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Actinomycetes Producing Anti-Methicillin Resistant *Staphylococcus aureus* from Soil Samples in Nakhon Si Thammarat

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Abstract

A total of 64 different isolates of *Actinomycetes* were isolated from soil samples in Nakhon Si Thammarat. The anti-MRSA activity of *Actinomycetes* isolates was examined using agar plug and agar well diffusion methods. It was found that 19 isolates of selected strains showed anti-MRSA activity, there are only 10 isolates, namely, BA2, CK7, CK9, CK11, CO4, WU6, WU7, WU10, WU11 and WU14 which can inhibit all MRSA strains. However, strains tested CK9, CO4, WU6, WU7 and WU10 exhibited the anti-MRSA activities on both agar plug and agar well diffusion methods. The taxonomic studies indicated that the isolates of *Actinomycetes* CK9, CO4, WU6, WU7 and WU10 belong to *Streptomyces* sp. CFJ2, *Streptomyces antibioticus* strain 1022-257, *Streptomyces flaveolus* strain NRRL B-1334, *Streptomyces psammoticus* strain NBRC 13971 and *Streptomyces* sp. b26, respectively.

Keywords: *Actinomycetes*, Anti-MRSA, MRSA, *Streptomyces* sp.

Introduction

Over the last few years, resistance of *Staphylococcus aureus* to many antibiotics, such as ß-lactams and glycopeptides has become a main cause of concern. Methicillin resistant *Staphylococcus aureus* or MRSA causes a health risk, especially in patients with severe underlying disease or immunosuppression [1,2]. The resistance of numerous MRSA to commonly used bioactive compounds is presently an urgent focus of research, and new anti-MRSA molecules are necessary to combat these MRSA [3,4].

Actinomycetes have the capability to produce numerous bioactive compounds such as antibiotics, anti-parasitics, pesticides, herbicides and enzymes [5] and are of considerable importance in agriculture, and the veterinary and pharmaceutical industries [6]. The majority of *Actinomycetes* are free living and saprophytic bacteria and are a widely distributed group of microorganisms in nature found in soil, water, air and tissues of animals or plants. They are filamentous Gram positive bacteria having high G + C (> 55 %) content in their DNA [7,8].

The objective of this work was to screen *Actinomycete* strains producing potent bioactive compounds against MRSA.

Materials and methods

Sampling procedure

Soil samples, 5 - 15 cm in depth were collected from Nakhon Si Thammarat, Thailand. Those samples were air dried at 30 °C in an incubator.

Isolation of *Actinomycetes* from soil samples

Isolation of *Actinomycetes* was performed using a spread plate technique on a yeast extract-malt extract (YM) agar (yeast extract 4.0 g, malt extract 5.0 g, glucose 4.0 g, agar 20 g and distilled water 1,000 ml) complemented with 50 µg/ml nystatin. One gram of each soil sample was suspended in 9 ml of sterile distilled water for ten-fold serial dilution. Each of aqueous dilution, 10^{-7} - 10^{-10}, were applied onto YM agar plates and incubated at 30 °C for 7 to 14 days. The fungi-like embedded colonies were selected and transferred to new YM agar plates and incubated at 30 °C for 7 days. The colonies from the YM agar plates containing pure cultures were stocked in 15 % glycerol and kept at –30 °C for further anti-MRSA activity determination.

Determination of anti-MRSA activity

Anti-MRSA activity was tested by agar plug method against 10 isolates of MRSA (142, 189, 239, 1424, 6780, 7234, 7535, 7613, 7645 and 8176) obtained as gift from Nakhon Si Thammarat Maharaj Hospital, Thailand. On YM agar plates, after incubation of the selected *Actinomycete* strains for 4 days at 30 °C, Mueller Hinton agar (MHA) plates were spread with isolates of MRSA. Selected *Actinomycete*s colonies were transferred onto the MHA plates and incubated for 24 h at 37 °C. The size of the inhibition zone was measured. Also, anti-MRSA activity was tested by agar well diffusion. Briefly, the culture broth was collected from 6 days *Actinomycete* culture YM. Then 100 µl of the isolates were loaded into well bored and MRSA (0.5 McFarland turbidity standard) swabbed MHA plates. The MHA plates were incubated for 24 h at 37 °C and the diameter of the inhibition zones were measured to the nearest whole millimeter (modified from [9]).

