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Growth of *Salinispora tropica* strains CNB440, CNB476, and NPS21184 in nonsaline, low-sodium media

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Abstract We recently described the development of a potassium-chloride-based salt formulation containing low sodium concentration (5.0 mM) to support the growth of *Salinispora tropica* strain NPS21184 and its production of salinosporamide A (NPI-0052). In order to determine whether the above low-sodium salt formulation can also support the growth of other *S. tropica* strains, we examined the growth of the type strain CNB440 and the parent strain CNB476, from which strain NPS21184 was derived as a single colony isolate. We demonstrated that good growth rate and yield of *S. tropica* strains CNB440 and CNB476, similar to *S. tropica* strain NPS21184 reported earlier, were detected in both agar and liquid media containing the potassium-chloride-based salt formulation with sodium concentration of 5.0 mM. Furthermore, we also detected good growth rate and yield of all three *S. tropica* strains on potassium-sulfate-based salt formulation agar medium containing both low-sodium (5.7 mM) and low-chloride (14 mM) content. This finding confirms the observation that the species of *S. tropica* does not have a seawater growth requirement but requirement for a specific combination of salts to provide a balance of salts and maintain a high enough ionic strength for growth.

Keywords Salinosporamide A · NPI-0052 · *Salinispora tropica* · Low-sodium salt formulation · Nonsaline fermentation · Marine actinomycete

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Introduction

The exploration of marine actinomycetes as a source for novel drug leads have been the focus of several recent review articles, and the tremendous diversity of marine actinomycetes is undisputable (Bull and Stach 2007; Fenical and Jensen 2006; Lam 2006; Ward and Bora 2006). 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; Udwary et al. 2007). 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 reported in its original description as MAR 1 (Mincer et al. 2002) and in the formal taxonomic description (Maldonado et al. 2005). Several strains of *S. tropica* have been shown to produce salinosporamide A (NPI-0052; Feling et al. 2003; Macherla et al. 2005; Reed et al. 2007), a potent proteasome inhibitor (Chauhan et al. 2005, 2006; Groll et al. 2006) that is currently undergoing phase I clinical studies for the treatment of patients with various cancers (Chauhan et al. 2006; Cusack et al. 2006).

The current production strain for manufacturing the clinical supply of NPI-0052 is *S. tropica* NPS21184, a single colony isolate derived directly from strain *S. tropica* CNB476 without any mutation and genetic modification (Tsueng et al. 2007). During salt formulation development to search for chemically defined salt formulations to replace the nondefined synthetic sea salt, Instant Ocean, for the manufacturing of NPI-0052, we found that *S. tropica* strain NPS21184 is able to grow in a peptone–yeast extract-based

medium containing 0.06 mM NaF, the only known sodium salt added to the medium (Tsueng and Lam 2008). Using inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) analysis, we were able to determine that the above peptone–yeast extract-based medium contains 5.0 mM sodium. The majority (~4.9 mM) of sodium was derived from the complex medium components peptone and yeast extract.

The ability of *S. tropica* NPS21184 to grow in medium containing 5.0 mM sodium, ~1% of the sodium content in seawater, hardly justifies that *S. tropica* strain NPS21184 requires sea water for growth. This observation contradicted the previous findings that *Salinispora* strains required seawater for growth (Mincer et al. 2002; Maldonado et al. 2005). However, *S. tropica* strain NPS21184 may represent a single variant that lost the seawater growth requirement property during isolation. In order to evaluate the seawater growth requirement of the species of *S. tropica*, we examined the growth of the type strain of *S. tropica*, strain CNB440 (Maldonado et al. 2005), and the parent strain of strain NPS21184, strain CNB476 (Jensen et al. 1991) in the above low-sodium medium. Since we have developed a low-chloride, sodium sulfate-based medium to support the growth of *S. tropica* strain NPS21184 (Tsueng et al. 2008), we also examined the ability of the three *S. tropica* strains to grow on the potassium-sulfate-based agar medium containing both low sodium and low chloride.

