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Molecular analysis of partially sequenced Ascomycin genes detected in a local *Streptomyces. sp* isolated from soil in Iraq.

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Abstract

Among 65 local *Streptomyces* isolates, one was found to harbor the immune suppressive compound ascomycin genes. This was confirmed by specific PCR amplification using primers designed for this purpose. The 16sRNA amplification proved that this isolate belong to *Streptomyces hygroscopicus* var *ascomyceticus.* Molecular analysis of the seven genes studied in this work including FCKWB, FCKWA, FCKWC, FCKWN, FCKWD, FCKWL, and FCKWO showed the presence of multiple ORFs in each one. The longest one was found in FCKWB with 22575 bp, and the shortest was found in FCKWO with length of 1032 bp. Long and high numbers of ORFs found in FCKWB, FCKWA, and FCKWC may suggest that these genes are candidate for cloning and production of different forms of FK520 (ascomycin) in other bacteria.

Keyword: *Streptomyces*, macrolides, immunosuppressive compounds, FK520

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Introduction

Streptomycetes are Gram-positive bacteria belonging to the order Actinomycetales and the family Streptomycetaceae; roughly, streptomycetes are represented by more than 570 different species (Kämpfer, 2006). In nature, streptomycetes have a quite widespread distribution and are found in soils of very different structure and chemistry, in surface waters, and in plants as rhizosphere colonizers or true endophytes. In different natural environments, they often play a major role in nutrient cycling. They may also have a strong influence in the population structure of environmental microbial communities due to their ability to produce a large set of secondary metabolites, many of which are of clinical and biotechnological importance (Strobel and Long, 1998). From the medical point of view, *Streptomyces* is the largest antibiotic-producing genus against clinical microorganisms (fungi and bacteria) and parasites. They also produce other clinically important bioactive compounds such as immunosuppressants (Vurukonda *et al*., 2018).

FK520 (ascomycinis macrocyclic amino acid-linked polyketides isolated from *Streptomyces hygroscopicus* subsp. *ascomyceticus*, and *Streptomyces* hygroscopicus. FK520 is an important therapeutics in immunosuppression and in combating inflammatory disease. Since its discovery, additional related compounds (Salituro GM, *et al.* 1995). The immunosuppressive mechanisms ofFK520 is distinct. The FK520 inhibits calcineurin. Calcineurin is a phosphatase required for the dephosphorylation of nuclear factor of activated T cells (NFAT), which then translocates to the nucleus and stimulates the release of IL-2 and other downstream cytokines (Andexera *et al.*, 2011).

FK506 and FK520 also accelerate the rate of nerve regeneration (Gold, 2000; Hamilton and Thomas, 2000), being very potent in promoting neurite outgrowth in PC12 cells, SHSY5Y cells, and primary neuronal cultures. The neurotrophic property of these compounds has been established in a variety of animal models including, sciatic nerve injury (Lee *et al.*, 2000), spinal cord injury (Emborg *et al.*, 2001).

Genes encoding the three polyketide synthase (PKS) subunits (fkbB, fkbC and fkbA) was studied (K. Wu *et al.*, 2000), but no extended studies were found concerning other genes involving this macrolide synthesis. This article focused on the analysis of other genes involving this immunosuppressive compound.

Materials and methods

Streptomyces isolates

A 65 *Streptomyces* isolates resemble the culture collection of Biotechnology Research Center was created, identified, and catalogued by Dr. Rebah N. Algafari (Algafari, 2014).

Cultivation medium

Streptomyces species were cultured on ISP4 broth medium (Atlas, 2005), which inoculated with 1^{10} spores and incubated at 30^0C in a shaker incubator at 150 rpm for 5 days. Growing cultures were collected by centrifugation and further subjected to DNA isolation.

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DNA isolation

DNA was isolated from each culture using Favorgen FATGK 001-2 DNA extraction kit following the company instructions. DNA yield was 125 ng with purity 1.9 – 2, and kept in deep freeze until used.

PCR primers

During this study, 28 primers were designed for Ascomycin clusters FKBP, FCKWB, FCKWL, FKWB, FKWC, FKWO, FKWN, FKWA, and FKWD depending on the GenBank: AF235504.1.

Table 1 shows the full details of each primer used in this study.

Table (1). Primers details used to amplify clusters of Ascomycin studied in this work.

