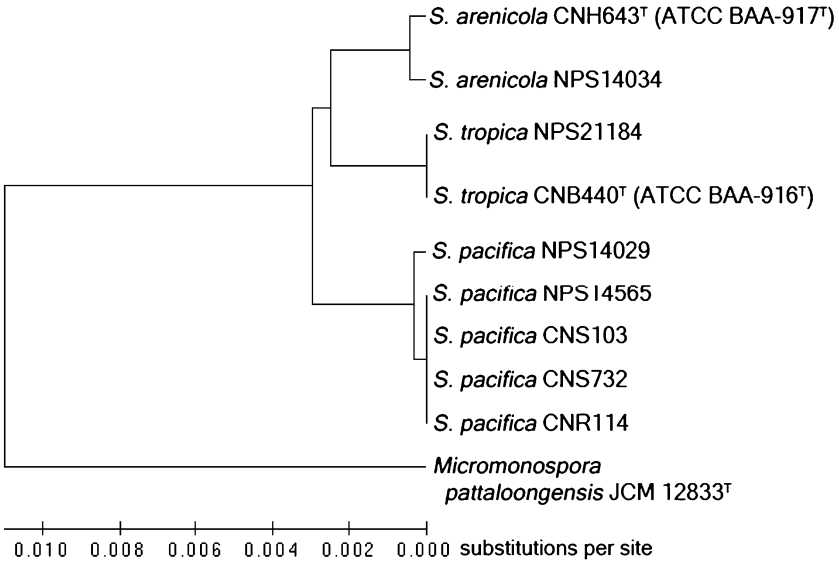

Fig. 1 Phylogenic tree of type strains, reference strains and strains used in this study. Neighbor-joining tree (Saitou and Nei 1987) based on nearly complete 16S rRNA gene sequences showing relationships between *Salinispora* strains

studies might be due to the use of different salt formulations and types of fermentations (solid versus submerged). In order to examine these possibilities, we determined the growth of the six *Salinispora* strains on M1-based agar media supplemented with different salt formulations and the results are shown in Table 1. All six *Salinispora* strains grew well on the sodium-based M1.Sieburth agar medium, as well as on the potassium-based M1.K-Sieburth, M1.SF1 (potassium chloride-based) and M1.SF3 (lithium chloride-based) agar media. The growth rate and yield of these six *Salinispora* strains on the above four agar media were similar, achieving maximum growth within two weeks of incubation. All six *Salinispora* strains did not grow on M1 agar medium prepared in deionized water or supplemented only with NaCl, KCl, or LiCl (Table 1), indicating that these *Salinispora* strains require a combination of ions, such as those present in salt formulations of SF1, SF2, SF3, and Sieburth, for growth.

Determination of monovalent and divalent cation concentrations in various media

In order to further understand the critical concentrations of sodium, potassium, calcium, and magnesium in supporting

Table 1 Growth of *Salinispora tropica*, *Salinispora arenicola*, and *Salinispora pacifica* in M1 liquid and agar media supplemented with different salt formulations

Media type	*S. tropica* CNB440	*S. tropica* NPS21184	*S. arenicola* CNH643	*S. arenicola* NPS14034	*S. pacifica* NPS14029	*S. pacifica* NPS14565
M1. SF1 liquid medium[a] (KCl-based)	5.97 mg/ml	6.63 mg/ml	5.63 mg/ml	5.74 mg/ml	4.46 mg/ml	4.92 mg/ml
M1. SF2 liquid medium[a] (NaCl-based)	6.45 mg/ml	6.31 mg/ml	5.81 mg/ml	5.38 mg/ml	4.72 mg/ml	5.14 mg/ml
M1. SF3 liquid medium[a] (LiCl-based)	6.23 mg/ml	6.18 mg/ml	5.68 mg/ml	5.30 mg/ml	5.16 mg/ml	5.25 mg/ml
M1.SF1 agar medium[b] (KCl-based)	+++	+++	+++	+++	+++	+++
M1.SF2 agar medium[b] (NaCl-based)	+++	+++	+++	+++	+++	+++
M1.SF3 agar medium[b] (LiCl-based)	+++	+++	+++	+++	+++	+++
M1.Sieburth agar medium[b] (sodium-based)	+++	+++	+++	+++	+++	+++
M1.K-Sieburth agar medium[b] (potassium-based)	+++	+++	+++	+++	+++	+++
M1.DI.agar medium[c]	–	–	–	–	–	–
M1.NaCl.agar medium[c]	–	–	–	–	–	–
M1.KCl.agar medium[c]	–	–	–	–	–	–
M1.LiCl.agar medium[c]	–	–	–	–	–	–

Key for growth measurement: – no growth, –/+ poor growth, + fair growth, ++ good growth, +++ very good growth