Materials and methods

Growth analysis by packed cell volume (PCV), culturing conditions for shake flask, extraction of cultures, high-performance liquid chromatography analysis, and ICP-DRC-MS analysis were described in Tsueng et al. (2008). The protocol for the washed cell experiment was described in Tsueng and Lam (2008).

Microorganism

Three *S. tropica* strains were used in this study. Strain CNB440 was isolated from a sediment sample collected from Chub Cay, Bahamas (Jensen et al. 1991). Strain CNB440 is the type strain for *S. tropica* with the fully described taxonomic identification (Maldonado et al. 2005) and the whole genome sequenced (Udwary et al. 2007). Strain CNB476 was isolated from a sediment sample collected from Cross Harbor, Abaco, Bahamas (Jensen et al. 1991). Strain NPS21184 is a single colony isolate derived from strain CNB476 (Tsueng et al. 2007). Strains CNB440, CNB476, and NPS21184 were deposited with the American Type Culture Collection (ATCC) and assigned the accession numbers ATCC BAA-916^T, PTA-5275, and PTA-6685, respectively.

Salt formulations

The composition of salt formulation I was described in Tsueng et al. (2008). For addition to the seed medium, salt formulation I consists of the following ingredients per liter of deionized water: 24 g of NaCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.69 g of KCl, 0.43 g of $CaCO_3$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 85.9 mg KBr, 21.5 mg H_3BO_3, 15.5 mg $SrCl_2$, 2.6 mg NaF, and 208 μg of $CoCl_2 \cdot 6H_2O$. For addition to the production medium, salt formulation I consists of the following ingredients per liter of deionized water: 24 g of NaCl, 0.69 g of KCl, 0.43 g of $CaCl_2 \cdot 2H_2O$, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF, and 52 μg of $CoCl_2 \cdot 6H_2O$.

The composition of salt formulation III was described in Tsueng and Lam (2008) and consists of the following ingredients in seed medium per liter of deionized water: 30 g of KCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCO_3$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF, and 208 μg of $CoCl_2 \cdot 6H_2O$. In the production medium, salt formulation III consists of the following ingredients per liter of deionized water: 30 g of KCl, 0.43 g of $CaCl_2 \cdot 2H_2O$, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF, and 52 μg of $CoCl_2 \cdot 6H_2O$.

Salt formulation IV is a potassium sulfate-based formulation consisting of the following ingredients per liter of deionized water: 48 g of K_2SO_4, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCO_3$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF, and 208 μg of $CoCl_2 \cdot 6H_2O$.

Growth and wash media

Seed medium SD2 supplemented with 30 g/l of Instant Ocean was described in Tsueng et al. (2008). Seed medium A1.Kc4C consists of the following ingredients per liter of deionized water: 10 g of starch, 2 g of peptone (USB), and 4 g of yeast extract (USB) and supplemented with salt formulation III. Agar medium A1.Kc4C has the same composition as the seed medium A1.Kc4C with addition of 17 g/l of agar (Difco) as a solidifying agent. Seed medium A1.Ks4C and agar medium A1.Ks4C have the same composition as seed medium A1.Kc4C and agar medium A1.Kc4C, respectively, except using 48 g per liter of K_2SO_4 instead of 30 g per liter of KCl (i.e., salt formulation IV).

Wash medium A1.K consists of the following ingredients per liter of deionized water: 10 g of starch, 2 g of peptone, 4 g of yeast extract, and 30 g of KCl (Sigma). Agar medium A1.K has the same composition as the wash medium A1.K with addition of 17 g/l agar (Difco) as a solidifying agent. Wash medium A1.Ks and agar medium A1.Ks have the same composition as Wash A1.K and agar

medium A1.K, respectively, except using 48 g per liter of K_2SO_4 instead of 30 g per liter of KCl.

Production medium SHY.KcMC consists of the following ingredients per liter of deionized water: 10 g of starch, 4 g of Hy Soy (Kerry Biosciences), 4 g of yeast extract, 1 g of $CaCO_3$ (Sigma), 40 mg of $Fe_2(SO_4)_3$ (Sigma), and 100 mg of KBr (Fisher) and supplemented with salt formulation III.