	Sequence (5'->3')	Start	Stop	Tm	Product length
Primer name	**FKBP1**				
Forward primer	CGACTACTCCGTCCAGGAGA	2028	2047	60	636
Reverse primer	CGGATCTTGACCTGGTCGTC	2663	2644	60	
Primer name	**FKBP2**				
Forward primer	GCATGTTCGTCAACACCCTG	935	954	59.76	965
Reverse primer	GGAGGTGTAGATGGCGTAGG	1899	1880	59.33	
Primer name	**FKBP3**				
Forward primer	CCTACGCCATCTACACCTCC	1880	1899	59.33	778
Reverse primer	TTGACCTGGTCGTCGATGC	2657	2639	60.08	
Primer name	**FCKWL1**				
Forward primer	GCGTCACCATGAAGACCGT	218	236	60.37	722
Reverse primer	GTCGAAGGCGATGTGGTCG	939	921	61.16	
Primer name	**FCKWL2**				
Forward primer	CGCGACATCAAGCGCATC	25	42	60.05	713
Reverse primer	GTCTTCGGCAGCTCGGTCTT	737	718	62.49	

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Primer name	**FKWB1**				
Forward primer	TACTTCAAGGTCCCGGACGA	2539	2558	59.82	1133
Reverse primer	GTCTTGCCCATCTCGACGAA	3350	3331	60.53	
Primer name	**FKWB2**				
Forward primer	GAGATGGGCAAGACCGACAT	2539	2558	59.82	812
Reverse primer	ACGGTGTTGGTGAACTCGAC	3350	3331	60.53	
Primer name	**FKWB3**				
Forward primer	CTGATGCTGGGCTACCACG	1093	1111	60.52	347
Reverse primer	TCGTCCGGGACCTTGAAGTA	1439	1420	60.25	
Primer name	**FKWB4**				
Forward primer	CTTCGTCGGCCACTCCATC	14859	14877	60.52	369
Reverse primer	TCGTGGTAGGTCAGGGACTC	15227	15208	60.32	
Primer name	**FKWB5**				
Forward primer	GATCGGCTCCCTGATCACCC	21252	21271	62.11	1290
Reverse primer	TCCATGTCGTCGAACAGCTC	22541	22522	60.11	
Primer name	**FKWB6**				
Forward primer	AGACCCTGATCGCCGACTA	19931	19949	59.77	482
Reverse primer	CTCGTGGTGGAAGGCGTAG	20412	20394	60.15	
Primer name	**FKW C1**				
Forward primer	TCATCGAGATGGGCAAGACC	9359	9378	59.53	1405
Reverse primer	CGGTCGATCAGGTCGAAGAT	10763	10744	59.34	
Primer name	**FKW C2**				
Forward primer	GACACCGTCTACGCCGAG	7975	7992	59.90	1410

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Reverse primer	GATGTCGGTCTTGCCCATCT	9384	9365	59.82	
Primer name	**FKW C3**				
Forward primer	ATGGCCGAGAACGACCTGAT	1	20	61.33	813
Reverse primer	GGACAGCTTCTCGACGACC	813	795	60.15	
Primer name	**FKW C4**				
Forward primer	GAGACCGGCTACTCCTACGG	3070	3089	61.17	529
Reverse primer	CCAGGACGAACTCGGTCAG	3598	3580	59.79	
Primer name	**FKW O1**				
Forward primer	ACTACGTCTCCGGCATCAAC	416	435	59.83	610
Reverse primer	ATGCCCTCGATCTCGACCA	1025	1007	60.46	
Primer name	**FKW O2**				
Forward primer	GTCGAGGCCCTGTCCATCTC	28	47	62.02	408
Reverse primer	GTTGATGCCGGAGACGTAGT	435	416	59.83	
Primer name	**FKW N1**				
Forward primer	CTGTCCCTGGCCCTGATCTA	1182	1201	59.25	1500
Reverse primer	AGGCGATCTCCTTGTTGGTG	2681	2662	60.53	
Primer name	**FKW N2**				
Forward primer	CCTGATCGACGACATCCGC	693	711	60.66	508
Reverse primer	GTAGGCGAACTCCAGGTAGG	1200	1181	59.25	
Primer name	**FKW N3**				
Forward primer	TACCTGGGCTCCGTCAAGT	11317	11335	60.23	1216
Reverse primer	GGTTGCGGTACCAGTAGGAC	12532	12513	60.11	
Primer name	**FKW N4**				