[a] Dry cell weight of the third-stage growth of the culture was determined after 5-day incubation at 28 °C and 250 rpm on a rotary shaker

[b] Growth of agar cultures of the second transfer was recorded after 3-week incubation at 28 °C

[c] Growth of agar cultures of the second transfer was recorded after 8-week incubation at 28 °C

the growth of different species of *Salinispora*, ICP-DRC-MS analysis was used to determine the amounts of the above four ions present in various media containing different salt formulations. While no discrete ions were added to medium M1.DI prepared in deionized water, medium M1.DI contains 5.0 mM sodium, 4.3 mM potassium, 0.16 mM calcium, and 0.21 mM magnesium based on ICP-DRC-MS analysis (Table 2). The metal ions present in medium M1.DI are derived from the complex medium components peptone and yeast extract (Tsueng and Lam 2008a). The concentrations of the above four ions represent the basal levels of these ions in the medium. The amount of sodium present in potassium chloride-based medium M1.SF1 (5.2 mM) and lithium chloride-based medium M1.SF3 (4.9 mM) is the same as the M1.DI medium, and is ~1% of the sodium content (487 mM) in seawater (Table 2). All six *Salinispora* strains grew well in both liquid and agar M1.SF1 and M1.SF3 media.

The basal level of potassium in the M1.DI medium is slightly lower than the basal level of sodium, at 4.3 mM, approximately 40% of the concentration of potassium in seawater (Table 2). The concentration of potassium present in potassium chloride-based M1.SF1 is 467 mM, similar to the concentration of sodium in seawater (Table 2). The concentrations of the divalent cations, calcium and magnesium, present in media M1.DI, M1.NaCl and M1.KCl are relatively low, at 0.16–0.18 and 0.20–0.21 mM, respectively (Table 2). The concentrations of calcium and magnesium in the potassium chloride-based M1.SF1 medium and seawater are 16–69-fold and 95–290-folds higher than in media M1.DI, M1.KCl, and M1.NaCl, respectively. None of the six *Salinispora* strains grew in M1.DI, M1.NaCl, and M1.KCl media (Table 1).

Examination of the effect of potassium, calcium, and magnesium on the growth of *S. tropica*, *S. arenicola*, and *S. pacifica*

We examined the growth of the two type strains *S. arenicola* CNH643 and *S. tropica* CNB440, and *S. pacifica* NPS14029 grown in a sodium chloride-based M1.SF1 media by varying the concentrations of potassium, calcium, and magnesium in the salt formulation in submerged cultures. As observed in agar cultures, *S. arenicola* CNH643, *S. tropica* CNB440, and *S. pacifica* NPS14029 did not grow in M1 medium supplemented only with 411 mM sodium in submerged cultures (Table 3). No growth of these three strains was observed even with the addition of 9.3 mM potassium in the medium, demonstrating the importance of the divalent cations, calcium, and magnesium, for supporting the growth of *Salinispora* (Table 3). The basal levels of calcium (0.16 mM) and magnesium (0.21 mM) present in the medium M1 (Table 2) were not enough to support the growth of *Salinispora*. We further examined the effect of different concentrations of added calcium in the salt formulations on the growth of *S. arenicola* CNH643, *S. tropica* CNB440, and *S. pacifica* NPS14029 grown in M1 medium containing 411 mM sodium, 9.3 mM potassium, and 17.5 mM magnesium in the salt formulation (i.e., varying the concentrations of calcium in salt formulation SF2). All three strains grew in this M1 medium without addition of calcium to the salt formulation, yielding DCW from 2.98 to 4.21 mg/ml (Table 3). We observed a trend of increasing growth yield of *S. pacifica* NPS14029 with increasing concentrations of calcium from 0 to 3 mM (2.98 to 4.63 mg/ml DCW) in the salt formulation while the growth yield of the other two strains leveling off at the added

Table 2 Medium conductivities and ICP-DRC-MS analysis on monovalent and divalent cation concentrations (mM) in different media

Media	Salt formulation	[Na]	[K]	[Ca]	[Mg]	[Li]	Conductivity (mS/cm)[a]
Seawater	Seawater	487	10	11	61	0.034	55.5
M1.DI	Deionized water	5.0	4.3	0.16	0.21	NT	1.19
M1.SF1	KCl-based ions supplement	5.2	467	2.6	20	NT	51.9
M1.SF2	NaCl-based ions supplement	426	15	1.2	19	NT	45.9
M1.SF3	LiCl-based ions supplement	4.9	16	2.3	18	131	14.6
M1.NaCl	411 mM NaCl	465	4.1	0.18	0.20	NT	43.5
M1.KCl	402 mM KCl	5.0	450	0.14	0.21	NT	52.0
SHY.DI	Deionized water	7.6	7.2	0.84	0.61	NT	1.81
SHY.SF4	No added sodium and potassium salts	9.4	8.0	3.3	21	0.0013	4.60
SHY.SF4+71 mM LiCl	No added sodium and potassium salts	8.6	7.3	3.6	20	84	11.2
SHY.SF4+118 mM LiCl	No added sodium and potassium salts	8.2	7.1	3.4	19	129	15.3
SHY.SF4+189 mM LiCl	No added sodium and potassium salts	8.7	7.4	3.6	19	213	23.5