Agar cultures

Sterile inoculation loops were used to transfer 30 μl washed cells to agar plates containing 20 ml of agar media A1.K, A1.Kc4C, A1.Ks, and A1.Ks4C. After inoculation, the edges of the agar plates were wrapped with parafilm to reduce evaporation. The agar plates were incubated at 28°C for 4 weeks to observe growth.

Determination of dry cell weight of culture

Dry cell weight (DCW) of culture was determined by centrifuging 5 ml of the culture in a tared 10-ml glass tube at 3,000 rpm for 15 min in a Beckman centrifuge (Allegra model 6). The supernatant was carefully removed using a pipette. The mycelia were centrifuge-washed with 5 ml deionized water, and the supernatant was removed using a pipette. The washed cell was dried in an oven at 70°C for 3 days before weighing and DCW determination.

Determination of conductivity of media

The conductivity of media was measured with an Oaklon® conductivity meter (con 11 series, Eutech Instruments, Singapore). All conductivity measurements were performed on autoclaved media.

Results

Examination of sodium concentrations in the defined salt formulation in supporting the growth of *Salinispora tropica* strains CNB440, CNB476, and NPS21184

We compared the minimal sodium concentration to support the growth of the three *S. tropica* strains by varying the sodium chloride concentration in the production medium SHY.KcMC containing the sodium chloride-based salt formulation I (Table 1). In our previous studies (Tsueng et al. 2008; Tsueng and Lam 2008), PCV was used to measure the growth of *S. tropica* strain NPS21184. Based on the PCV data (Table 1), no growth of any *S. tropica* strains was observed when sodium chloride was excluded from the salt formulation. Even though no sodium chloride was added to the salt formulation, the medium contained 31 mM sodium as determined by ICP-DRC-MS analysis (Table 1). The sodium in the medium was derived from the complex nitrogen sources (~10 mM, Tsueng et al. 2008) and 5% inoculum from the seed culture (~20 mM). When 86 mM NaCl was added to the salt formulation (actual sodium concentration by ICP-DRC-MS analysis was 114 mM, Table 1), *S. tropica* strains CNB440 and NPS21184 achieved the maximum growth yield as determined by the PCV (5% to 6%), while small amount of growth (1% PCV) were observed in strain CNB476. Strain CNB476 requires a higher concentration of sodium than strains CNB440 and NPS21184, at 120 mM (148 mM actual concentration), to achieve a good growth yield of 5% PCV.

PCV is a semiquantitative method to measure growth. In order to obtain a definitive condition to support the growth of *S. tropica*, we examined the DCW of the culture, which is a

Table 1 The growth yield of the day 6 production cultures of *S. tropica* strains CNB440, CNB476, and NPS21184 grown in production medium containing salt formulation I with different concentrations of NaCl

Amount of sodium added to the salt formulation (mM)	Amount of sodium determined by ICP-DRC-MS analysis (mM)	Conductivity (mS/cm)[a]	DCW (mg/ml)[b]			PCV (%)		
			CNB440	CNB476	NPS21184	CNB440	CNB476	NPS21184
0	31	8.29	−0.58	−0.77	−0.71	0	0	0
8.6	39	9.09	−0.56	−0.57	−0.45	0	0	0
17	49	10.2	−0.45	−0.63	−0.39	0	0	0
34	63	11.3	0.08	−0.67	−0.10	0	0	0
51	81	13.6	0.86	−0.70	−0.27	2.0	0	0
68	98	15.2	3.12	−0.32	5.59	3.0	0	4.5
86	114	16.6	8.25	0.34	7.74	6.5	1.0	6.0
120	148	20.8	7.15	4.67	7.19	6.0	5.0	6.0

[a] millisiemens per centimeter

[b] Since the media contain the insoluble calcium carbonate and we also need to take into account of the 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. The negative values of DCW indicated the lysis of the inoculated mycelial cells

more quantitative and reliable measurement of growth than PCV. Since the media contain insoluble calcium carbonate and we also need to take into account the DCW of the inoculum, dry weight of the culture right after inoculation served as the "culture blank." The dry weight of the culture blank was subtracted from the DCW of the culture after growth to reflect the real growth of the culture after inoculation. While the DCW data correlate well with the PCV data in general, the DCW data demonstrated that lysis of cell from inoculum (negative DCW values, Table 1) occurred in media containing low sodium and low ionic strength (see next section) that was not detected using the PCV data (below the detection limit of 0.25%). The DCW data also showed a more accurate difference in the growth yield of strain CNB476 when compared to strains CNB440 and NPS21184 at 120 mM added sodium medium (148 mM actual value), than the PCV data. The growth of strain CNB476 in the above medium was 65% and 83% of strains CNB440 and NPS21184, by DCW and PCV analysis, respectively.