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Forward primer	CCGTTCCAGCACAAGGACTA	2416	2435	59.68	530
Reverse primer	ATGTCCTCGAAGCGGTCGTA	2945	2926	61.03	
Primer name	**FKW N5**				
Forward primer	GAGTCCTACTGGTACCGCAAC	12511	12531	60.14	1472
Reverse primer	TCGGTCTCGACCAGGAAGAA	13982	13963	60.25	
Primer name	**FKW D1**				
Forward primer	TCAAGCTGGTCACCAACGAC	99	117	60.45	337
Reverse primer	GTGGAGTACAGCGGGATGAC	435	416	60.04	
Primer name	**FKW D2**				
Forward primer	CAAGACCCTGACCGACGAC	513	531	60.08	407
Reverse primer	GGTTGGCGGTGGAGTACAG	919	901	60.38	

Classification of *Streptomyces* strain

Three primers were designed to classify *Streptomyces* strain harboring Ascomycin gene depending on information provided by NCBI GenBank: FJ799176.1. Primers details are listed in table 2.

Table (2). Primers details for 16 sRNA used to classify *Streptomyces* strain harboring Ascomycin gene.

	Sequence (5'->3')	Start	Stop	Tm	Product length
Primer name	**SH16-1**				
Forward primer	CTTAACCCCGGGTCTGCATT	558	577	60.03	825
Reverse primer	TCGGGTGTTACCGACTTTCG	1382	1363	60.04	
Primer name	**SH16-2**				
Forward primer	ATTAGTGGCGAACGGGTGAG	55	74	60.11	553
Reverse primer	CCCCTACCGAACTCTAGCCT	607	588	60.11	
Primer name	**SH16-3**				
Forward primer	TTCAGCAGGGAAGAAGCGAG	398	417	60.04	988

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Reverse primer	GCTTCGGGTGTTACCGACTT	1385	1366	60.32	

PCR amplification conditions

DNA from all 65 *Streptomyces* isolates was subjected to amplification by PCR type Lab Net (USA) using primers designed for Ascomycin in this study. Amplification conditions were as follow.

Initial denaturation	94 ^{0}C	5 minutes	1 cycle
Denaturation	94 ^{0}C	45 sec	35 cycles
Annealing	62 ^{0}C	1 minute	
Extension	72 ^{0}C	1.30 minute	
Final extension	72 ^{0}C	10 minutes	1 cycle
Hold	4 ^{0}C	∞	

DNA sequencing

PCR product showed bands during gel electrophoresis were sent for sequencing by Macrogen biotech Corp. DATA received from the company were analyzed afterward.

DATA analysis

DNA sequences obtained from PCR products were analyzed using available tools provided by NCBI at https://www.ncbi.nlm.nih.gov.

Results

PCR technique is considered a fast, reliable, and highly specific method to identify an organism with specific secondary metabolite when information about the gene(s) involving production of this compound is available. We used this approach for rapid identification of *Streptomyces* strain harboring genes responsible for Ascomycin production. Preliminary identification of FK520 is shown in figure 1.

Figure (1). Amplification different gene of ascomycin clusters.

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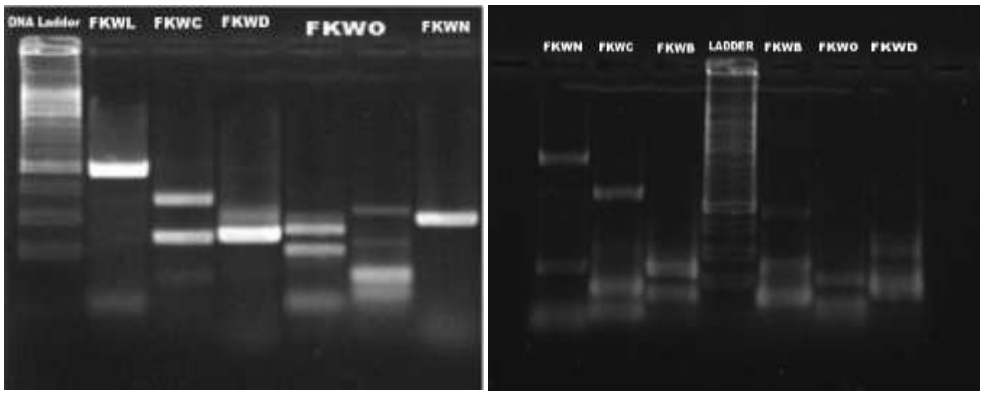

Among the 65 *Streptomyces* tested, only one gave specific bands for ascomycin and was selected for this study.