Taxonomy of active *Actinomycete* isolates

The nucleotide sequence of the 16S rRNA gene for active *Actinomycete* isolates were identified following the directions given by the KU-vector custom DNA synthesis service unit, Department of Microbiology, Faculty of Science, Kasetsart University, Thailand. The obtained nucleotide sequences were compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov).

Results and discussion

Isolation of *Actinomycetes*

Sixty-four isolates of *Actinomycetes* were recovered from soil samples collected in Nakhon Si Thammarat, Thailand, using YM agar complemented with 50 µg/ml of nystatin for preliminary isolation as shown in **Table 1**. The YM agar seems to be specific and suitable for *Actinomycetes*, which use it as a carbon, nitrogen and energy source [10].

The research for new bioactive compounds, especially from *Actinomycetes* desires several types or over thousands of isolates in order to discover new bioactive compounds of pharmaceutical interest. The research will be more promising if diverse *Actinomycetes* are sampled and screened. Thus, soils were specifically collected under identified organic soil samples. This is based on several previous studies that *Actinomycetes* diversity may be influenced by the diversity of these bacteria which grow profusely in the humus and leaf litter layer [11].

Table 1 Sampling sources.

No.	Sampling sources	*Actinomycetes* code	Isolates number
1	Tesco Lotus, Thasala	LT1-LT2	2
2	Wangmatcha, Walailak University	WU1-WU2	2
3	Academic building 1, Walailak University	WU3-WU5	3
4	Scientific and technological equipment building 7, Walailak University	WU6	1
5	Walailak University gateway	WU7-WU15	9
6	Scientific and technological equipment building 5, Walailak University	WU16	1
7	Academic building 4, Walailak University	WU17-WU19	3
8	Thasala hospital	TH1	1
9	Eaknakhon road, Thawang	ER1-ER3	3
10	Thaksin community	TC1-TC2	2
11	Banana plantation, Changklang	BA1-BA2	2
12	Sewage treatment area, Changklang	KW1-KW5	5
13	Thoongthalad gateway	TT1-TT2	2
14	Cattle farm, Chulabhorn	CO1-CO6	6
15	Poultry farm, Chulabhorn	CK1-CK12	12
16	Bamboo plantation, Chulabhorn	MA1-MA8	8
17	Sunantha waterfall	SW1	1

Anti-MRSA activity of *Actinomycete* isolates

The Anti-MRSA activities of 64 *Actinomycetes* were subjected to primary screening using the agar plug method. It was observed that 19 isolates (approximately 30 %) were shown to have a very efficient *in vitro* anti-MRSA activity against isolates of MRSA as shown in **Table 2**. Altogether 19 putative isolates were subjected to secondary screening by agar well diffusion to further test the capability of primarily screened organisms. Approximately 5 isolates (~8 %) of *Actinomycetes* (CK9, CO4, WU6, WU7 and WU10) showed anti-MRSA activity, and gave an inhibition zone ranging from 13.52 to 25.68 mm as shown in **Table 3**. The putative isolates of primary screening did not show the activity in the secondary screening. This is because *actinomycete* isolates are often found to show activity on agar but not in the liquid culture [12].

Table 2 Anti-MRSA activities of *Actinomycetes* (agar plug method).

