CHAPTER V

Results

5.1 Isolation and characterization of *sakA* and *atfA* genes from *Penicillium marneffei*

P. marneffei sakA gene was amplified from genomic DNA of *P. marneffei* strain F4 using primers *SakA*-WF and *SakA*-WR. An approximately 1.7-kb amplified product (Figure 5.1) was sequenced for nucleotide analysis. Nucleotide blast program from NCBI was used to analyze the sequence. The blast result revealed that the 1.7-kb amplified product of the *P. marneffei* strain F4 contained the *sakA* open reading frame including 1,532 nucleotides interrupted by eight introns. The 1,068 nucleotide mRNA predicted a 355-amino acid protein with a molecular mass of 40.712 kDa with high similarity to kinases from the stress-activated MAPK family (Figure 5.2). This protein revealed 100% identical to the analogous *P. marneffei* ATCC 18224 (EEA25700), 94% identical to *A. fumigatus* Af293 SakA (EAL90626), 86% identical to HogA of *A. nidulans* FGSC A4 (AAF81523), 85% identical to MAP kinase Sty1 of *S. pombe* 972h (NP_592843) and 81% identical to Hog 1 of *S. cerevisiae* (CAA97680) (Figure 5.3). The conserved TGY phosphorylation lip found in the stress Hog1/Spc1/p38 MAPK family was shown at amino acid 171-173 (Figure 5.2).

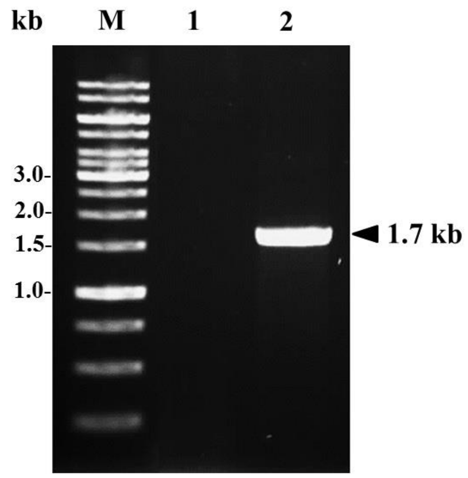

Figure 5.1 Amplification of *P. marneffei sakA* gene. *P. marneffei* genomic DNA was amplified by primers SakA-WF and SakA-WR. M represents 1 kb DNA ladder. Lane 1 contains negative control and lane 2 contains an amplified product of *P. marneffei sakA*, indicated by arrow head.

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1      ATG GCG GAG TTC GTC CGT GCC CAA ATT TTT GGC ACA ACC TTT GAG   45
1       M   A   E   F   V   R   A   Q   I   F   G   T   T   F   E    15

46     ATC ACC AGC AGA TAC ACC GAT CTC CAA CCT GTC GGA ATG GGT GCA   90
16      I   T   S   R   Y   T   D   L   Q   P   V   G   M   G   A    30

91     TTT GGC CTG GTC TGT TCT GCG AGG GAT CAA TTG ACT GGT CAA CCG   135
31      F   G   L   V   C   S   A   R   D   Q   L   T   G   Q   P    45

136    GTC GCT GTA AAG AAG ATA ATG AAG CCG TTC AGT ACT CCG GTA CTG   180
46      V   A   V   K   K   I   M   K   P   F   S   T   P   V   L    60

181    TCA AAA CGC ACA TAC CGA GAA CTC AAG CTT TTG AAA CAT CTA CGG   225
61      S   K   R   T   Y   R   E   L   K   L   L   K   H   L   R    75

226    CAT GAG AAT GTC ATT AGC CTC AGC GAC ATC TTC ATC TCA CCA TTG   270
76      H   E   N   V   I   S   L   S   D   I   F   I   S   P   L    90

271    GAA GAC ATT TAC TTC GTC ACC GAG CTC CTA GGC ACT GAC TTG CAC   315
91      E   D   I   Y   F   V   T   E   L   L   G   T   D   L   H    105

316    CGA CTC CTT ACC TCG CGT CCT CTT GAA AAA CAA TTC ATT CAG TAT   360
106     R   L   L   T   S   R   P   L   E   K   Q   F   I   Q   Y    120

361    TTC CTC TAT CAG ATT TTA CGC GGC TTG AAA TAT GTC CAC TCC GCT   405
121     F   L   Y   Q   I   L   R   G   L   K   Y   V   H   S   A    135

406    GGC GTT GTC CAC CGT GAT CTC AAA CCC AGC AAC ATT CTG ATC AAC   450
136     G   V   V   H   R   D   L   K   P   S   N   I   L   I   N    150

451    GAA AAT TGC GAT TTG AAA ATC TGT GAC TTT GGT CTA GCT CGA ATC   495
151     E   N   C   D   L   K   I   C   D   F   G   L   A   R   I    165

496    CAA GAT CCT CAG ATG ACG GGA TAC GTG TCA ACG AGA TAC TAC CGT   540
166     Q   D   P   Q   M   T   G   Y   V   S   T   R   Y   Y   R    180

541    GCC CCC GAA ATC ATG CTC ACA TGG CAG AAA TAT GAT GTC GAG GTG   585
181     A   P   E   I   M   L   T   W   Q   K   Y   D   V   E   V    195

586    GAT ATC TGG AGT GCC GGC TGT ATC TTT GCC GAA ATG CTG GAT GGA   630
196     D   I   W   S   A   G   C   I   F   A   E   M   L   D   G    210

631    AAG CCT CTT TTC CCT GGA AAA GAC CAT GTC CAC CAG TTT TCG ATC   675
211     K   P   L   F   P   G   K   D   H   V   H   Q   F   S   I    225

676    ATC ACC GAG CTT CTA GGC ACT CCT CCT GAC GAT GTC ATT GAA ACC   720
226     I   T   E   L   L   G   T   P   P   D   D   V   I   E   T    240

721    ATT TGC AGT GAA AAT ACC CTC CGA TTT GTA CAG TCT CTA CCA AAG   765
241     I   C   S   E   N   T   L   R   F   V   Q   S   L   P   K    255

766    CGC GAA CGC CAA CCA CTT GCT GCT AAG TTC AAG AAT GCT GAT CCA   810
256     R   E   R   Q   P   L   A   A   K   F   K   N   A   D   P    270

811    GCA GCT ATC GAC TTA CTC GAG CGG ATG CTC GTC TTT GAC CCA AAG   855
271     A   A   I   D   L   L   E   R   M   L   V   F   D   P   K    285

856    AAG CGA ATT CGT GCC GGA GAT GCG CTT GCC CAT GAA TAT CTT GCT   900
286     K   R   I   R   A   G   D   A   L   A   H   E   Y   L   A    300

901    CCA TAC CAT GAC CCC ACC GAC GAA CCA GTT GCC GAT GAG AAG TTC   945
301     P   Y   H   D   P   T   D   E   P   V   A   D   E   K   F    315

946    GAC TGG TCG TTT AAT GAC GCT GAT TTG CCC GTG GAC ACT TGG AAG   990
316     D   W   S   F   N   D   A   D   L   P   V   D   T   W   K    330

991    ATC ATG ATG TAC TCG GAG ATA TTG GAC TAT CAT AAC ATT GAC CAA   1035
331     I   M   M   Y   S   E   I   L   D   Y   H   N   I   D   Q    345