Morphological characteristics

The selected *Streptomyces* isolate was cultured on different types of media, like *Streptomyces* agar (Himedia, India, ISP4, and ISP2) for morphological characterization. The isolate appeared light grey, powdery aerial mycelia, with smooth edges and white substrate mycelia. Figure 2 shows the morphology of the *Streptomyces* isolate used in this study.

Figure (2). Growth of Streptomyces sp. Under study on ISP4 medium. Colonies appear light grey in aerial mycelia, smooth edges, and white substrate mycelia.

Growth on carbon and nitrogen sources

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Streptomyces isolate was tested for growth on different carbon sources. Results obtained are listed in table 3.

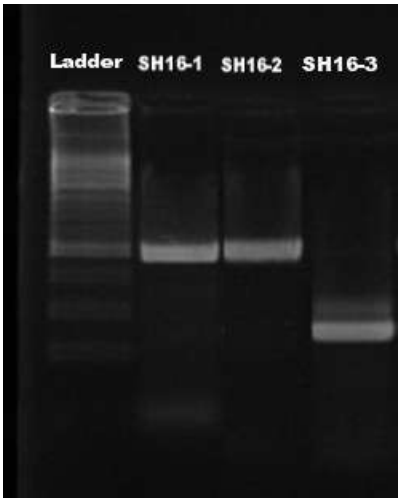
Table (3). Growth of *Streptomyces* isolate on different carbon and nitrogen sources.

No.	Carbon source	Growth	Nitrogen source	Growth
1.	Glycerol	Good	Alanine	Good
2.	Mannitol	Good	Aspartic acid	Good
3.	D(+) trehalose	Good	Histidine	Good
4.	Mannose	Good	Glutamine	Good
5.	D(+) xylose	Fair	Glycine	Good
6.	Raffinose	Good	Arginine	Good
7.	Lactose	Fair	Proline	Good
8.	Sucrose	Fair	Valine	Good
9.	Sorbitol	Fair	Leucine	Good
10.	L – Rahmnose	Weak	Urea	Fair
11.	Inulin	Weak	Phenylalanine	Fair
12.	Sorbose	Non	Glutamic acid	weak

Molecular classification of *Streptomyces* isolate

Three primers were designed to identify *Streptomyces* isolate subjected to this study depending on 16sRNA gene. The design mainly depended on previously sequenced *Streptomyces hygroscopicus* 16sRNA gene. Result is shown in figure (3).

Figure (3). Amplification of 16sRNA gene from *Streptomyces* isolate under study.



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Analysis of partially sequenced genes

Seven genes from ascomycin cluster were sequenced and analyzed. The analysis blast them against the similar once at NCBI website to determine the similarity between them, identification of their open reading frames (ORFs) included within each sequence to establish the involvement of each gene the biochemical synthesis of F520 in the culture.

DATA obtained are summarized in table 4.

Table (4). Summary of molecular analysis of genes constructing ascomycin cluster.

Gene name	FKBP	No. of ORFs			12
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	+	**1**	**<1**	**>4485**	**4485 | 1494**
ORF4	-	2	3113	2778	336 | 111
ORF2	-	2	4322	4017	306 | 101
ORF10	-	2	1169	918	252 | 83
ORF11	-	2	821	651	171 | 56
ORF5	-	2	2669	2511	159 | 52
ORF9	-	2	1658	1530	129 | 42
ORF7	-	2	2153	2031	123 | 40
ORF6	-	2	2468	2352	117 | 38
ORF12	-	2	119	>3	117 | 38
ORF8	-	2	1979	1890	90 | 29

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Gene name	FKBP	No. of ORFs			12
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF3	-	2	3392	3315	78 | 25

Gene name	FCKWB		No. of ORFs		41
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF1	**+**	**1**	**<1**	**>22575**	**22575 | 7524**
ORF24	-	2	10361	9240	1122 | 373
ORF7	-	2	19859	18852	1008 | 335
ORF9	-	2	18251	17259	993 | 330
ORF19	-	2	13496	12516	981 | 326
ORF26	-	2	8639	7665	975 | 324
ORF35	-	2	3839	3057	783 | 260
ORF36	-	2	3041	2349	693 | 230
ORF17	-	2	14678	14097	582 | 193
ORF3	-	2	22346	21783	564 | 187
ORF32	-	2	5360	4842	519 | 172