NT Not tested

[a] Millisiemens per centimeter

Table 3 Effect of different concentrations of calcium, magnesium and potassium on the growth of *Salinispora tropica, Salinispora arenicola*, and *Salinispora pacifica* in M1 medium containing modified sodium chloride-based SF2 salt formulation

Base medium	Ca in salt formulation (mM)	Mg in salt formulation (mM)	K in salt formulation (mM)	Dry cell weight (mg/ml)[a]		
				S. arenicola CNH643	*S. tropica* CNB440	*S. pacifica* NPS14029
SHY	0	0	0	4.81	3.14	0.13
M1	0	0	0	0.0	−0.02	0.03
M1	0	0	9.3	0.16	−0.04	−0.15
M1	0	17.5	9.3	3.94	4.21	2.98
M1	0.375	17.5	9.3	3.97	4.63	3.52
M1	0.75	17.5	9.3	3.98	4.33	4.32
M1	1.5	17.5	9.3	4.21	4.71	4.41
M1	3.0	17.5	9.3	4.02	4.68	4.63
M1	3.0	17.5	0	4.16	4.84	4.54

[a] Since the media contain the insoluble calcium carbonate and we also need to take into account of DCW of the inoculum, dry weight of the culture right after inoculation was determined to serve as the "culture blank." The dry weight of the culture blank was subtracted from the DCW of the culture before entering the values in the table. Since only small amount of calcium carbonate present in the media and the utilization of calcium carbonate by *Salinispora* in the media was low, the amount of calcium carbonate consumed at the end of the fermentation had negligible effect on the DCW determination. The negative values of DCW indicated the lysis of the inoculated mycelial cells

calcium concentration of 0.375 mM (Table 3). All three strains achieved maximal growth yield without the addition of potassium to the salt formulation when 3.0 mM calcium and 17.5 mM magnesium were included in the salt formulation (Table 3).

The effect of different concentrations of magnesium in the salt formulation on the growth of *S. arenicola* CNH643, *S. tropica* CNB440, and *S. pacifica* NPS14029 grown in M1 medium containing 411 mM sodium, 9.3 mM potassium, and 3.0 mM calcium in the salt formulation (modified M1. SF2) was examined. *S. arenicola* CNH643 and *S. tropica* CNB440 grew well in the above M1 medium without addition of any magnesium, achieving DCW of 3.52 mg/ml and 3.88 mg/ml, respectively (Table 4). While the maximum growth yield of these two strains was achieved at the added magnesium concentration of 1.10 mM (Table 4), the above data suggested that the basal level of 0.21 mM magnesium in the medium was sufficient to support substantial growth of *S. arenicola* CNH643 and *S. tropica* CNB440. *S. pacifica* NPS14029 has a more stringent growth requirement for magnesium as growth was not observed when magnesium was omitted from the salt formulation. We also observed a trend of increasing growth yield of *S. pacifica* NPS14029 with increasing concentrations of magnesium from 0 to 17.5 mM in the salt formulation, yielding DCW from 0.13 to 4.63 mg/ml (Table 4).

Table 4 Effect of different concentrations of magnesium on the growth of *Salinispora tropica, Salinispora arenicola*, and *Salinispora pacifica* in M1 medium containing modified sodium chloride-based SF2 salt formulation

Mg in salt formulation (mM)	Ca in salt formulation (mM)	K in salt formulation (mM)	Dry cell weight (mg/ml)[a]		
			S. arenicola CNH643	*S. tropica* CNB440	*S. pacifica* NPS14029
0	3.0	9.3	3.52	3.88	0.13
1.10	3.0	9.3	4.78	4.39	0.91
2.19	3.0	9.3	4.63	4.27	0.98
4.38	3.0	9.3	4.60	4.57	1.61
8.75	3.0	9.3	4.81	5.00	3.00
17.5	3.0	9.3	4.02	4.68	4.63

[a] Since the media contain the insoluble calcium carbonate and we also need to take into account of DCW of the inoculum, dry weight of the culture right after inoculation was determined to serve as the "culture blank." The dry weight of the culture blank was subtracted from the DCW of the culture before entering the values in the table. Since only small amount of calcium carbonate present in the media and the utilization of calcium carbonate by *Salinispora* in the media was low, the amount of calcium carbonate consumed at the end of the fermentation had negligible effect on the DCW determination