1036   TCC GCC GAT CCC GCC CAA GTT CCT TTA CAG TAA   1068
346     S   A   D   P   A   Q   V   P   L   Q   *
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Figure 5.2 The nucleotide and deduced amino acid sequences of *P. marneffei sakA* gene. Nucleotide and amino acid numbers are indicated on both sides. The conserved TGY phosphorylation lip is highlighted.

```
P. marneffei F4 SakA ---MAEFVRAQIFGTTFEITSRYTDLQPVGMGAFGLVCSARDQLTGQPVAVKKIMKPFST 57
P. marneffei SakA    ---MAEFVRAQIFGTTFEITSRYTDLQPVGMGAFGLVCSARDQLTGQPVAVKKIMKPFST 57
A. fumigatus SakA    ---MAEFVRAQIFGTTFEITSRYTDLQPVGMGAFGLVCSARDQLTGQPVAVKKIMKPFST 57
A. nidulans HogA     ---MAEFVRAQIFGTTFEITSRYTDLQPVGMGAFGLVCSARDQLTAQPVAVKKIMKPFST 57
S. pombe Sty1        ---MAEFIRTQIFGTCFEITTRYSDLQPIGMGAFGLVCSAKDQLTGMNVAVKKIMKPFST 57
S. cereviseae Hog1   MTTNEEFIRTQIFGTVFEITNRYNDLNPVGMGAFGLVCSATDTLTSQPVAIKKIMKPFST 60
                          **:*:***** ****.**.**:*:*********** * **.  **:*********

P. marneffei F4 SakA PVLSKRTYRELKLLKHLRHENVISLSDIFISPLEDIYFVTELLGTDLHRLLTSRPLEKQF 117
P. marneffei SakA    PVLSKRTYRELKLLKHLRHENVISLSDIFISPLEDIYFVTELLGTDLHRLLTSRPLEKQF 117
A. fumigatus SakA    PVLSKRTYRELKLLKHLRHENIISLSDIFISPLEDIYFVTELLGTDLHRLLTSRPLEKQF 117
A. nidulans HogA     PVLSKRTYRELKLLKHLRHENIISLSDIFISPLEDIYFVTELLGTDLHRLISSRPLEKQF 117
S. pombe Sty1        PVLAKRTYRELKLLKHLRHENIISLSDIFISPFEDIYFVTELLGTDLHRLLTSRPLETQF 117
S. cereviseae Hog1   AVLAKRTYRELKLLKHLRHENLICLQDIFLSPLEDIYFVTELQGTDLHRLLQTRPLEKQF 120
                     .**:*****************:*.*.***:**:********* *******: :****.**

P. marneffei F4 SakA IQYFLYQILRGLKYVHSAGVVHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 177
P. marneffei SakA    IQYFLYQILRGLKYVHSAGVVHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 177
A. fumigatus SakA    IQYFLYQILRGLKYVHSAGVVHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 177
A. nidulans HogA     IQYFLYQIMRGLKYVHSAGVVHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 177
S. pombe Sty1        IQYFLYQILRGLKFVHSAGVIHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 177
S. cereviseae Hog1   VQYFLYQILRGLKYVHSAGVIHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR 180
                     :*******:****:******:***************************************

P. marneffei F4 SakA YYRAPEIMLTWQKYDVEVDIWSAGCIFAEMLDGKPLFPGKDHVHQFSIITELLGTPPDDV 237
P. marneffei SakA    YYRAPEIMLTWQKYDVEVDIWSAGCIFAEMLDGKPLFPGKDHVHQFSIITELLGTPPDDV 237
A. fumigatus SakA    YYRAPEIMLTWQKYDVEVDIWSAGCIFAEMLEGKPLFPGKDHVNQFSIITELLGTPPDDV 237
A. nidulans HogA     YYRAPEIMLTWQKYDAKVDVWSAACIFAEMLLGAPLFPGKDHVNQFSIITELLGTPPDDV 237
S. pombe Sty1        YYRAPEIMLTWQKYNVEVDIWSAGCIFAEMIEGKPLFPGRDHVNQFSIITELLGTPPMEV 237
S. cereviseae Hog1   YYRAPEIMLTWQKYDVEVDIWSAGCIFAEMIEGKPLFPGKDHVHQFSIITDLLGSPPKDV 240
                     **************:.:**:***.******: * *****:***:******:***:** :*

P. marneffei F4 SakA IETICSENTLRFVQSLPKRERQPLAAK-------FKNADP-------AAIDLLERMLVFD 283
P. marneffei SakA    IETICSENTLRFVQSLPKRERQPLAAK-------FKNADP-------AAIDLLERMLVFD 283
A. fumigatus SakA    IQTICSENTLRFVKSLPKRERQPLANK-------FKNADP-------EAVDLLERMLVFD 283
A. nidulans HogA     IQTICSENTLRFVKSLPKREPQDLAKLPKFLALVHPDKKPEEDEDYKNTINLLKAMLVYN 297
S. pombe Sty1        IETICSKNTLRFVQSLPQKEKVPFAEK-------FKNADP-------DAIDLLEKMLVFD 283
S. cereviseae Hog1   INTICSENTLKFVTSLPHRDPIPFSER-------FKTVEP-------DAVDLLEKMLVFD 286
                     *:****:***:** ***:::    ::          .   .*          :::**: ***::

P. marneffei F4 SakA PKKRIRAGDALAHEYLAPYHDPTDEPVADEKFDWSFNDADLPVDTWKIMMYSEILDYHNI 343
P. marneffei SakA    PKKRIRAGDALAHEYLAPYHDPTDEPVADEKFDWSFNDADLPVDTWKIMMYSEILDYHNI 343
A. fumigatus SakA    PKKRIRAGEALAHEYLSPYHDPTDEPEAEEKFDWSFNDADLPVDTWKIMMYSEILDFHNI 343
A. nidulans HogA     PKDRISAEAALAAPYLAPYHDETDEPVAEEKFDWSFNDADLPVDTWKIMMYSEILDFHNI 357
S. pombe Sty1        PRKRISAADALAHNYLAPYHDPTDEPVADEVFDWSFQDNDLPVETWKVMMYSEVLSFHNM 343
S. cereviseae Hog1   PKKRITAADALAHPYSAPYHDPTDEPVADAKFDWHFNDADLPVDTWRVMMYSEILDFHKI 346
                     *:.** *  ***  * :**** **** *:  *** *:* ****:**::*****:*.:*::

P. marneffei F4 SakA DQSA---------DPAQVPLQ--------------------------------------- 355
P. marneffei SakA    DQSA---------DPAQVPLQ--------------------------------------- 355
A. fumigatus SakA    DQGN---------DAGQVLMEGGVAQAQQNYA---------------------------- 366
A. nidulans HogA     DQGG---------DINPALVEG-AGLNQQGFQ---------------------------- 379
S. pombe Sty1        DN-----------ELQS------------------------------------------- 349
S. cereviseae Hog1   GGSDGQIDISATFDDQVAAATAAAAQAQAQAQAQVQLNMAAHSHNGAGTTGNDHSDIAGG 406
                     .            :

P. marneffei F4 SakA -----------------------------
P. marneffei SakA    -----------------------------
A. fumigatus SakA    -----------------------------
A. nidulans HogA     -----------------------------
S. pombe Sty1        -----------------------------
S. cereviseae Hog1   NKVSDHVAANDTITDYGNQAIQYANEFQQ 435
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Figure 5.3 The multiple alignment of fungal Hog1/SakA amino acid sequences. *P. marneffei* strain F4 SakA amino acid sequence was aligned with SakA of *P. marneffei* ATCC 18224 (EEA25700), *A. fumigatus* Af293 SakA (EAL90626), HogA of *A. nidulans* FGSC A4 (AAF81523), Sty1 of *S. pombe* 972h (NP_592843) and Hog 1 of *S. cerevisiae* (CAA97680). The conserved TGY phosphorylation lip is highlighted.

P. marneffei atfA gene was amplified from genomic DNA of *P. marneffei* strain F4 using primers *AtfA*-WF and *AtfA*-WR. An approximately 1.68-kb amplified product (Figure 5.4) was sequenced for nucleotide analysis. Nucleotide blast program from NCBI was used to analyze the sequence. The blast result revealed that the 1.68-kb amplified product of the *P. marneffei* strain F4 contained the *atfA* open reading frame including 1,536 nucleotides with three introns. The 1,230 nucleotide mRNA predicted a 409-amino acid protein with a molecular mass of 43.5 kDa with high similarity to bZip transcription factor (Figure 5.5). This protein revealed 99.76% identical to the analogous *P. marneffei* ATCC 18224 (EEA27441), 67.24% identical to *A. fumigatus* Af293 AtfA (EAL92448), 65.77% identical to *A. oryzae* RIB40 AtfA (XP_001819834), 65.04% identical to AtfA of *A. nidulans* FGSC A4 (CBF83765) and 29.83% identical to Atf1 of *S. pombe* 972h- (NP_595652) (Figure 5.6). The conserved basic-leucine zipper (bZip) domain found in the bZip transcription factor family was shown at amino acid 352-405. Comparing to *P. marneffei* ATCC 18224, there is a base transition in the *atfA* gene of *P. marneffei* strain F4 at nucleotide position 949 from guanine to adenine resulting in changing of amino acid residue of the AtfA protein at position 317 from alanine to threonine.

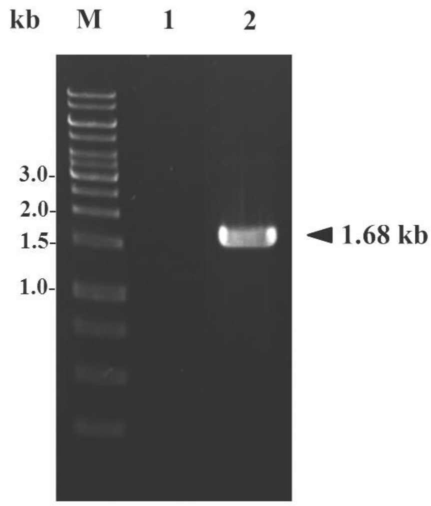


Figure 5.4 Amplification of *P. marneffei atfA* gene. *P. marneffei* genomic DNA was amplified by primers *AtfA*-WF and *AtfA*-WR. M represents 1 kb DNA ladder. Lane 1 contains negative control and lane 2 contains an amplified product of *P. marneffei atfA*, indicated by arrow head.

1	ATG ATC ACC TAT TCT TCA GCA AAT ACA AAA GAC CAG AAG CCT GCG	45
1	Met Ile Thr Tyr Ser Ser Ala Asn Thr Lys Asp Gln Lys Pro Ala	15
46	ACC GAC ACT GAA GCG TCT CAA TCA CTT CCA CCG CCG CCC CGC CCA	90
16	Thr Asp Thr Glu Ala Ser Gln Ser Leu Pro Pro Pro Pro Arg Pro	30
91	GCT GCT TCT GGT CCG GGC GAT ACA CCT GAC TAC TTC AAC TCG CTC	135
31	Ala Ala Ser Gly Pro Gly Asp Thr Pro Asp Tyr Phe Asn Ser Leu	45
136	CAC AAC CCA TTC TCC CTC GAG CCC AAC CCG TTC GAG CAG TCT TTT	180
46	His Asn Pro Phe Ser Leu Glu Pro Asn Pro Phe Glu Gln Ser Phe	60
181	GGC AAC CCA TCA GCT GAA ACC CCG GGG AAG TCA TTG CTT CCC CCA	225
61	Gly Asn Pro Ser Ala Glu Thr Pro Gly Lys Ser Leu Leu Pro Pro	75
226	GTC GCA TCC ATC ACG TCT CCT GCA CTG CCC GGA GCA AGC TCA GCC	270
76	Val Ala Ser Ile Thr Ser Pro Ala Leu Pro Gly Ala Ser Ser Ala	90
271	GGC TAT AAC TGG CCC AAC TCT TTA CGC TCC GGA CCG CTG AGT CCT	315
91	Gly Tyr Asn Trp Pro Asn Ser Leu Arg Ser Gly Pro Leu Ser Pro	105
316	GCT ATG CTG GCG GGC CCT ACT GGT GCC GAC TAT TTT GAC AGT ATC	360
106	Ala Met Leu Ala Gly Pro Thr Gly Ala Asp Tyr Phe Asp Ser Ile	120
361	GGA CGC GGC TTT CCA ACT CCC AAC GAG TCT TCA TTA CGA ACG GGG	405
121	Gly Arg Gly Phe Pro Thr Pro Asn Glu Ser Ser Leu Arg Thr Gly	135
406	TTG ACC CCT GGT GGT GGT GGG TCA ATG TTC CCA GCC CCG AGT CCT	450
136	Leu Thr Pro Gly Gly Gly Gly Ser Met Phe Pro Ala Pro Ser Pro	150
451	AAT ACT CAG GCG ATC CTT TCT CAA CTT CAA AGC GGT GGC GCG ACT	495
151	Asn Thr Gln Ala Ile Leu Ser Gln Leu Gln Ser Gly Gly Ala Thr	165
496	CCT TCG ACT CTC GAG TTC CAC CGC ACT GCC TTA AAC GCA GCC AAG	540
166	Pro Ser Thr Leu Glu Phe His Arg Thr Ala Leu Asn Ala Ala Lys	180
541	CGC AGC GGT TTC AAT GCA CCT ACG TCT AAC CCG ACT AGC GAT CCA	585
181	Arg Ser Gly Phe Asn Ala Pro Thr Ser Asn Pro Thr Ser Asp Pro	195
586	GAG CAG CTC CAG AAC ATG GAC AAG AAG ACT GCA CCA CCT GCC GTC	630
196	Glu Gln Leu Gln Asn Met Asp Lys Lys Thr Ala Pro Pro Ala Val	210
631	GAT CAG TTC ACG CAC CAC GAT GCC GCT GAT GCA GCC AAC GGC CTC	675
211	Asp Gln Phe Thr His His Asp Ala Ala Asp Ala Ala Asn Gly Leu	225
676	TTC ATG CTT GCG AAG GGT GGC CAG CCC AAC AAT GAC GCG TTC GCG	720
226	Phe Met Leu Ala Lys Gly Gly Gln Pro Asn Asn Asp Ala Phe Ala	240
721	GCA GCC GCG AAG CCT GTG GAC ATT CCT GAT ACG AAA CGA ACG ACG	765
241	Ala Ala Ala Lys Pro Val Asp Ile Pro Asp Thr Lys Arg Thr Thr	255
766	CGA AAT GCT CAC AAT TCG GTG AGC AGT GGT CGC GAA ATG ACT GCT	810
256	Arg Asn Ala His Asn Ser Val Ser Ser Gly Arg Glu Met Thr Ala	270
811	GAG GGT TCC GAC AGT CAA GGC GAG CAA GCC AAG CCA GCA TCG AAA	855
271	Glu Gly Ser Asp Ser Gln Gly Glu Gln Ala Lys Pro Ala Ser Lys	285
856	GGT AAA GGC AAG AAG AAC ACG TCC ACC AAA CAG ACC TCC ACC GTC	900
286	Gly Lys Gly Lys Lys Asn Thr Ser Thr Lys Gln Thr Ser Thr Val	300
901	AAT GGC CGT CGC AAG GCA GAA GAA GCG CCC AAG GGT TCC AAT AAG	945
301	Asn Gly Arg Arg Lys Ala Glu Glu Ala Pro Lys Gly Ser Asn Lys	315
946	AGG ACC AAA ATG AAT AAT GGA CCT ATG GAG GTG ACT CCT GAA GAG	990
316	Arg Thr Lys Met Asn Asn Gly Pro Met Glu Val Thr Pro Glu Glu	330
991	GAG TCG GAC GAT GAA GAC ATG AAG GAC GAA CTG AAT GGC AAA GAC	1035
331	Glu Ser Asp Asp Glu Asp Met Lys Asp Glu Leu Asn Gly Lys Asp	345
1036	CCG AAG AAG ATG ACT GAT GAA GAG AAG CGA AAG AAT TTC TTG GAG	1080
346	Pro Lys Lys Met Thr Asp Glu Glu Lys Arg Lys Asn Phe Leu Glu	360
1081	AGG AAT CGT GTC GCG GCG CTC AAA TGT CGT CAA CGT AAG AAG CAG	1125
361	Arg Asn Arg Val Ala Ala Leu Lys Cys Arg Gln Arg Lys Lys Gln	375
1126	TGG CTT GCA AAT CTC CAA GCC AAG GTC GAG CTC TTC ACA ACG GAA	1170
376	Trp Leu Ala Asn Leu Gln Ala Lys Val Glu Leu Phe Thr Thr Glu	390
1171	AAT GAT GCT TTA ACG GCG ACC GTC ACT CAA CTA CGG GAA GAA ATA	1215
391	Asn Asp Ala Leu Thr Ala Thr Val Thr Gln Leu Arg Glu Glu Ile	405
1216	GTC TCG GTC CTT TGA 1230	
406	Val Ser Val Leu End	

Figure 5.5 The nucleotide and deduced amino acid sequences of *P. marneffei atfA* gene. Nucleotide and amino acid numbers are indicated on both sides and those that are different from *P. marneffei* ATCC 18224 are highlighted.