Actinomycetes isolates	Diameter of the inhibition zone (mm)									
	MRSA isolates									
	142	**189**	**239**	**1424**	**6780**	**7234**	**7535**	**7613**	**7645**	**8176**
BA1	-	-	-	-	4.67±0.36	-	-	-	-	-
BA2	5.61±0.23	7.01±0.24	6.58±0.18	8.30±0.10	6.79±0.33	7.67±0.40	4.88±0.52	7.28±0.13	6.14±0.19	7.29±0.06
CK7	5.88±0.05	6.44±0.29	4.19±0.05	6.42±0.05	5.58±0.11	4.94±0.02	5.19±0.07	6.13±0.01	5.40±0.01	5.21±0.30
CK9	7.24±0.16	7.28±0.10	7.46±0.19	7.25±0.06	8.21±0.14	5.20±0.11	6.18±0.06	5.23±0.14	8.90±0.03	3.73±0.17
CK11	4.09±0.12	5.94±0.09	4.95±0.03	3.99±0.03	3.05±0.07	5.41±0.04	5.27±0.38	4.08±0.13	3.16±0.09	2.91±0.13
CO4	19.99±0.19	20.44±0.12	20.52±0.04	21.35±0.28	18.69±0.47	20.56±0.06	18.78±0.31	20.35±0.17	21.46±0.04	21.40±0.21
LT1	-	-	-	4.65±0.10	-	5.23±0.09	-	-	-	-
TH1	-	-	-	3.26±0.07	-	-	-	-	-	4.90±0.16
WU3	8.17±0.33	11.10±0.54	7.31±0.04	-	11.88±0.17	7.30±0.34	-	7.50±0.03	12.27±0.54	11.47±0.08
WU4	-	9.40±0.58	10.60±0.20	-	8.70±0.15	-	-	2.82±0.18	7.59±0.25	9.87±0.18
WU5	3.56±0.23	7.46±0.25	4.11±0.13	-	8.65±0.09	-	7.37±0.06	7.09±0.07	-	-
WU6	7.89±0.08	8.68±0.13	10.60±0.02	7.81±0.08	12.32±0.25	6.81±0.25	6.67±0.37	9.54±0.18	7.29±0.08	9.50±0.08
WU7	15.91±0.10	16.09±0.04	14.04±0.13	16.41±0.11	15.04±0.10	12.46±0.14	18.73±0.40	17.79±0.31	18.80±0.11	17.39±0.03
WU8	4.26±0.15	-	8.08±0.08	-	-	6.54±0.02	-	4.28±0.06	-	-
WU9	-	-	-	-	-	-	-	-	-	4.45±0.20
WU10	5.33±0.21	7.08±0.09	10.48±0.16	6.68±0.20	5.76±0.08	5.31±0.04	6.44±0.06	5.57±0.30	5.48±0.05	6.44±0.10
WU11	8.65±0.22	9.32±0.08	10.09±0.02	7.56±0.08	7.88±0.17	7.38±0.23	9.79±0.06	8.43±0.16	8.86±0.10	9.37±0.06
WU12	-	5.93±0.05	4.45±0.12	5.23±0.28	6.59±0.01	6.55±0.17	4.60±0.58	-	5.42±0.18	-
WU14	8.53+0.30	9.31+0.22	9.97+0.06	7.63±0.05	7.98±0.33	7.37±0.16	9.67±0.40	8.63±0.11	9.49±0.08	8.87±0.76

Table 3 Anti-MRSA activities of *Actinomycetes* (agar well diffusion method).

Actinomycetes isolates	Diameter of the inhibition zone (mm)									
	MRSA isolates									
	142	**189**	**239**	**1424**	**6780**	**7234**	**7535**	**7613**	**7645**	**8176**
CK9	18.83±0.41	17.65±0.60	19.17±1.17	16.76±0.34	16.75±0.52	16.92±0.40	16.41±0.09	13.52±0.08	15.73±0.01	16.82±0.46
CO4	24.51±0.04	23.88±0.18	25.68±0.35	24.21±0.10	22.40±0.21	23.71±0.04	25.11±0.11	23.24±0.37	23.55±0.32	25.56±0.08
WU6	22.38±0.09	20.77±0.29	20.99±0.35	22.32±0.08	21.79±0.32	21.96±0.26	23.00±0.02	22.08±0.11	21.81±0.37	21.38±0.25
WU7	20.84±0.51	22.29±0.52	19.67±0.10	20.53±0.07	20.04±0.19	22.44±0.04	20.41±0.25	18.84±0.35	23.08±0.69	19.48±0.88
WU10	19.67±0.12	18.86±0.36	18.82±0.57	19.36±0.30	19.98±0.13	18.80±0.06	20.14±0.03	18.90±0.01	20.95±0.04	19.41±0.08

Taxonomy of active *Actinomycete* isolates

Based on the morphological and microscopic studies, we found that the *Actinomycete* isolates, CO4 and WU7, produced a yellow pigment on the YM plate (**Figure 1**) and all of them showed different types of spore morphology as shown in **Figure 2**. In order to identify the strain of *Actinomycete*, the analysis of the partial nucleotide sequence for the 16S rRNA gene of each *Actinomycete*, CK9, CO4, WU6, WU7 and WU10 was carried out using a 16S rRNA universal primer. The result strongly suggested that the isolates CK9, CO4, WU6, WU7 and WU10 showed high similarity with the 16S rRNA gene of *Streptomyces* sp. CFJ2, *S. antibioticus* strain 1022-257, *S. flaveolus* strain NRRL B-1334, *S. psammoticus* strain NBRC 13971 and *Streptomyces* sp. b26, respectively as summarized in **Table 4**.