Examination of the growth yield of *S. tropica*, *S. arenicola*, and *S. pacifica* grown in medium SHY containing 411 mM NaCl

We reported the use of the growth medium SHY for fermenting the industrial strain *S. tropica* NPS21184 in the manufacturing of NPI-0052 (Tsueng et al 2008), a potent proteasome inhibitor, currently undergoing clinical evaluation for the treatment of cancer (Chauhan et al. 2006). Medium SHY contains higher levels of carryover ions than medium M1. The basal levels of sodium, potassium, calcium, and magnesium in medium SHY.DI prepared in deionized water are 7.6, 7.2, 0.84, and 0.61 mM, respectively (Table 2). We examined the growth of *S. arenicola* CNH643, *S. tropica* CNB440, and *S. pacifica* NPS14029 grown in SHY medium supplemented only with 411 mM sodium chloride (SHY.NaCl). We observed good growth of *S. arenicola* CNH643 (4.81 mg/ml) and *S. tropica* CNB440 (3.14 mg/ml) grown in medium SHY.NaCl but not *S. pacifica* NPS14029 (Table 3). The above observation demonstrates the importance of carryover of ions from different media in affecting the growth of *Salinispora* and further supported the more stringent magnesium growth requirement for *S. pacifica* NPS14029.

Examination of ionic strength of media in supporting the growth of *S. arenicola* and S. *pacifica* in sodium chloride-based media

In an earlier study using a sodium chloride-based SD2 seed medium and the sodium chloride-based SHY growth media (SHY.SF2), we demonstrated that three *S. tropica* strains, including the type strain CNB440, have a growth requirement for ionic strength (Tsueng and Lam 2008b). Quantifiable growth of the three *S. tropica* strains, as determined by DCW, was detected in growth media with conductivity ranging from 15.2 mS/cm to 20.8 mS/cm. All three *S. tropica* strains did not grow in media with conductivity less than 13.6 mS/cm. Hence, we examined the growth requirement for ionic strength of two *S. arenicola* strains (CNH643 and NPS14034) and two *S. pacifica* strains (NPS14029 and NPS14565) grown in the same sodium chloride-based SHY media containing different concentrations of sodium chloride as used in the previous study (Tsueng and Lam 2008b), to create media with different ionic strengths. The SHY-based media contain 9.3 mM potassium, 3.0 mM calcium and 17.5 mM magnesium to meet the growth requirement of these ions for *S. arenicola* and *S. pacifica*. The medium contained a basal level of 31 mM sodium derived from the complex medium nitrogen sources and inoculum from seed culture, and a basal medium conductivity of 8.29 mS/cm (Tsueng and Lam 2008b). The two *S. pacifica* strains did not grow in media with conductivity of less than 15.2 mS/cm, yielding DCW of 2.19 and 2.63 mg/ml in medium with conductivity of 15.2 mS/cm (Fig. 2) and therefore, demonstrated a growth requirement for ionic strength similar to *S. tropica*. The two *S. arenicola* strains showed less stringent growth requirement for ionic strength than the other two *Salinispora* species, demonstrating growth of 2.33 and 2.42 mg/ml DCW in medium with the lowest tested conductivity of 8.29 mS/cm (Fig. 2). There is a trend to increasing growth yield for all four strains with increasing ionic strength of the media, yielding maximum DCW of all four strains in media with the highest conductivity tested at 20.8 mS/cm (Fig. 2).

Examination of ionic strength of media in supporting the growth of *S. tropica*, *S. arenicola* and *S. pacifica* in lithium chloride-based media

In order to confirm the effect of the medium ionic strength on the growth of *Salinispora*, we examined the growth yields of the three *Salinispora* species, *S. arenicola* CNH643, *S. tropica* CNB440 and *S. pacifica* NPS14029, in the lithium chloride-based SHY.SF4 media, to dissociate any potential effect of sodium on the growth of *Salinispora*. In this study, salt formulation SF4 with no added sodium and potassium salts was used. Even though discrete sodium and potassium were not added to medium SHY.SF4, the medium contains 9.4 mM sodium and 8.0 mM potassium (Table 2). Four different concentrations (0, 71, 118 and 189 mM) of lithium chloride were added to the SHY.SF4 medium to create media with different ionic strengths (Table 2). In this study, we used the same lithium chloride-based SHY media for both the