Examination of ionic strength of media in supporting the growth of *Salinispora tropica* strains CNB440, CNB476, and NPS21184

We have previously demonstrated that *S. tropica* strain NPS21184 grew well in media containing 5.0 mM sodium with proper ionic supplements in the media (Tsueng and Lam 2008). However, *S. tropica* strains did not grow in medium containing 63 mM sodium (medium with 34 mM sodium added, Table 1) in this study. We did not balance the ionic strength of the media with potassium chloride or potassium sulfate (such as in salt formulations III and IV) and the media with lower concentrations of sodium chloride have lower ionic strength (conductivity) as shown in Table 1. This demonstrated the importance of maintaining certain ionic strength in the medium in order to support the growth of *S. tropica*. From the DCW data (Table 1), we clearly observed that strain CNB440 was most tolerable to the low ionic strength medium without cell lysis, while strain CNB476 was the least tolerable. Lysis of strains CNB440, NPS21184, and CNB476 was detected in media with conductivity of 10.2, 13.6, and 15.2 mS/cm, respectively. Furthermore, measurable growth of strains CNB440, NPS21184, and CNB476 was detected in media with conductivity of 13.6, 15.2, and 16.6 mS/cm, respectively.

Examination of production of NPI-0052 by *Salinispora tropica* strains CNB440, CNB476, and NPS21184 in the defined salt formulation containing different concentrations of sodium chloride

Strain NPS21184 produces 2.5-fold and 3.0-fold more NPI-0052 than strains CNB476 and CNB440, respectively (Fig. 1).

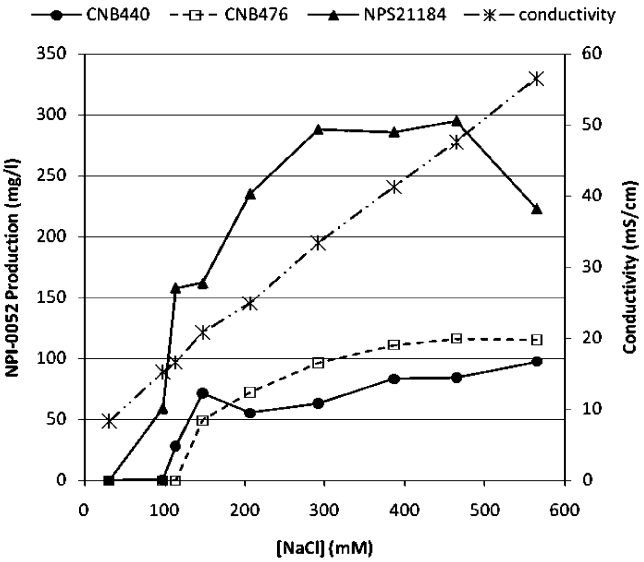

Fig. 1 Effect of sodium concentrations and ionic strength (conductivity) on the production of NPI-0052 by *S. tropica* strains CNB440, CNB476, and NPS21184

The maximal production of NPI-0052 by strains NPS21184, CNB476, and CNB440 was 295, 116, and 97 mg/l, respectively, and was detected in media with sodium concentrations ranging from 292 to 387 mM and conductivities between 33.4 and 41.4 mS/cm (Fig. 1). The lowest medium concentration of sodium and conductivity to support the production of NPI-0052 by strains CNB440 and NPS21184 was 98 mM and 15.2 mS/cm, respectively. Strain CNB476 required higher sodium concentration (148 mM) and conductivity (20.8 mS/cm) in the medium than strains CNB440 and NPS21184 for production of NPI-0052.