```
PmF4            -------------------------MITYSSANTKD-QKP-------ATDTEASQSLPPP 27
Pm              -------------------------MITYSSANTKD-QKP-------ATDTEASQSLPPP 27
Afu             ---CDLYPAVGMTSHLASSMEG-IFLIRFLPCQPRDATNPPLQHAQHKPDVDPQTSLAPP 56
Aor             MSAAVASAVTGTLTHRADTINTSPMDAKKNALETEDSQAPSSETKNIKSDVDPQTSLAPP 60
Ani             -MSAAVASAVSTTLPSNPTAHSSPMDAKKNSVKMDNEASS--ETKEQKTDGEPQTSLAPP 57
Spo             --MSPSPVNTSTEPASVAAVSNGNATASSTQVPENNQSDSFAPPSNNSQQNQQSSTIAPN 58
                                                   :   .         : : . ::.*

PmF4            PRPAASGPGDTPDYFNSLHNPFS------LEPNPFEQSFGN-----PSAETP-------- 68
Pm              PRPAASGPGDTPDYFNSLHNPFS------LEPNPFEQSFGN-----PSAETP-------- 68
Afu             PRPAVTTATDTPDYFNSVHNPFS------LEPNPFEQSFGG----GSQSETP-------- 98
Aor             PRPAITSAADTPDYFNSVHNPFS------LEPNPFEQSFGGGTSGGSSGETP-------- 106
Ani             SRPNPSAATDTPDYFNSVHNPFA------LEPNPFEQSFGG-----GSGETP-------- 98
Spo             GGAGSVANANPADQSDGVTPSFVGSLKLDYEPNPFEHSFGSTASVGQGNPSLNRNPSLSN 118
                  .      :..*  :.:  .*        ******:***.         :

PmF4            ---------GKSLLPPVASITSP----ALPGASSAG--YNWPNSLR---SGPLSPAMLAG 110
Pm              ---------GKSLLPPVASITSP----ALPGASSAG--YNWPNSLR---SGPLSPAMLAG 110
Afu             ---------GKSLLPPVAALTSP----ALPGTSSTGG-YNWSNSLR---SGPLSPAMLAG 141
Aor             ---------GKSILPPVASLTSP----ALPGTSSAGGGYSWSNSLR---SGPLSPAMLAG 150
Ani             ---------GKSILPPVASITSP----ALPGTSSAGGAYNWSNSLR---SGPLSPAMLAG 142
Spo             IPSGVPPAFARTLLPPVSSIASPDILSGAPGIASPLGYPAWSAFTRGTMHNPLSPAIYDA 178
                         .:::****::::**    . ** :*.     *.   *    .*****:  .