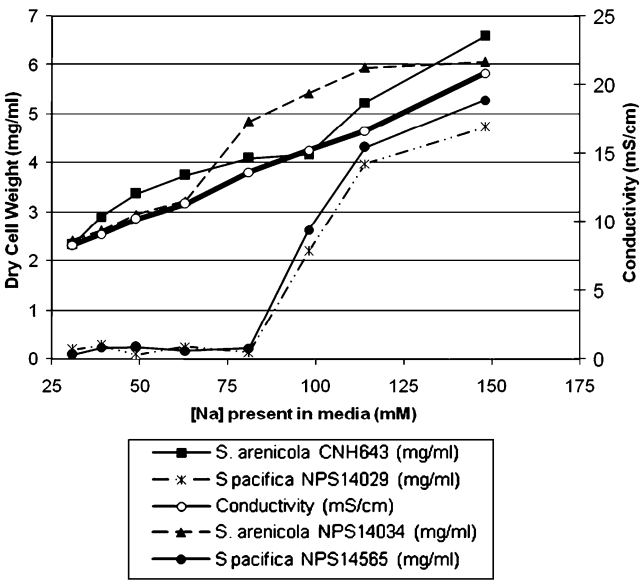

Fig. 2 Effect of sodium concentrations and medium ionic strength (conductivity) on the growth yields of *Salinispora arenicola* (CNH643 and NPS14034) and *Salinispora pacifica* (NPS14029 and NPS14565)

seed and the growth media so that there is no carryover of any undesirable ions to the final growth medium for DCW determination. *S. tropica* CNB440 and *S. pacifica* NPS14029 demonstrated a similar growth requirement for ionic strength in the lithium chloride-based media as in the sodium chloride-based media, with good growth yield for both strains (5.97 and 3.89 mg/ml) observed in medium with conductivity of 15.3 mS/cm (Table 5). *S. tropica* and *S. pacifica* did not grow in lithium chloride-based media with conductivity of 11.2 mS/cm or lower (Table 5). No real, quantifiable growth of *S. arenicola* CNH643 (0.27 mg/ml) was observed in medium without addition of lithium chloride, resulting the lowest medium conductivity tested at 4.60 mS/cm. Maximal growth of *S. arenicola* CNH643 was achieved in media containing 71 mM and 118 mM lithium chloride with medium conductivities of 11.2 mS/cm (6.99 mg/ml DCW) and 15.3 mS/cm (6.88 mg/ml DCW), respectively (Table 5). Increasing the concentration of lithium chloride in the medium to 189 mM reduced the growth yield of *S. arenicola* CNH643 significantly to 1.23 mg/ml but has no effect on the growth of *S. tropica* CNB440 and *S. pacifica* NPS14029, showing that *S. tropica* CNB440 and *S. pacifica* NPS14029 are more tolerant to lithium at high concentration than *S. arenicola* CNH643.

Discussion

A significant amount of data have been accumulated to demonstrate that marine Gram-negative bacteria, mainly belonging to the Proteobacteria Gamma division, require sodium for growth through a wide range of functions such as sodium-dependent active transport, pH regulation and flagellar motility requiring the sodium motive force (Dimroth 1987; Nakamura et al. 1992; Kogure 1998; Häse et al. 2001; McCarter 2001). Sodium is also required for membrane stabilization and maintaining a suitable osmotic pressure in the medium to support the growth of marine Gram-negative bacteria (MacLeod and Matula 1962; MacLeod 1965; Unemoto et al. 1973). There have been only a few publications to illustrate the importance of potassium, magnesium, and calcium in supporting the growth of marine Gram-negative bacteria (MacLeod and Onofrey 1956; MacLeod and Matula 1961; MacLeod 1965; Unemoto et al. 1977). The knowledge regarding the marine Gram-positive bacteria growth requirement for ionic strength is significantly lacking, especially for marine actinomycetes, and hinders the progress in the isolation of new marine actinomycetes. In this paper, we reported our findings on the examination of the marine actinomycete *Salinispora* growth requirement for monovalent cations and divalent cations, as well as the growth requirement for ionic strength.

In this study, we demonstrate that an absolute sodium requirement to support growth of *Salinispora* has not been established. All three species of *Salinispora*, *S. arenicola*, *S. tropica*, and *S. pacifica* can use potassium or lithium to replace sodium in the media to support maximum growth and to yield similar DCW as compared with these strains grown in sodium chloride-based medium (Table 1). We did not reproduce the finding of Mincer et al. (2002) of the specificity for sodium in supporting the growth of *S. arenicola* and *S. tropica* even though same media were used. We demonstrate that *S. arenicola*, *S. tropica*, and *S. pacifica* grow well in M1 agar media containing Sieburth salt formulations supplemented with sodium salts or replacement of sodium salts by potassium salts (Table 1). Two of the strains used in this study, *S. arenicola* CNH643 and *S. tropica* CNB440, were the same strains used by Mincer et al. (2002), therefore, eliminate the possibility of strain variance differences. Our data illustrate that all three species of *Salinispora* require divalent cations, magnesium, and calcium, for growth. *S. pacifica* apparently has more stringent growth requirement for the divalent cations than *S. arenicola* or *S. tropica*. Due to the carryover of sodium and potassium in millimolar quantities from the complex nitrogen sources in the media used in this study (Tsueng and Lam 2008a), we have not established the absolute requirement for sodium and potassium in the medium, if any, to support the growth of *Salinispora*. To determine the absolute concentrations of the above monovalent and divalent cations for supporting the growth of *Salinispora*, a defined medium needs to be developed in which we can control the exact amounts of the four cations present.