Growth of washed cells of *Salinispora tropica* strains CNB440, CNB476, and NPS21184 in agar media containing low sodium ion (5.0 mM) or both low sodium (5.7 mM) and low chloride (14 mM) ions determined by ICP-DRC-MS analysis

Washed cells of strains CNB440, CNB476, and NPS21184 from the first seed cultures were used to examine the growth yield of *S. tropica* strains in the agar media containing either low levels of sodium (agar medium Kc4C) or low levels of sodium and chloride (agar medium Ks4C) with salt formulations containing the proper ionic strength (conductivity of 51.9 and 53.7 mS/cm, Table 2). The seed cultures of three *S. tropica* strains were centrifuge-washed twice in either wash medium A1.K (containing 30 g/l KCl) or A1.Ks (containing 48 g/l K_2SO_4) with no discrete sodium salt to reduce carryover of the sodium ion to the agar cultures. Even though no discrete sodium salt was added to wash media A1.K and A1.Ks, ICP-DRC-MS analysis demonstrated that both wash media A1.K and A1.

Table 2 ICP-DRC-MS analysis on key ion concentration (mM) in different media

Media	[Na]	[Cl]	[K]	[Mg]	[Co]	[S]	[Ca]	[Fe]	Conductivity (mS)
A1.K	5.0	368	441	0.21	5.1×10^{-5}	<0.42	0.14	0.008	52.0
A1.KS	5.0	0.9	637	0.21	5.1×10^{-5}	369	0.28	0.009	53.3
A1.Kc4C	5.0	379	458	20	1.2×10^{-3}	30	2.7	0.009	51.9
A1.Ks4C	5.7	14	649	23	1.2×10^{-3}	421	4.0	0.011	53.7

Ks contained 5.0 mM sodium ion (Table 2). The sodium ion was derived from the complex medium components peptone and yeast extract as determined by ICP-DRC-MS analysis (Tsueng and Lam 2008). As expected, the sodium concentration in media A1.Kc4C and A1.Ks4C was similar to wash media A1.K and A1.Ks, from 5.0 to 5.7 mM (Table 2). The chloride concentration in A1.Ks4C medium was also very low, at 14 mM (Table 2). All three *S. tropica* strains grew well in agar medium A1.Kc4C after 2 weeks of incubation, and excellent growth was achieved after 3 weeks of incubation (Table 3). While good growth of strains CNB440 and CNB476 was observed in A1.Ks4C agar medium, strain NPS21184 grew poorly in A1.Ks4C agar medium for the first 3 weeks before good growth was observed after 4 weeks of incubation. After 3 weeks of incubation, the growth of three strains was transferred to the corresponding fresh agar medium A1.Kc4C and A1.Ks4C to further eliminate the carryover of sodium ions from any extraneous sources. The growth of three *S. tropica* strains after the second transfer on agar media A1.Kc4C and A1.Ks4C is summarized in Table 3. Good growth of all three strains was observed on agar medium A1.Kc4C of the second transfer after 1 week of incubation. The growth rates of all three strains were very similar in A1.Kc4C agar medium. However, strains CNB440 and CNB476 grew much faster than strain NPS21184 in A1.Ks4C agar medium, achieving good growth after 1 week of incubation. It took 3 weeks of incubation before good growth of strain NPS21184 occurred. The reduction in growth rate of strain NPS21184 on agar medium A1.Ks4C of the second transfer might be due to the poor inoculum from the first transfer. The growth of the three *S. tropica* strains after 3 weeks of incubation on agar media A1.Kc4C and A1.Ks4C are shown in Figs. 2 and 3, respectively. No growth of three *S. tropica* strains was observed on agar media A1.K and A1.Ks at the first transfer after 4 weeks of incubation. The above observation demonstrated that high ionic strength (conductivity of 52.0 to 53.3 mS/cm, Table 2) in the media alone cannot support growth of *S. tropica* in low sodium media. The presence of proper concentrations of other ions, such as magnesium (~20 mM) and calcium (2.7 to 4.0 mM), are required to support growth in low sodium media (Table 3).