PmF4            PTG-ADYFDS-----------IGRGFPTPNESSLRTGLTPGGGGSMFPAPSPNTQAILSQ 158
Pm              PTG-ADYFDS-----------IGRGFPTPNESSLRTGLTPGGGGSMFPAPSPNTQAILSQ 158
Afu             PAGGNDYFDS-----------IGRGFPTPNESSLRTGLTPGGGGSMFPAPSPNSQAILQQ 190
Aor             PTGSSDYFDS-----------IGRGFPTPNESSLRTGLTPGGGGSMFPAPSPNSQALLQQ 199
Ani             PAGGSDYFDS-----------IGRGFPTPNESSLRTGLTPGGGGSMFPAPSPNSQALLNQ 191
Spo             TLR-PDYLNNPSDASAAARFSSGTGFTPGVNEPFRSLLTPTGAG--FPAPSPGTANLLGF 235
                .    **::.            * **..  :..:*: *** *.*  ******.:  :*

PmF4            LQSGGATPSTLEFHRTALNAAKRSGFNAPTSNPTSDPE-----QLQNMDKKTAPP-AVDQ 212
Pm              LQSGGATPSTLEFHRTALNAAKRSGFNAPTSNPTSDPE-----QLQNMDKKTAPP-AVDQ 212
Afu             LQSGGATPSTIEFHRTALSAAKKNGLSGPTSNPTADSE-G--VHSSSMDIKSSQPASVDP 247
Aor             LQSGGATPSTIEFHRTALNAAKKNALNGPTSNPTSDPEQA--SQNTNMDMKPNQP---DP 254
Ani             LQNGGATPSTIEFHRTALNVKKNG--IAPTSNPTGEGDQVPQNITTTMDIKPAQP-ATVD 248
Spo             HTFDSQFPDQYRFTPRDGKPPVVNGTNGDQSDYFGANAAVHGLCLLSQVPDQQQKLQQPI 295
                   ..  *.  .*     .    .   .  *:  .           .   .

PmF4            FTHHDAADAANGLFMLAKGGQPNNDAFA-AAAKPVDIPDTKR-----TTRNAHNSVS--- 263
Pm              FTHHDAADAANGLFMLAKGGQPNNDAFA-AAAKPVDIPDTKR-----TTRNAHNSVS--- 263
Afu             FTHHDAADAANGLFMLAKGGQAN--QF--VSNQSSVPPHTIQG--NNQSRDADRRGSQNV 301
Aor             FGHHDAADAANGLFMLAKGGQANPNQFA-VSNQSSIPPQNIQN--NDQARDSDRRTS--- 308
Ani             FGPHDAADAANGLFMLAKGGQSTANQFAAVSNQTAIPPQTLQTSEILQDQNAARRQSVNV 308
Spo             SSENDQAASTTANNLLKQTQQQTFPDSIRPSFTQNTNPQAVTGTMNPQASRTQQQPMYFM 355
                   :* * ::..  :* :  * .       :      *.            : .

PmF4            -------------------SGREMTAEGSDSQGEQAKPASKGKGKKNTSTKQTSTVNGRR 304
Pm              -------------------SGREMTAEGSDSQGEQAKPASKGKGKKNTSTKQTSTVNGRR 304
Afu             NGP-------------VASSREMSGDASDMQTEQAKPSAKGKGKKNTASKSGTTANNRR 347
Aor             NG------------------GRETSGDVSDVQGEQAKPATKGK-KKNTATKTSGAANNRR 349
Ani             NG--------------VANTREPSGDGSE-QSEQAKP-ARGRGKRNTSTKASSTGN-RR 350
Spo             GSQQFNGMPSVYGDTVNPADPSLTLRQTTDFSGQNAENGSTNLPQKTSNSDMPTANSMPV 415
                                          : :: . ::*:  : .  ::.: :.   : .

PmF4            KAEEAP-KGSNKRTK--MNNGPMEVTPEE-ESDDEDMK-----DELNGKDPKKMTDEEKR 355
Pm              KAEEAP-KGSNKRAK--MNNGPMEVTPEE-ESDDEDMK-----DELNGKDPKKMTDEEKR 355
Afu             KADDTPAKGPNKKAKTSVSSGSAEPPSEAGDSEDEDDM-----KKKSQTDTKKMTDEEKR 402
Aor             KADDAPVKGSNKKAK--LSSGSTEPPSDAGDSEEEEEQ-----KKKSQSDSKKMTDEEKR 402
Ani             KTDDST-QGSNKRTKLNNGAASTESPSEG-ESEEEEQQPA---QKKKAGDTKKMTDEEKR 405
Spo             KLENGTDYSTSQEPSSNANNQSSPTSSINGKASSESANGTSYSKGSSRRNSKNETDEEKR 475
                * :: .  ...:...   .  .   ..   .:..*.       .  .  :.*: ******

PmF4            KNFLERNRVAALKCRQRKKQWLANLQAKVELFTTENDALTATVTQLREEIVSVL------ 409
Pm              KNFLERNRVAALKCRQRKKQWLANLQAKVELFTTENDALTATVTQLREEIVSVL------ 409
Afu             RNFLERNRVAALKCRQRKKQWLANLQAKVELFTTENDALTATVTQLREEIVNLKTLLLAH 462
Aor             KNFLERNRVAALKCRQRKKQWLANLQAKVELFTSENDALTATVTQLREEIVNLKTLLLAH 462
Ani             KNFLERNRVAALKCRQRKKQWLANLQAKVELFTSENDALTTTVTQLREEIVNLKTLLLAH 465
Spo             KSFLERNRQAALKCRQRKKQWLSNLQAKVEFYGNENEILSAQVSALREEIVSLKTLLIAH 535
                :.****** *************:*******:: .**: *:: *: ******.:


PmF4            --------------------------------------------------
Pm              --------------------------------------------------
Afu             KDCPVSQAQGLGSLMMNGMSTGFDPHPYNIANNMGMQPGAPIPTQGMRR- 511
Aor             KDCPVSQAQGLGPLMMNGMSAGFDPHPYNIPNNMGMQPGAPIPTQGLRRQ 512
Ani             KDCPVSQAQG---LIWN------PNPPYT--------------------- 485
Spo             KDCPVAKSNSAAVATSVIGSGDLAQRINLGY------------------- 566
```

Figure 5.6 The multiple alignment of fungal AtfA/Atf1 amino acid sequences. *P. marneffei* strain F4 AtfA amino acid sequence (PmF4) was aligned with *P. marneffei* ATCC 18224 (Pm) AtfA (EEA27441), *A. fumigatus* Af293 (Afu) AtfA (EAL92448), *A. oryzae* RIB40 (Aor) AtfA (XP_001819834), AtfA of *A. nidulans* FGSC A4 (Ani) (CBF83765) and Atf1 of *S. pombe* 972h- (Spo) (NP_595652). The conserved basic-leucine zipper (bZip) domain is highlighted.

5.2 Analysis of *sakA* and *atfA* gene expressions during phase transition and stress condition

To investigate the expression of *sakA* and *atfA* in *P. marneffei*, RNA was isolated from wild type vegetative hyphae grown for 3 days in SDB at 25°C, conidia (asexual development) collected from cultures grown for 10 days on MEA at 25°C and filtered through sterile glass wool and yeast cells grown for 6 days in BHI broth at 37°C. The *sakA* and *atfA* transcripts were detected by reverse transcriptase (RT)-PCR using primers *SakA*F and *SakA*355-R for *sakA* transcript (Table1) and primers *AtfA*F-RT and *AtfA*R-RT for *atfA* transcript (Table 2). Very low amount of *sakA* transcript was detected during asexual development (conidia) when compared with the 18S rRNA loading control and was increased during vegetative hyphal growth (mycelia) at 25°C and during yeast growth at 37°C (Figure 5.7). The amount of *sakA* transcript was increased in conidia under heat shock at 39°C and oxidative stress with H_2O_2, whereas *sakA* transcripts in both mycelia and yeast were slightly increased under oxidative stress (Figure 5.7).

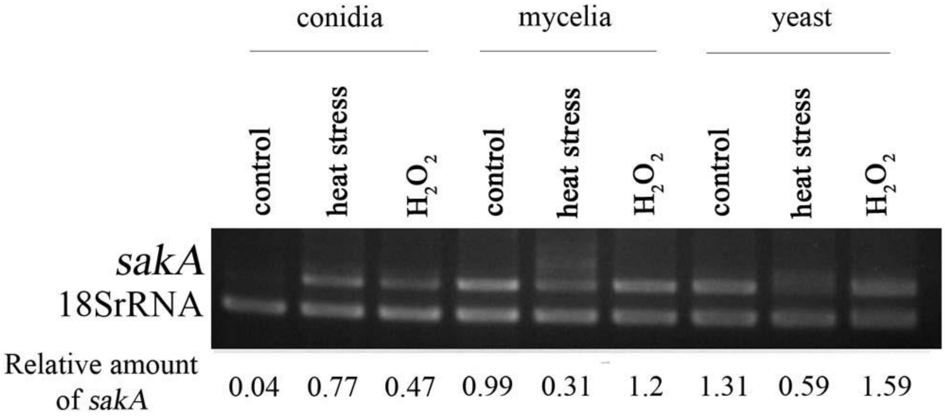


Figure 5.7 *sakA* expression during phase transition. RNA was isolated from *P. marneffei* strain F4 collected from cultures grown for 10 days on MEA at 25°C and filtered through sterile glass wool (conidia), 3 days in SDB at 25°C (mycelia), and 6 days in BHI broth at 37°C (yeast) under normal condition (control) or heat stress at 39ºC or 1 mM H_2O_2 for 1 hour. PCR product of 18S rRNA using primers Pm1 and Pm2 (Table 1) was used as loading control of each growth phase. Relative amount of *sakA* is an average value from two-independent experiments.

For *atfA* gene expression, comparing with 18S rRNA loading control, *atfA* transcript determined during asexual development (conidia) was less than those cells during vegetative hyphal growth (mycelia) at 25°C and during yeast growth at 37°C similar to *sakA* transcript (Figure 5.8). Under stress conditions, the amount of *atfA* transcript was not increased in conidia, mycelia and yeast under both heat shock at 39°C and oxidative stress with one mM H_2O_2 (Figure 5.8).

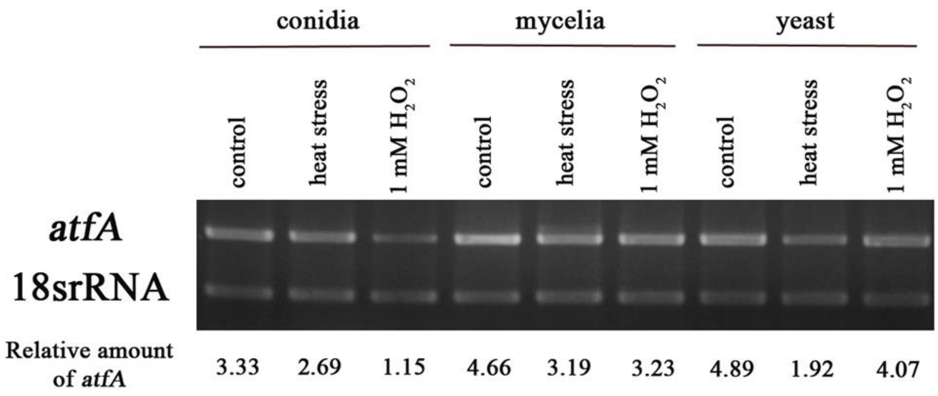


Figure 5.8 *atfA* expression during phase transition. RNA was isolated from *P. marneffei* strain F4 cells including conidia collected from cultures grown for seven days on PDA at 25°C , three days in SDB at 25°C (mycelia), and six days in BHI broth at 37°C (yeast). PCR product of 18S rRNA using primers Pm1 and Pm2 (Table 1) was used as loading control of each growth phase.

5.3 Functional analysis of *sakA* and *atfA* genes

5.3.1 Generations of *P. marneffei sakA* and *atfA* mutant strains