Table 5 The growth yields of *Salinispora arenicola* CNH643 *Salinispora tropica* CNB440 and *Salinispora pacifica* NPS14029 grown in lithium chloride-based media at different ionic strengths

[Lithium] (mM)	Conductivity (mS/cm)[a]	Dry cell weight (mg/ml)[b]		
		S. arenicola CNH643	*S. tropica* CNB440	*S. pacifica* NPS14029
0	4.60	0.27	−0.60	0.18
71	11.2	6.99	0.12	0.07
118	15.3	6.88	5.97	3.89
189	23.5	1.23	5.86	4.83

[a] Millisiemens per centimeter

[b] Since the media contain the insoluble calcium carbonate and we also need to take into account of DCW of the inoculum, dry weight of the culture right after inoculation was determined to serve as the "culture blank." The dry weight of the culture blank was subtracted from the DCW of the culture before entering the values in the table. Since only small amount of calcium carbonate present in the media and the utilization of calcium carbonate by *Salinispora* in the media was low, the amount of calcium carbonate consumed at the end of the fermentation had negligible effect on the DCW determination. The negative values of DCW indicated the lysis of the inoculated mycelial cells

Nonetheless, our data establish that 5.0 mM sodium, ~1% of the sodium content in seawater, is sufficient to provide maximum growth yield of all three species of *Salinispora* when the medium was supplemented with salt formulation SF1 or SF3 (Table 1). Since all three species of *Salinispora* grew well in the modified M1.SF2 (sodium chloride-based salt formulation) medium without addition of potassium salt (Table 3), we also established that 4.3 mM potassium in the medium is sufficient to provide maximum growth yield of *Salinispora*.

While demonstrating that all three species of *Salinispora* require magnesium and calcium for growth, our finding also suggests that there is an interaction between magnesium and calcium in supporting the growth of *S. arenicola* and *S. tropica*. The presence of a high concentration of magnesium in the medium reduced the requirement for calcium for growth and the presence of a high concentration of calcium in the medium lowered the amount of magnesium required for growth. This type of interaction between magnesium and calcium in supporting the growth of Gram-negative marine bacteria has been reported (MacLeod and Onofrey 1956; MacLeod 1965). Our data demonstrated that *S. pacifica* has a more stringent requirement for magnesium and calcium for growth than *S. arenicola* and *S. tropica*. The maximum growth yield of *S. pacifica* was obtained in the medium containing the salt formulation with the highest concentration of magnesium (17.5 mM) and calcium (3.0 mM) tested. The interaction between magnesium and calcium in supporting the growth of *S. pacifica* was also observed, albeit significantly less than the effect observed in *S. arenicola* and *S. tropica*.

In our previous studies using sodium chloride-based media, we demonstrated that in addition to the balance of salts in the medium, *S. tropica* also has a growth requirement for ionic strength (Tsueng and Lam 2008a, b). The data from our current study, using the same sodium chloride-based media and the lithium chloride-based media, demonstrated that all three species of *Salinispora* have growth requirement for a certain medium ionic strength (8.29 to 15.2 mS/cm), much higher than the medium ionic strength of the typical media, ISP1 and ISP2 (0.97 to 1.29 mS/cm), supporting the growth of terrestrial actinomycetes (Tsueng and Lam 2008b). Thus, *Salinispora* has a certain growth requirement for ionic strength, a trait for a marine actinomycete, which distinguishes it from terrestrial actinomycetes. We also determined that *S. arenicola* has a significantly lower growth requirement for medium ionic strength than *S. tropica* and *S. pacifica*. Quantifiable amounts of growth of two *S. arenicola* strains (2.33 and 2.42 mg/ml), including the type strain CNH643, were obtained in the sodium chloride-based medium with conductivity of 8.29 mS/cm, however, *S. arenicola* CNH643 did not show any real growth in the lithium chloride-based medium with conductivity of 4.60 mS/cm. Therefore, *S. arenicola* has a growth requirement for the medium ionic strength between 4.60 to 8.29 mS/cm. *S. tropica* and *S. pacifica* have a very similar growth requirement for ionic strength in both media, with quantifiable growth observed in medium with ionic strength of 15.2 mS/cm. The reason we selected lithium for the ionic strength growth requirement study is to dissociate any potential effect of sodium on the growth of *Salinispora*. Lithium does not have any specific biological function in bacteria and it is toxic to bacteria at moderate to high concentrations (Wackett et al. 2004). It present at a low concentration (0.034 mM) in seawater (Table 2). We also observed that lithium is more toxic to *S. arenicola* than to *S. tropica* and *S. pacifica*, inhibiting the growth of *S. arenicola* by 82% at 189 mM but without effect on the growth of *S. tropica* and *S. pacifica*. The inhibitory effect is due to the lithium toxicity and not the high medium ionic strength since *S. arenicola* grew well in media (M1.SF1 and M1.SF2) with medium ionic strength of 45.9 mS/cm and 51.9 mS/cm (Table 2).