Growth of washed cells of *Salinispora tropica* strains and production of NPI-0052 in liquid media containing low sodium seed (5.0 mM) and production (11.0 mM) media

The washed cells of all three *S. tropica* strains were also used to inoculate into the liquid second seed media A1.Kc4C and A1.Ks4C to examine growth in submerged cultures in low-sodium and low-sodium and low-chloride media. None of the three strains grew in A1.Ks4C liquid medium, possibly due to the high pH (>8) of the cultures, and this portion of the experiment was terminated. After 2 to 3 days incubation in the A1.Kc4C medium, the second seed cultures were inoculated into a third seed medium A1.Kc4C to further eliminate the carryover of sodium ion from any extraneous sources. Both the second and third seed cultures were incubated for 5 days before determination of PCV and DCW (Table 4). Good growth of strains CNB440 (4.92 to 5.95 mg/ml DCW), CNB476 (5.20 to 6.73 mg/ml DCW), and NPS21184 (4.77 to 5.70 mg/ml DCW) was observed in the second and third seed-submerged cultures growing in A1.Kc4C medium containing 5.0 mM sodium. We did not observe any growth of the three *S. tropica* strains in seed medium supplemented only with 30 g/l of

Table 3 Growth of *Salinispora strains* CNB440, CNB476, and NPS21184 grown on low-sodium agar (Kc4C) and low-sodium and low-chloride agar (Ks4C)

Medium	CNB440				CNB476				NPS21184			
	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
Kc4C (first transfer)	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
Kc4C (second transfer)	++	++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
Ks4C (first transfer)	++	++	++	++	+++	+++	+++	+++	−	−/+	−/+	++
Ks4C (second transfer)	+++	+++	+++	+++	++	++	+++	+++	−/+	+	++	+++

Key: − no growth, −/+ poor growth, + fair growth, ++ good growth, +++ very good growth

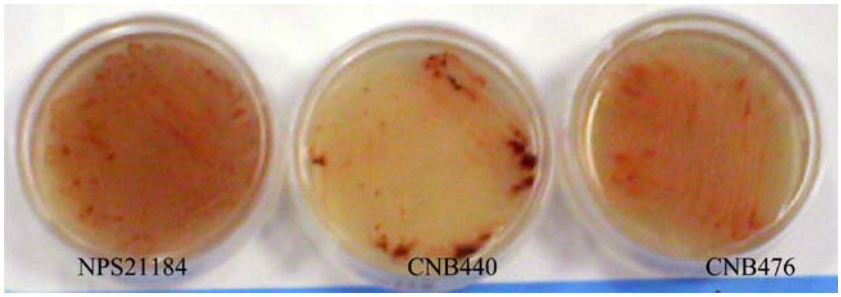

Fig. 2 Growth of washed cells of *Salinispora tropica* strains NPS21184, CNB440, and CNB476 on agar medium A1. Kc4C containing 5.0 mM sodium after 3 weeks of incubation (second transfer)

KCl, confirming the observation of the agar media's finding that proper concentrations of the supplementary ions together with the high ionic strength are required to support growth.

The third seed cultures were inoculated into production medium SHY.KcMC containing 11 mM sodium ion (Tsueng and Lam 2008) to determine the production of NPI-0052 by the three *S. tropica* strains. Maximal production of NPI-0052 by strains CNB440, CNB476, and NPS21184 in the low-sodium SHY.KcMC medium was 67, 147, and 176 mg/l, respectively (Fig. 4).