To determine the functions of *sakA* gene in *P. marneffei*, the *sakA* deletion strain was constructed by replacing the open reading frame of the *sakA* gene with the *hph* cassette. After two DNA fragments containing the 5'and 3' flanking regions of *sakA* gene and the truncated sequence of the *hph* cassette were transformed into *P. marneffei* F4 wild type strain, there were four transformants grew on selective agar containing hygromycin denoted M1, M2, M3 and M4 (Figure 5.9). The transformants lacking the *sakA* gene were screened by PCR using primers specific to *sakA* and *hph* genes. The results revealed that only one transformant, M4 did not contain *sakA* gene in its genome DNA (Figure 5.10). This transformant was used as a *sakA* mutant strain for further analysis.

To prove the absence of *sakA* expression in the mutant, mRNAs were extracted from hyphal cells of the wild type and the mutant and were reverse transcribed to cDNA. PCR amplification was done using primers specific to the *sakA* mRNA (*SakA*F and *SakA*344-R; Table 1) and control 18S rRNA (Pm1 and Pm2; Table 1). The results showed that there was no expression of *sakA* gene in the mutant (Figure 5.11). In addition, Southern blot hybridization was used to indicate the copy of the *hph* gene that was integrated into the mutant genomic DNA (Figure 5.12). The hybridizations were performed at 55ºC using *Eco*RI-digested genomic DNA from the wild type and the *sakA* mutant and hybridized with probe specific to the *sakA* gene (Figure 5.13) and probe that is specific to the *hph* gene and 3' flanking region of the *sakA* gene (Figure 5.14). The results demonstrated that the mutant (M4) contained only one copy of the *hph* gene that was correctly integrated within the *sakA*. However, a single band found in the mutant when used probe specific to the *hph* gene and 3' flanking region of the *sakA* gene was bigger than expected (Figure 5.14B). This may be the result from incomplete digestion of the mutant genomic DNA by *Eco*RI before Southern blot hybridization.

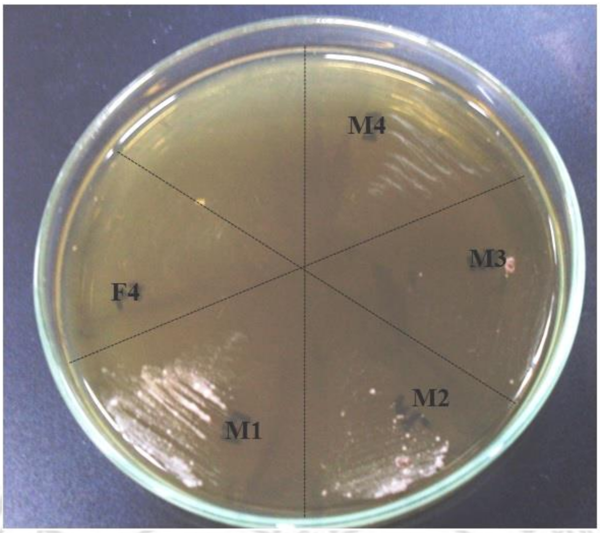


Figure 5.9 Growth of *P. marneffei* wild type and *sakA* mutant strains on selective agar. *P. marneffei* wild type strain F4 and *sakA* mutant strains (M1, M2, M3, M4) were inoculated on brain heart infusion agar (BHA) containing 200 µg/ml hygromycin and incubated at 37ºC for 3 days.

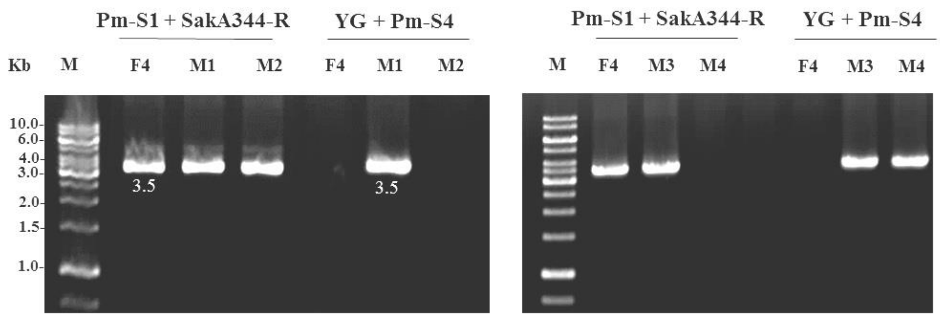


Figure 5.10 Confirmation of *P. marneffei sakA* mutant strains by PCR amplification. *P. marneffei* wild type (F4) and *sakA* mutant (M1, M2, M3, M4) genomic DNA were amplified by primers specific to *sakA* gene (*SakA*-F+Pm-S4 and PmS1+*SakA*344-R) and primers specific to both *sakA* and hph genes (Pm-S1+HY and YG+Pm-S4). M represents 1 kb DNA ladder. Sizes of PCR products are indicated.

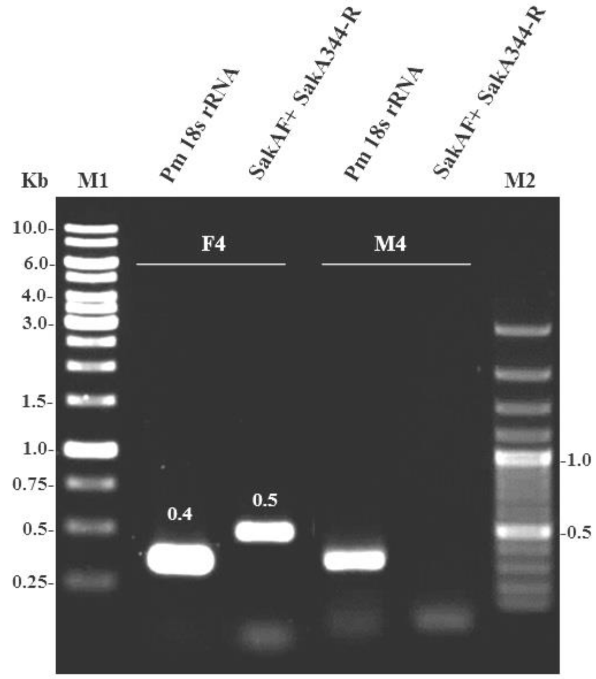


Figure 5.11 Expression of *sakA* gene in *P. marneffei* wild type and *sakA* mutant. *P. marneffei* wild type (F4) and *sakA* mutant (M4) cDNA were amplified by primers specific to 18S rRNA (Pm1 and Pm2) and primers specific to *sakA* gene (SakA-F and SakA344-R). M1 and M2 represent 1 kb DNA ladder and 100 bp ladder plus, respectively. Sizes of PCR products are indicated.

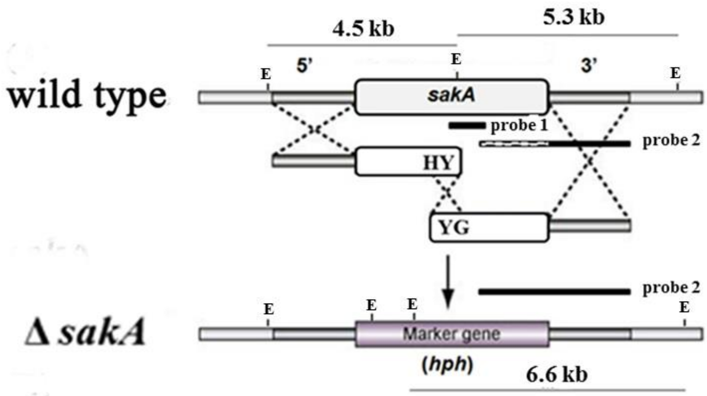


Figure 5.12 Strategy for Southern blot hybridization of the *sakA* mutant. The *Eco*RI-digested genomic DNA of the wild type and *sakA* mutant (Δ*sakA*) was hybridized with probe 1 or probe 2. E represents *Eco*RI. The black boxes indicate the position of probe 1 and probe 2 amplified by primers specific to the *sakA* gene (*SakA*F and *SakA*344R) and primers specific to the *hph* gene and 3' flanking region of the *sakA* gene (YG and Pm-S4), respectively.

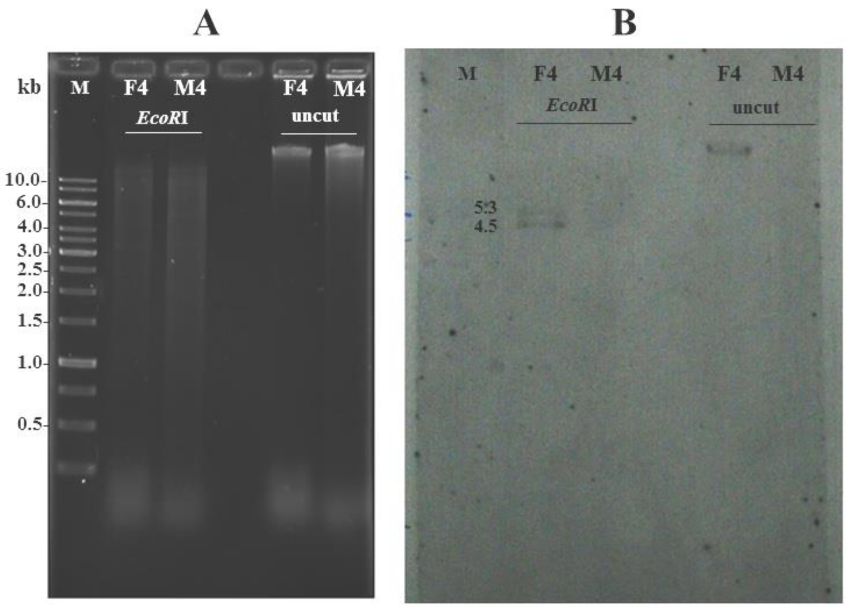


Figure 5.13 Southern blot hybridization of *P. marneffei sakA* mutant using probe specific to the *sakA* gene. (A) Genomic DNA of the wild type (F4) and the *sakA* mutant (M4) was digested with *Eco*RI and separated by agarose gel electrophoresis comparing to undigested DNA (uncut). (B) Digested and undigested genomic DNA was probed with probe 1 shown in Figure 5.12. M represents 1 kb DNA ladder. Probe 1 (a 650 bp fragment of *sakA*) hybridized with 4.5 kb and 5.3 kb fragments of *Eco*RI-digested DNA and undigested DNA from only the F4 strain.