In several previous studies, the *S. antibioticus* and *S. flaveolus* were antibacterial substances producing strains, actinomycins and polycyclic ethers, respectively. These antibacterial substances were inhibited Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* [13-16]. It was also reported that *S. psammoticus* produced polyketide antibiotics effective against MRSA [17]. However, the benefits of active *Actinomycete* isolates and the bioactive compound characterization are subject to further studies.

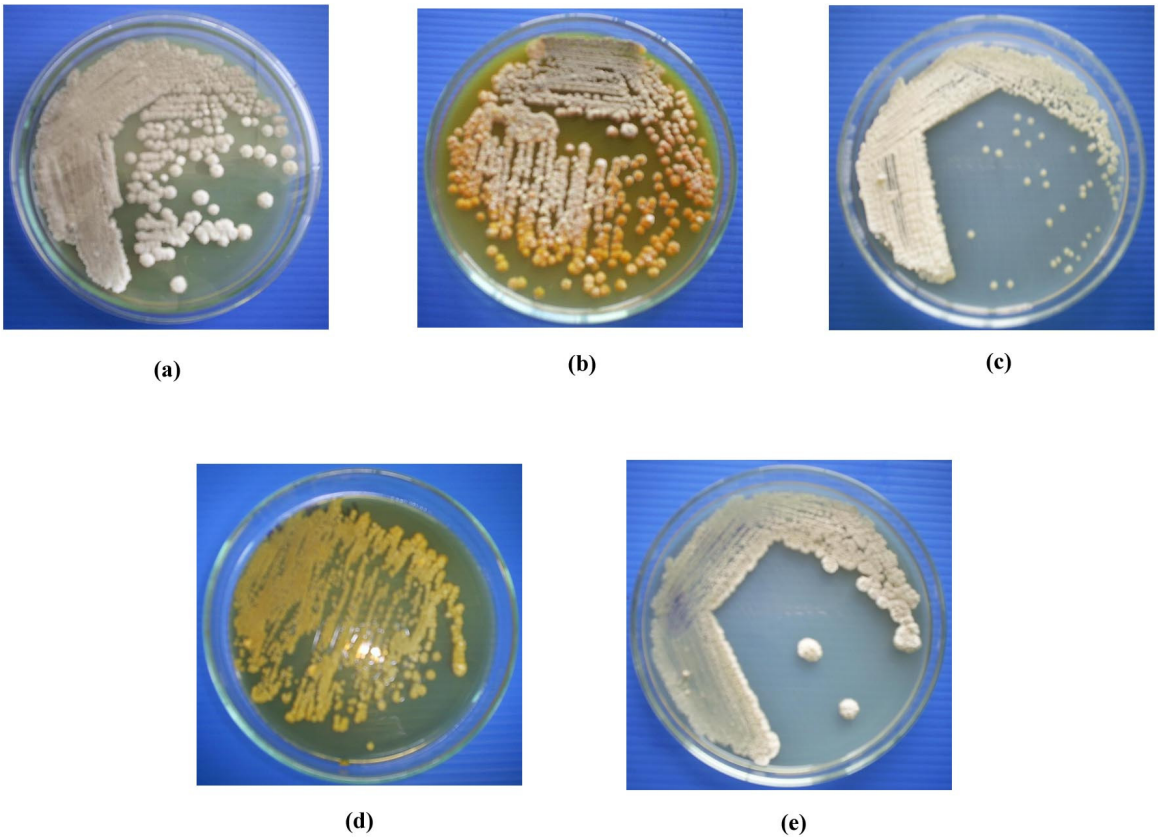

Figure 1 The colony of *Actinomycetes* CK9 (a), CO4 (b), WU6 (c), WU7 (d) and WU10 (e).