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Gene name	FCKWB		No. of ORFs		41
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF13	-	2	16031	15546	486 | 161
ORF28	-	2	7322	6885	438 | 145
ORF5	-	2	20828	20415	414 | 137
ORF25	-	2	9125	8736	390 | 129
ORF34	-	2	4325	3939	387 | 128
ORF18	-	2	13982	13596	387 | 128
ORF8	-	2	18737	18351	387 | 128
ORF40	-	2	779	420	360 | 119
ORF12	-	2	16487	16164	324 | 107
ORF37	-	2	2324	2013	312 | 103
ORF6	-	2	20249	19947	303 | 100
ORF21	-	2	11990	11697	294 | 97
ORF39	-	2	1391	1104	288 | 95
ORF33	-	2	4724	4440	285 | 94
ORF15	-	2	15173	14895	279 | 92

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Gene name	FCKWB		No. of ORFs		41
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF14	-	2	15488	15219	270 | 89
ORF30	-	2	6221	5955	267 | 88
ORF10	-	2	17042	16785	258 | 85
ORF23	-	2	11051	10815	237 | 78
ORF20	-	2	12482	12249	234 | 77
ORF22	-	2	11561	11388	174 | 57
ORF2	-	2	22538	22374	165 | 54
ORF27	-	2	7508	7353	156 | 51
ORF38	-	2	1958	1809	150 | 49
ORF29	-	2	6662	6546	117 | 38
ORF31	-	2	5780	5670	111 | 36
ORF41	-	2	215	105	111 | 36
ORF11	-	2	16688	16587	102 | 33
ORF16	-	2	14876	14781	96 | 31
ORF4	-	2	21779	21702	78 | 25

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Gene name	FCKWL		No. of ORFs		3
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF1	**+**	**1**	**<1**	**>1035**	**1035 | 344**
ORF2	-	2	989	255	735 | 244
ORF3	-	2	227	>3	225 | 74

Gene name	FKWC		No. of ORFs		25
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF1	**+**	**1**	**<1**	**>10773**	**10773 | 3590**
ORF7	-	2	8792	7983	810 | 269
ORF21	-	2	1964	1446	519 | 172
ORF10	-	2	6797	6279	519 | 172
ORF24	-	2	1034	537	498 | 165
ORF13	-	2	5867	5376	492 | 163
ORF5	-	2	9650	9183	468 | 155
ORF15	-	2	4757	4290	468 | 155
ORF16	-	2	4268	3825	444 | 147

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Gene name	FKWC		No. of ORFs		25
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF14	-	2	5276	4839	438 | 145
ORF25	-	2	401	>3	399 | 132
ORF3	-	2	10388	9993	396 | 131
ORF18	-	2	3527	3186	342 | 113
ORF19	-	2	2825	2559	267 | 88
ORF8	-	2	7655	7392	264 | 87
ORF6	-	2	9044	8847	198 | 65
ORF4	-	2	9863	9681	183 | 60
ORF11	-	2	6161	5991	171 | 56
ORF22	-	2	1328	1158	171 | 56
ORF2	-	2	10646	10503	144 | 47
ORF12	-	2	5987	5877	111 | 36
ORF9	-	2	7217	7107	111 | 36
ORF20	-	2	2384	2274	111 | 36
ORF23	-	2	1154	1044	111 | 36

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Gene name	FKWC		No. of ORFs		25
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF17	-	2	3659	3573	87 | 28

Gene name	FKWO	No. of ORFs			5
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	+	**1**	**<1**	**>1032**	**1032 | 343**
ORF5	-	2	269	>3	267 | 88
ORF3	-	2	821	654	168 | 55
ORF4	-	2	611	462	150 | 49
ORF2	-	2	1025	894	132 | 43

Gene name	FKWN	No. of ORFs			8
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	+	**1**	**<1**	**>2739**	**2739 | 912**
ORF4	-	2	2033	1485	549 | 182
ORF8	-	2	479	>3	477 | 158
ORF2	-	2	2555	2289	267 | 88

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Gene name	FKWN	No. of ORFs			8
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF3	-	2	2267	2085	183 | 60
ORF5	-	2	1373	1197	177 | 58
ORF7	-	2	707	552	156 | 51
ORF6	-	2	875	774	102 | 33