We presented a preliminary examination of the growth requirement for monovalent and divalent ions, and the growth requirement for ionic strength of the marine actinomycete *Salinispora*. The above findings only scratch the surface of our understanding of nutritional growth requirement for ions of marine actinomycetes. Significant efforts in this area of research should be rigorously pursued so that we can utilize the new information for the isolation of novel marine actinomycetes and also to understand the adaptation and ecological roles of actinomycetes in the marine environment.

References

Avetisyan AV, Dibrov PA, Semeykina AL, Skulachev VP, Sokolov MV (1991) Adaptation of *Bacillus* FTU and *Escherichia coli* to alkaline conditions: the Na^+-motive respiration. Biochim Biophys Acta 1098:95–104

Bryant MP, Robinson IM, Chu H (1959) Observations on the nutrition of *Bacteroides succinogenes* – a ruminal cellulolytic bacterium. J Dairy Sci 42:1831–1847

Bull AT, Stach JEM (2007) Marine actinobacteria: new opportunities for natural product search and discovery. Trends Microbiol 15:491–499

Caldwell DR, Hudson RF (1974) Sodium, an obligate growth requirement for predominant rumen bacteria. Appl Microbiol 27:549–552

Caldwell DR, Keeney M, Barton JS, Kelley JF (1973) Sodium and other inorganic growth requirement of *Bacteroides amylophilus*. J Bacteriol 114:782–789

Chauhan D, Hideshima T, Anderson KC (2006) A novel proteasome inhibitor NPI-0052 as an anticancer therapy. Brit J Cancer 95:961–965

Chun J, Bae KS, Moon EY, Jung S-O, Lee HK, Kim S-J (2000) *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete isolated from a saltern. Int J Syst Evol Microbiol 50:1909–1913

Dimroth P (1987) Sodium ion transport decarbolyases and other aspects of sodium ion cycling in bacteria. Microbiol Rev 51:320–340

Fenical W, Jensen PR (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. Nat Chem Biol 2:666–673

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tonb JF, Dongherty BA, Merrick JM et al (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzea* RD. Science 269:496–512

Fujiwara-Nagata E, Eguchi M (2004) Significance of Na^+ in the fish pathogen, *Vibrio anguillarum*, under energy depleted condition. FEMS Microbiol Lett 234:163–167

Goldman M, Deibel RH, Niven CF Jr (1963) Interrelationship between temperature and sodium chloride on growth of lactic acid bacteria isolated from meat-curing brines. J Bacteriol 85:1017–1021

Häse CC, Fedorova ND, Galperin MY, Dibrov PA (2001) Sodium ion cycle in bacterial pathogens: evidence from cross-genome comparisons. Microbiol Mol Biol Rev 65:353–370

Hayashi M, Nakayama Y, Unemoto T (1996) Existence of Na^+-translocating NADH-quinone reductase in *Haemophilus influenzae*. FEBS Lett 381:174–176

Hunter SH (1972) Inorganic nutrition. Annu Rev Microbiol 26:313–346

Imhoff JF (2001) True marine and halophilic anoxygenic phototrophic bacteria. Arch Microbiol 176:243–254

Jensen PR, Lauro FM (2008) An assessment of actinobacterial diversity in the marine environment. Antonie Van Leeuwenhoek 94:51–62

Johnson FH, Harvey EN (1938) Bacterial luminescence, respiration and viability in relation to osmotic pressure and specific salts of sea water. J Cell Comp Physiol 11:213–232

Kogure K (1998) Bioenergetics of marine bacteria. Curr Opin Biotechnol 9:278–282

Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol 9:245–251

Larsen H (1962) Halophilism. In: Gunsalus IC, Stanier RY (eds) The bacteria: a treatise on structure and function, vol 4. Academic Press, Inc, New York, pp 297–342

McCarter LL (2001) Polar flagellar motility of the *Vibrionaceae*. Microbiol Mol Biol Rev 65:445–462

MacLeod RA (1965) The questions of the existence of specific marine bacteria. Bacteriol Rev 29:9–23

MacLeod RA (1968) On the role of inorganic ions in the physiology of marine bacteria. In: Droop MR, Ferguson Wood EJ (eds) Advances in microbiology of the sea, vol 1. Academic Press, London, pp 95–126