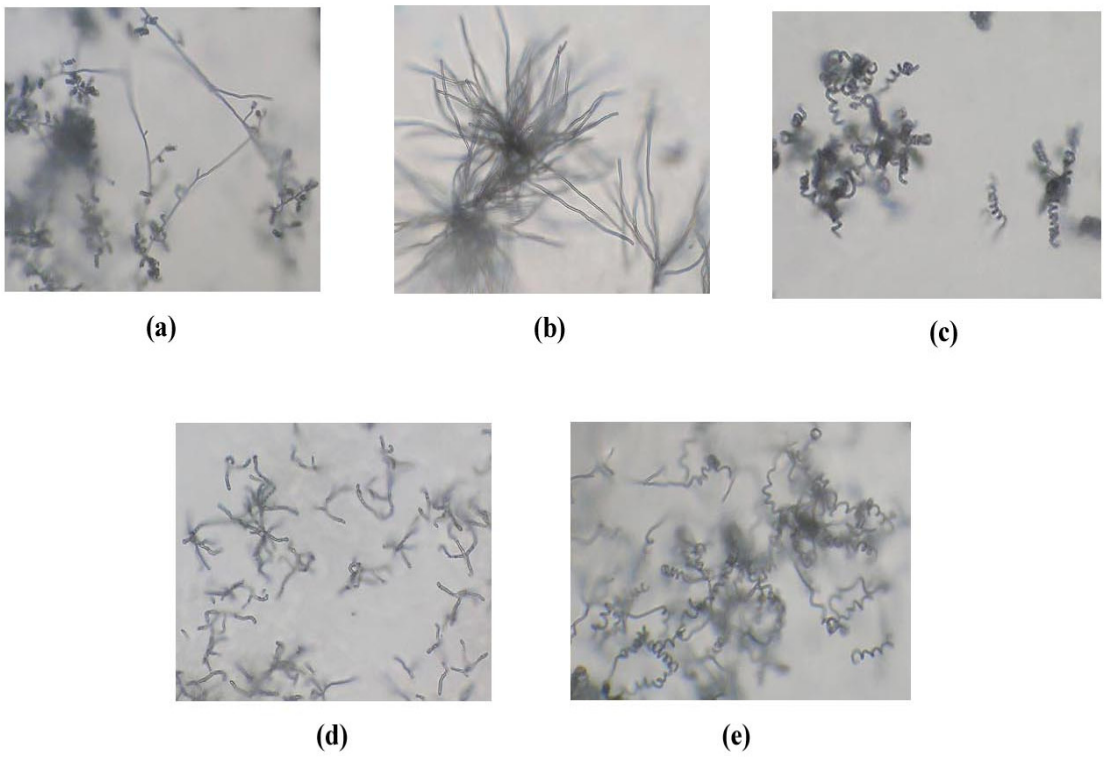

Figure 2 The spore morphology of *Actinomycetes* CK9 (a), CO4 (b), WU6 (c), WU7 (d) and WU10 (e) under a light microscope (400X).

Table 4 The nucleotide sequence analysis of the *Actinomycetes* 16S rRNA gene.

***Actinomycetes* isolates**	**Closets sequence**	**% Similarity**	**Accession number**
CK9	*Streptomyces* sp. CFJ2 Length = 1476 Score = 1152 bits (581), Expect = 0.0 Gaps = 0/581 (0 %) Strand = Plus/Plus	581/581(100 %)	EU100402
CO4	*Streptomyces antibioticus* strain 1022-257 Length = 1490 Score = 1328 bits (670), Expect = 0.0 Gaps = 0/670 (0 %) Strand = Plus/Plus	670/670 (100 %)	EF063450
WU6	*Streptomyces flaveolus* strain NRRL B-1334 Length = 1481 Score = 1189 bits (600), Expect = 0.0 Gaps = 0/600 (0 %) Strand = Plus/Plus	600/600 (100 %)	EF654098
WU7	*Streptomyces psammoticus* strain: NBRC 13971 Length = 1461 Score = 1215 bits (613), Expect = 0.0 Gaps = 0/621 (0 %) Strand = Plus/Plus	619/621 (99 %)	AB184554
WU10	*Streptomyces* sp. b26 Length = 1465 Score = 1176 bits (593), Expect = 0.0 Gaps = 0/593 (0 %) Strand = Plus/Plus	593/593 (100 %)	EU260042

Conclusions

Five isolates of *Actinomycete*s, CK9, CO4, WU6, WU7 and WU10, were shown to have a good potential to inhibit the growth of 10 clinical isolates of MRSA. They were identified as *Streptomyces* sp. CFJ2, *S. antibioticus* strain 1022-257, *S. flaveolus* strain NRRL B-1334, *S. psammoticus* strain NBRC 13971 and *Streptomyces* sp. b26, respectively.

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