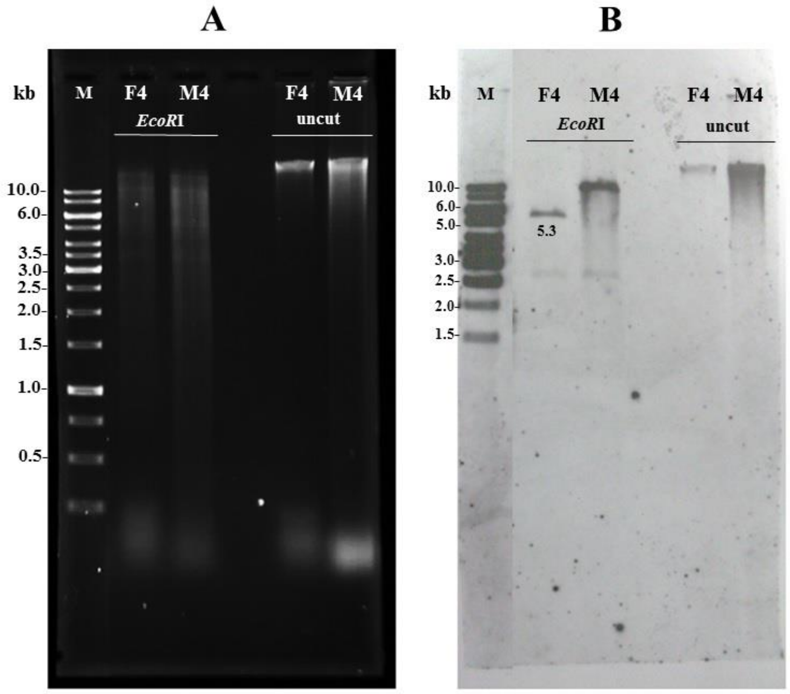


Figure 5.14 Southern blot hybridization of *P. marneffei sakA* mutant using probe the *hph* gene and 3' flanking region of the *sakA* gene. (A) Genomic DNA of the wild type (F4) and the *sakA* mutant (M4) was digested with *Eco*RI and separated by agarose gel electrophoresis comparing to undigested DNA (uncut). (B) Digested and undigested genomic DNA was probed with probe 2 shown in Figure 5.12. M represents 1 kb DNA ladder. Probe 2 (a 3.5 kb fragment of *hph* and 3'flanking region of *sakA*) hybridized with a 5.3 kb and approximately 10 kb fragment of *Eco*RI-digested DNA from the F4 and M4 strains, respectively.

The *atfA* mutant strain of *P. marneffei* was constructed by replacing the open reading frame of the *atfA* gene with the *hph* cassette using modified split marker method (see materials and methods). After two DNA fragments containing the 5'and 3' flanking regions of *atfA* gene and the truncated sequence of the *hph* cassette were transformed into *P. marneffei* F4 wild type strain, there were five transformants grew on selective agar containing hygromycin denoted SBΔatfA, SCΔatfA, SDΔatfA, SEΔatfA and SFΔatfA. PCR amplification using primers specific to *atfA* gene (*AtfA*-WF and *AtfA*-WR) demonstrated that only SCΔatfA did not contain *atfA* gene (Figure 5.15). To confirm that there is no expression of *atfA* in the mutant, mRNAs were extracted from cells of the wild type and the mutant and were reverse transcribed to cDNA. PCR amplification was done using primers specific to the *atfA* mRNA (*AtfA*F-RT and *AtfA*R-RT; Table 2) and 18S rRNA (Pm1 and Pm2; Table 1). The results showed that there is the expression of 18S mRNA but not *atfA* gene in the conidia, mold and yeast cells of the mutant (Figure 5.16). Southern blot hybridization was done to indicate the copy of the *hph* gene that was integrated into the mutant genomic DNA (Figure 5.17). The hybridizations were performed at 55°C using *Eco*RV-digested genomic DNA from the wild type and the *atfA* mutant and used probe specific to the *hph* gene and 3' flanking region of the *atfA* gene. The results demonstrated that the mutant (SCΔatfA) contained only one copy of the *hph* gene that was correctly integrated within the *atfA* (Figure 5.18). Therefore, this mutant was used for further study.

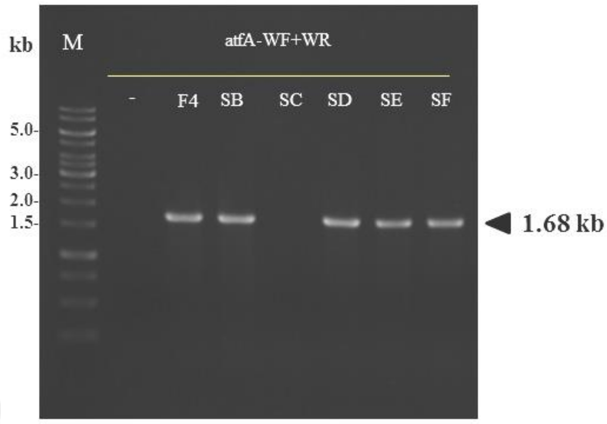


Figure 5.15 Confirmation of *P. marneffei atfA* mutant strains by PCR amplification. *P. marneffei* wild type (F4) and *atfA* mutant (SB, SC, SD, SE, SF) genomic DNA were amplified by primers specific to *atfA* gene (atfA-WF+WR). M represents 1 kb DNA ladder. (-) represents negative control and size of PCR product is indicated.

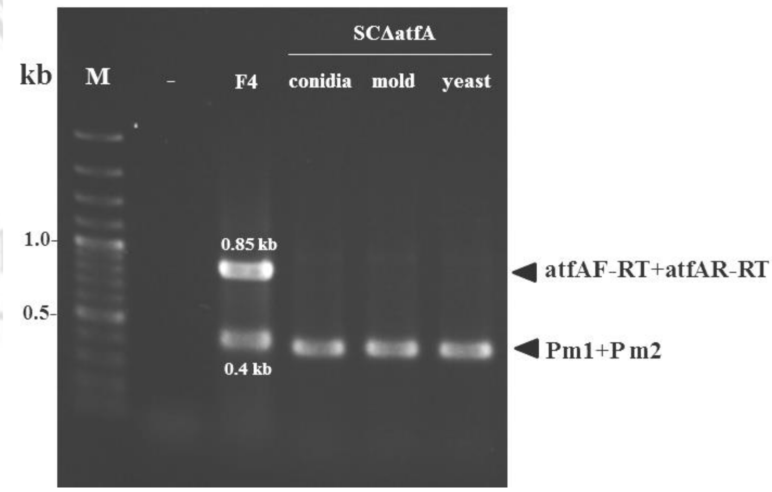


Figure 5.16 Expression of *atfA* gene in *P. marneffei atfA* strain. *P. marneffei* wild type (F4) and *atfA* mutant (SCΔatfA) cDNA were amplified by primers specific to *atfA* gene (*Atf*AF-RT+*Atf*AR-RT) and 18S rRNA (Pm1+Pm2). M represents 100 bp ladder plus. (-) represents negative control and sizes of PCR products are indicated.

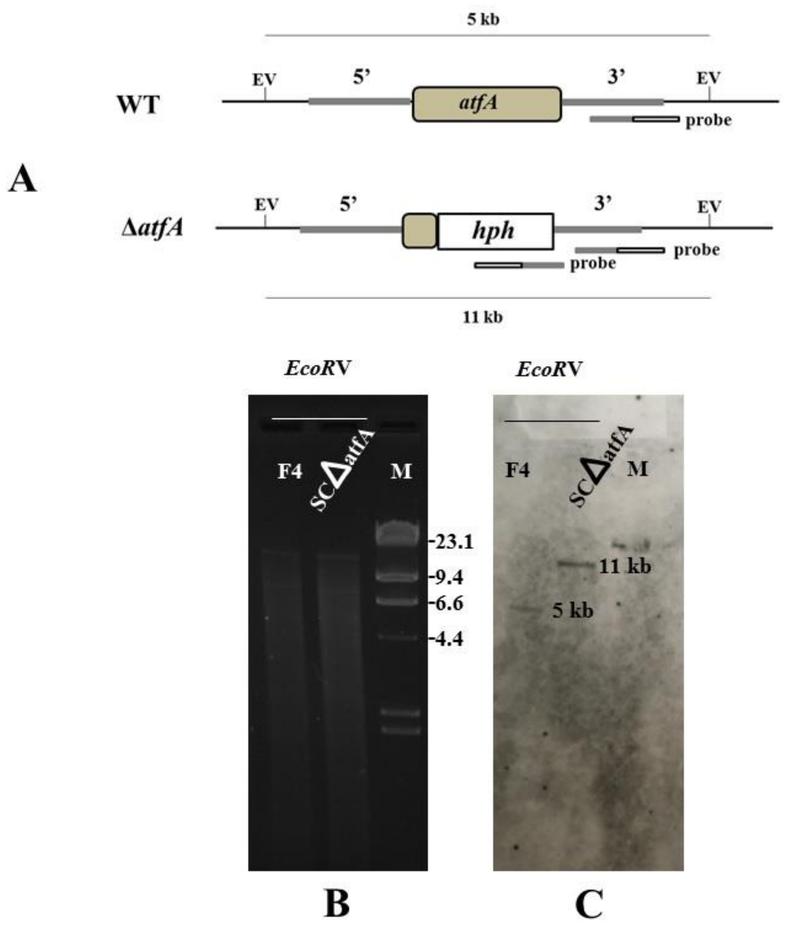


Figure 5.17 Southern blot hybridization of the *atfA* mutant. (A) Diagram for Southern blot hybridization. The predicted results of hybridization are shown. EV represents *Eco*RV. The grey and white boxes indicate the position of probe amplified by primers specific to the *hph* gene and 3' flanking region of the *atfA* gene (YG and *AtfA*-A4). (B)The *Eco*RV-digested genomic DNA of the wild type and *atfA* mutant (Δ*atfA*) was separated by agarose gel electrophoresis and hybridized with probe indicated in Figure 5.17A at 55ºC (C). M represents λ *Hin*DIII marker.