MacLeod RA (1971) Salinity: bacteria, fungi, and blue-green algae. In: Kinne O (ed) Marine ecology, vol 1. Environmental factors, Part 2. Wiley Interscience, London, pp 689–703

MacLeod RA, Matula TI (1961) Solute requirements for preventing lysis of some marine bacteria. Nature 192:1209–1210

MacLeod RA, Matula TI (1962) Nutrition and metabolism of marine bacteria. XI. Some characteristics of the lytic phenomenon. Can J Microbiol 8:883–896

MacLeod RA, Onofrey E (1956) Nutrition and metabolism of marine bacteria. VI. Quantitative requirements for halides, magnesium, calcium and iron. Can J Microbiol 3:753–759

Maldonado LA, Fenical W, Jensen PR, Kauffman CA, Mincer TJ, Ward AC, Bull AT, Goodfellow M (2005) *Salinispora arenicola* gen. nov., sp. nov. and *Salinispora tropica* sp. nov., obligate marine actinomycetes belonging to the family *Micromonosporaceae*. Int J Syst Evol Microbiol 55:1759–1766

Mincer TJ, Jensen PR, Kauffman CA, Fenical W (2002) Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. Appl Environ Microbiol 61:3695–3700

Nakamura T, Kawasaki S, Unemoto T (1992) Roles of K^+ and Na^+ in pH homeostatis and growth of marine bacterium *Vibrio alginolyticus*. J Gen Microbiol 138:1271–1276

Oh S, Kogure K, Ohwada K, Simidu U (1991) Correlation between possession of a respiration-dependent Na^+ pump and Na^+ requirement for growth of marine bacteria. Appl Environ Microbiol 57:1844–1846

Oh D-C, Williams PG, Kauffman CA, Jensen PR, Fenical W (2006) Cyanosporasides A and B, chloro- and cyano-cyclopenta[α] indene glycosides from the marine actinomycete "*Salinispora pacifica*". Org Lett 8:1021–1024

Oh DC, Gontang EA, Kauffman CA, Jensen PR, Fenical W (2008) Salinipyrones and pacificanones, mixed-precursor polyketides from the marine actinomycete *Salinispora pacifica*. J Nat Prod 71:570–575

Pratt D (1974) Salt requirements for growth and function of marine bacteria. In: Colwell RR, Morita RY (eds) Effect of the ocean environment on microbial activities. University Park Press, Baltimore, pp 3–15

Rürgen H-J, Hentzschel G (1980) Mineral salt requirements of *Bacillus globisporus* subsp. *marinus* strains. Arch Microbiol 126:83–86

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstruction phylogenic trees. Mol Biol Evol 4:406–425

Sakazaki RS, Iwanami S, Fukumi H (1963) Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticous*. I. Morphological, cultural and biochemical properties and its taxonomical position. Jap J Med Sci Biol 16:161–188

Sieburth JM (1979) Sea microbes. Oxford University Press, New York

Sistrom WR (1960) A requirement for sodium in the growth of *Rhodopseudomonas spheroides*. J Gen Microbiol 22:778–785

Tsueng G, Lam KS (2008a) A low-sodium-salt formulation for the fermentation of salinosporamides by *Salinispora tropica* strain NPS21184. Appl Microbiol Biotechnol 78:821–826

Tsueng G, Lam KS (2008b) Growth of *Salinispora tropica* strains CNB440, CNB476, and NPS21184 in nonsaline, low-sodium media. Appl Microbiol Biotechnol 80:873–880

Tsueng G, Teisan S, Lam KS (2008) Defined salt formulations for the growth of *Salinispora tropica* strain NPS21184 and the production of salinosporamide A (NPI-0052) and related analogs. Appl Microbiol Biotechnol 78:827–832

Udwary DW, Zeigler L, Asolkar RN, Singan V, Lapidus A, Fenical W, Jensen PR, Moore BS (2007) Genome sequence reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. Proc Natl Acad Sci USA 104:10376–10381

Unemoto T, Tsuruoka T, Hayashi M (1973) Role of Na^+ and K^+ in preventing lysis of a slightly halophilic *Vibrio alginolyticus*. Can J Microbiol 19:563–571

Unemoto T, Hayashi M, Terao K (1977) Lysis of halophilic *Vibrio alginolyticus* and *Vibrio costicolus* induced by chaotropic anions. Biochim Biophys Acta 500:425–431

Unemoto T, Akagawa A, Mizugaki M, Hayashi M (1992) Distribution of Na^+-dependent respiration and a respiration-driven electrogenic Na^+ pump in moderately halophilic bacteria. J Gen Microbiol 138:1999–2005

Wackett LP, Dodge AG, Ellis LBM (2004) Microbial genomics and the periodic table. Appl Environ Microbiol 70:647–655

Ward AC, Bora N (2006) Diversity and biogeography of marine actinobacteria. Curr Opin Microbiol 9:1–8