Discussion

The results shown here confirm our previous finding that *S. tropica* does not require seawater for growth, specifically by showing supporting data from the type strain CNB440 and the parent strain of strain NPS21184, strain CNB476. Both CNB strains can be grown on submerged and agar cultures containing the potassium-chloride-based (salt formulation III) medium with low sodium concentration at 5.0 mM, ~1% of the sodium content in seawater (450 to 500 mM), and ~30-fold lower than the sodium content in mammalian cell cultures (145 mM; Boder and Hull 1986). The growth of three *S. tropica* strains on the potassium-sulfate-based (salt formulation IV) agar medium containing 5.7 mM sodium and 14 mM chloride further substantiated our observation that *S. tropica* does not require seawater for growth since the potassium-sulfate-based medium does not resemble seawater in both sodium and chloride contents. While we did not observe any growth of the three *S. tropica* strains in the liquid medium A1.Ks4C probably due to high culture pH, by adjusting the medium pH to neutral or to slightly acidic, growth of the three *S. tropica* strains might be detected in this low-sodium and low-chloride liquid medium. We have not yet established the lowest concentration of sodium in the medium required to support growth of *S. tropica* since the majority of sodium is derived from the complex nitrogen sources in the medium. The only known sodium salt added to the media is 0.06 mM NaF. Furthermore, similar growth data for the three *S. tropica* strains were observed by replacing NaF with KF thus demonstrating the ability of the three *S. tropica* strains grown in media with no discrete sodium salt (unpublished data). Since sodium constitutes about 1% of the DCW of microorganisms (Nagodawithana 1998), we might expect a certain amount of sodium in the medium, at least 0.45 mM, to support the growth of 1 mg/ml DCW of the microorganism in submerged culture. To determine the absolute concentration of sodium, if any, for supporting the growth of *S. tropica*, a defined medium needs to be developed in which we can control the exact amount of sodium added. The accurate measurement of ions in the media by ICP-DRC-MS analysis is also a critical tool to help us understand the nutritional ion requirements necessary to support the growth of *S. tropica*.

The findings in this and the previous studies (Tsueng and Lam 2008; Tsueng et al. 2008) contradicted the conclusion of Mincer et al. (2002) and Maldonado et al. (2005) that *S. tropica* requires seawater for growth, particularly the requirement for sodium ion. However, no specific concentration of sodium growth requirement for *S. tropica* was reported in these publications. Maldonado et al. (2005) reported that the seawater growth requirement of the other *Salinispora* species, *S. arenicola*, is 25–50% seawater, approximately equivalent to 125 to 250 mM sodium concentration. A limited description of the protocol used

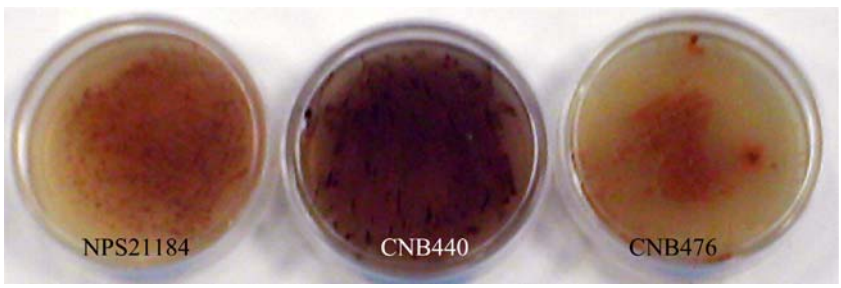

Fig. 3 Growth of washed cell of *Salinispora tropica* strains NPS21184, CNB440, and CNB476 on agar medium A1. Ks4C containing 5.7 mM sodium after 3 weeks of incubation (second transfer)

Table 4 Growth of *Salinispora tropica* strains CNB440, CNB476, and NPS21184 in liquid media Kc4C and SHY.Kc4C as determined by DCW

Media	CNB440	CNB476	NPS21184
	DCW (mg/ml)	DCW (mg/ml)	DCW (mg/ml)
A1.Kc4C (second seed)[a]	5.95	5.20	4.77
A1.Kc4C (third seed)[b]	4.92	6.73	5.70