Gene name	FKWA		No. of ORFs		39
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF1	**+**	**1**	**<1**	**>19188**	**19188 | 6395**
ORF12	-	2	14474	13401	1074 | 357
ORF33	-	2	3842	3009	834 | 277
ORF30	-	2	5855	5067	789 | 262
ORF20	-	2	10457	9672	786 | 261
ORF23	-	2	8750	8115	636 | 211
ORF32	-	2	4460	3897	564 | 187
ORF25	-	2	7745	7227	519 | 172

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Gene name	FKWA		No. of ORFs		39
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF15	-	2	12206	11691	516 | 171
ORF11	-	2	15080	14613	468 | 155
ORF5	-	2	17546	17088	459 | 152
ORF36	-	2	2066	1620	447 | 148
ORF2	-	2	19142	18702	441 | 146
ORF22	-	2	9329	8892	438 | 145
ORF9	-	2	15821	15423	399 | 132
ORF6	-	2	16973	16581	393 | 130
ORF38	-	2	896	510	387 | 128
ORF28	-	2	6563	6177	387 | 128
ORF39	-	2	368	>3	366 | 121
ORF8	-	2	16220	15861	360 | 119
ORF17	-	2	11282	10935	348 | 115
ORF27	-	2	6962	6678	285 | 94
ORF37	-	2	1295	1011	285 | 94

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Gene name	FKWA		No. of ORFs		39
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF16	-	2	11573	11289	285 | 94
ORF24	-	2	8054	7791	264 | 87
ORF13	-	2	13064	12801	264 | 87
ORF34	-	2	2693	2433	261 | 86
ORF31	-	2	4724	4470	255 | 84
ORF35	-	2	2360	2112	249 | 82
ORF4	-	2	17741	17550	192 | 63
ORF19	-	2	10688	10500	189 | 62
ORF29	-	2	6035	5856	180 | 59
ORF21	-	2	9515	9360	156 | 51
ORF3	-	2	18137	17994	144 | 47
ORF18	-	2	10919	10782	138 | 45
ORF7	-	2	16442	16314	129 | 42
ORF14	-	2	12626	12516	111 | 36
ORF26	-	2	7184	7080	105 | 34

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Gene name	FKWA		No. of ORFs		39
Label	Strand		Frame	Start Stop	Length (nt | aa)
ORF10	-	2	15212	15111	102 | 33

Gene name	FKWD	No. of ORFs			5
Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	**+**	**1**	**<1**	**>1164**	**1164 | 387**
ORF2	-	2	1025	708	318 | 105
ORF5	-	2	239	>3	237 | 78
ORF4	-	2	356	261	96 | 31
ORF3	-	2	485	393	93 | 30

Discussion

Streptomycetes are an important type of bacteria, they known with their ability to produce novel types of secondary metabolites that form the back bone of health, agricultural, and pharmacology industry. Ascomycin (FK520) is considered one of these novel compounds for its ability to reduce inflammation, prevent organ transplantation rejection. Cluster of ascomycin is composed of set of genes that play a role in this compound formation, and *Streptomyces hygroscopicus* var ascomyceticus is considered the known producer for this compound. The use of specific PCR amplification to determine which isolate is harboring ascomycin cluster proved to be reliable, fast, time and effort saving. All 65 *Streptomyces* isolates were investigated for their ability to produce this compound within 48 working hours without the use of chemical investigation for FK520 which may be time consuming with high cost.

The use of gene walking technique for which primers designed in this were used proved to give high precision results with less time. Each primer was designed to amplify specific part of the genes studied in this work, and data obtained were assembled for final gene sequence. However, sequence alignment of genes sequenced during this research with NCBI reserved data gave about 87% similarity suggesting genes

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encoding for ascomycin may come with different sequences, even when the local *Streptomyces* sp. Studied during this work gave 96% similarity with *Streptomyces hygroscopicus* 16sRNA at NCBI site.

Seven genes were studied during this work to establish their suggested role in ascomycin synthesis. This came from determination of ORFs contained in their sequence. The analysis came from the number of ORFs, the number of amino acids that will form enzymes involving ascomycin synthesis, number of ORFs, and length of the ORFs. This arranged the genes studied as follow from the longest ORF to the shortest: FCKWB, FCKWA, FCKWC, FCKWN, FCKWD, FCKWL, and FCKWO respectively. We suggest that, genes with long and high number of ORFs might be the candidate for cloning since they form the functional enzymes that play the key role in ascomycin synthesis.

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