[a] Five-day-old cultures were used for PCV and DCW determination

[b] Two-day-old second seed culture of strain NPS21184 was used to inoculate the third seed culture. Three-day-old second seed cultures of strains CNB440 and CNB476 were used to inoculate the third seed cultures. Five-day-old third seed cultures were used for PCV and DCW determination

to determine the seawater growth requirements of *Salinispora* was provided by Maldonado et al. (2005). In their study, ISP 2 agar and modified Bennett's agar supplemented with 0.5% (*w*/*v*) mannitol, 0.5% (*w*/*v*) soybean flour, and 3.5% (*w*/*v*) sodium chloride were used as media for the cultural and morphological studies. If the above basal media did not contain the proper concentration of ions to support the growth of *Salinispora*, simply substituting different concentrations of sodium chloride and seawater by Maldonado et al. (2005) cannot be expected to support the growth of *Salinispora*. The carbon and nitrogen sources in the media used by Maldonado et al. (2005) were also different from the media used in this study. This might have also contributed to the difference in supporting the growth of *S. tropica*. The Mincer et al. (2002) study provided the experimental protocol for determining the seawater growth requirement for *Salinispora*. The carbon and nitrogen sources (yeast extract and peptone) and the concentrations used in the medium by Mincer et al. (2002) are exactly the same as in this study; therefore, they both contain 5 mM sodium. The major difference between the media used in these two studies was the compositions of the salt formulations. The salt formulation of Sieburth (1979) was used in the Mincer et al. (2002) study and may not contain the proper composition to support the growth of *S. tropica*. This might explain the discrepancy in the observation of the growth of *S. tropica* between the two studies. From the current and previous studies (Tsueng et al. 2008; Tsueng and Lam 2008), we clearly demonstrate that specific combinations of salts, in addition to maintaining a high ionic strength in the medium, are required to support the growth of *S. tropica*, even at low sodium concentration of 5.0 mM.

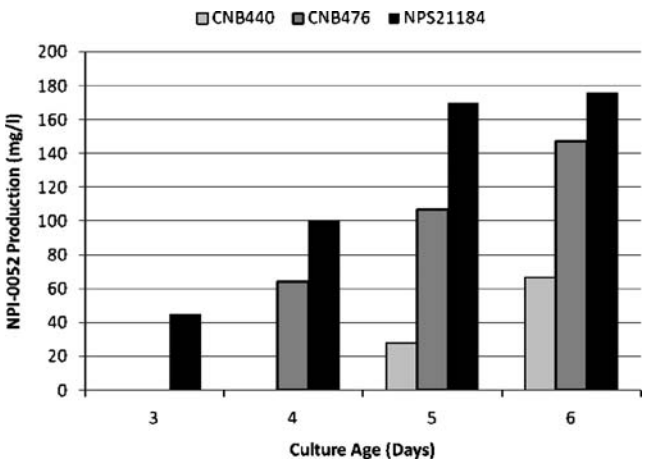

Fig. 4 Production of NPI-0052 by washed cell of *Salinispora tropica* strains CNB440, CNB476, and NPS21184 grown in production medium containing 11 mM sodium ion based on ICP-DRC-MS analysis

The three *S. tropica* strains have different sensitivity to the medium ionic strength for maintenance of viability and growth. The type strain CNB440 can survive, even though without new growth, in medium with a conductivity of 11.3 mS/cm and with measurable growth in medium at or above a conductivity of 13.6 mS/cm. As a reference point, two common media that support the growth of many terrestrial actinomycetes, ISP 1 and ISP 2 (Shirling and Gottlieb 1966), when prepared in deionized water, have conductivity of 1.29 and 0.97 mS/cm, respectively, significantly lower than the medium ionic strength required to support the growth of *S. tropica*. All three S. *tropica* strains did not grow with both ISP 1 and ISP 2 media prepared in deionized water (unpublished data). Therefore, we present the first convincing evidence in this study that *S. tropica* is indeed a true marine actinomycete by demonstrating that it requires a certain medium ionic strength as one of the criteria for growth. That is, *S. tropica* has a certain osmotic pressure requirement for growth, a trait for a marine actinomycete, and distinguishes it from terrestrial actinomycete, which has not been published before. Strain CNB476 has a more stringent medium ionic strength growth requirement, at medium conductivities of 16.6 mS/cm and above. We only examined the growth of three *S. tropica* strains in this study, and all three strains have different medium ionic strength requirements for growth. As there is very limited knowledge of the nutritional ion requirement in supporting the growth of marine actinomycetes, understanding this nutritional requirement will help us to isolate new marine actinomycetes and also to understand the adaptation and ecological roles of actinomycetes in the marine environment.

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