5.3.2 Constructions of *P. marneffei sakA* and *atfA* complemented strains

To confirm the function of the *sakA* gene, *sakA* complemented strains were constructed. Plasmid pJLsakA containing promoter, *sakA* coding sequence and 3’ region of *sakA* was transformed into the *sakA* mutant strain (M4). Four clones denoted YSC1, YSC2, YSC8 and YSC9 were selected on selective agar containing 200 µg/ml hygromycin and 2 µg/ml bleomycin (Figure 5.18). These clones revealed colonies similar to that of wild type strain at both 25ºC and 37ºC (Figure 5.19) and contained both *sakA* and *hph* genes (Figure 5.20). However, Southern blot analysis showed that only YSC1 contained one copy of *sakA* gene and the expressions of *sakA* gene in conidia, mold and yeast cells of YSC1 were confirmed by RT-PCR using primers *SakA*F and *SakA*-344R (Figure 5.21).

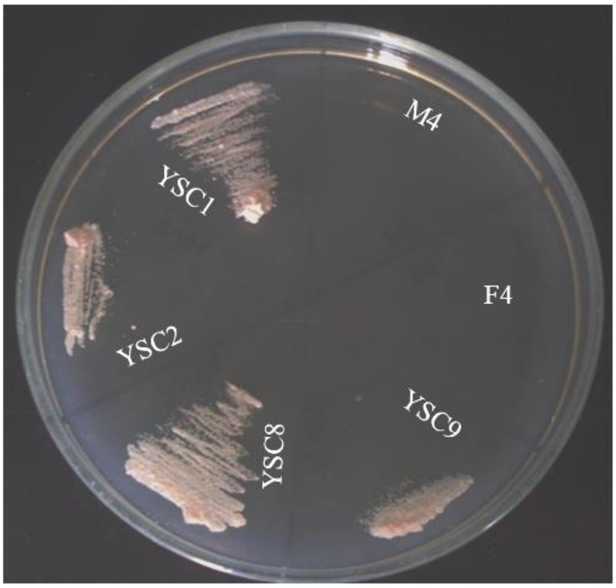
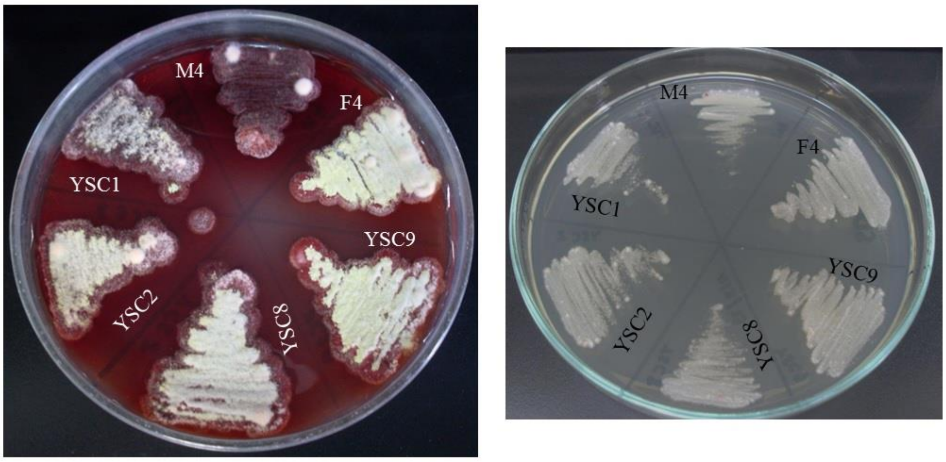


Figure 5.18 Growth *P. marneffei* wild type, *sakA* mutant and complemented strains on selective agar. *P. marneffei* wild type (F4), *sakA* mutant (M4) and complemented strains (YSC1, YSC2, YSC8, YSC9) were inoculated on Sabouraud dextrose agar (SDA) containing 200 µg/ml hygromycin and 2 µg/ml bleomycin and incubated at 37ºC for 7 days.



PDA 25°C, 3 d **SDA 37°C, 3 d**

Figure 5.19 Colonies of *P. marneffei sakA* complemented strains comparing to wild type and mutant strains. *P. marneffei* wild type (F4), *sakA* mutant (M4) and complemented strains (YSC1, YSC2, YSC8, YSC9) were inoculated on potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) and incubated at 25ºC and 37ºC for 3 days, respectively.

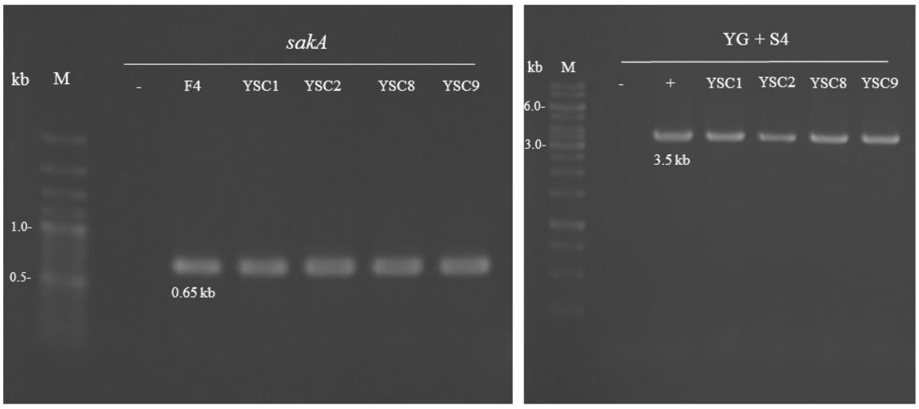


Figure 5.20 Confirmation of *P. marneffei sakA* complemented strains by PCR amplification. *P. marneffei* wild type (F4) and *sakA* complemented strain (YSC1, YSC2, YSC8, YSC9) genomic DNA were amplified by primers specific to *sakA* gene (*SakA*F and *SakA*-344R; *sakA*) and primers specific to *hph* gene (YG and *SakA*-S4; YG+S4). M represents 1 kb DNA ladder. (-) represents negative control and sizes of PCR products are indicated.

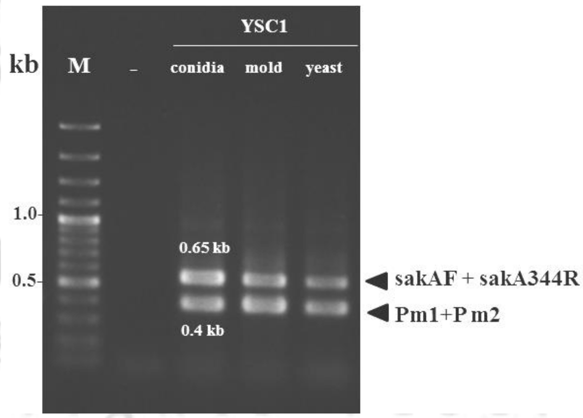


Figure 5.21 Expression of *sakA* gene in *P. marneffei sakA* complemented strain. *P. marneffei sakA* complemented strain (YSC1) cDNA were amplified by primers specific to *sakA* gene (*SakA*F+*SakA*-344R) and 18S rRNA (Pm1+Pm2). cDNA was synthesized from RNA extracted from conidial, hyphal and yeast cells. M represents 100 bp ladder plus. (-) represents negative control and sizes of PCR products are indicated.

For *atfA* complemented strains, plasmid pJLatfA containing promoter, *atfA* coding sequence and 3’region of *atfA* was transformed into the *atfA* mutant strain (SCΔatfA). Three clones denoted AC1, AC2 and AC3 were selected on selective agar containing 200 µg/ml hygromycin and 2 µg/ml bleomycin (Figure 5.22A). All of them contained *atfA* gene in their genomic DNA (Figure 5.22B). Southern blot analysis showed that only AC1 contained one copy of *atfA* gene and the expression of *atfA* gene in AC1 was confirmed by RT-PCR using primers specific to *atfA* mRNA (Figure 5.23).

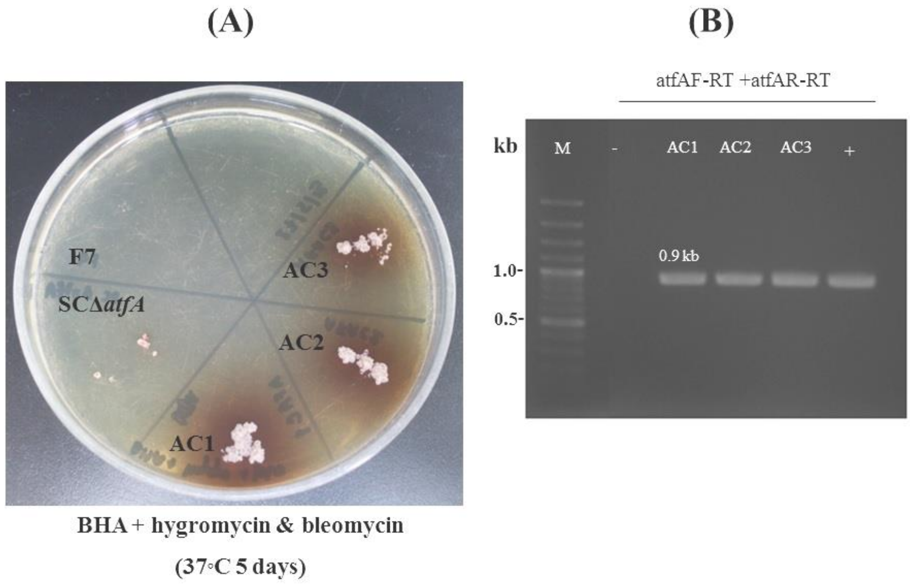


Figure 5.22 Colonies and the presence of *atfA* gene of *P. marneffei atfA* complemented strains. (A) *P. marneffei* wild type (F4), *atfA* mutant (SCΔatfA) and complemented strains (AC1, AC2, AC3) were inoculated on BHA containing 200 µg/ml hygromycin and 2 µg/ml bleomycin and incubated at 37ºC for 5 days. (B) The *atfA* complemented strain genomic DNA were amplified by primers specific to *atfA* gene (*Atf*AF-RT + *Atf*AR-RT). M represents 100 bp kb ladder plus. (-) and (+) represent negative and positive controls and size of PCR product is indicated.

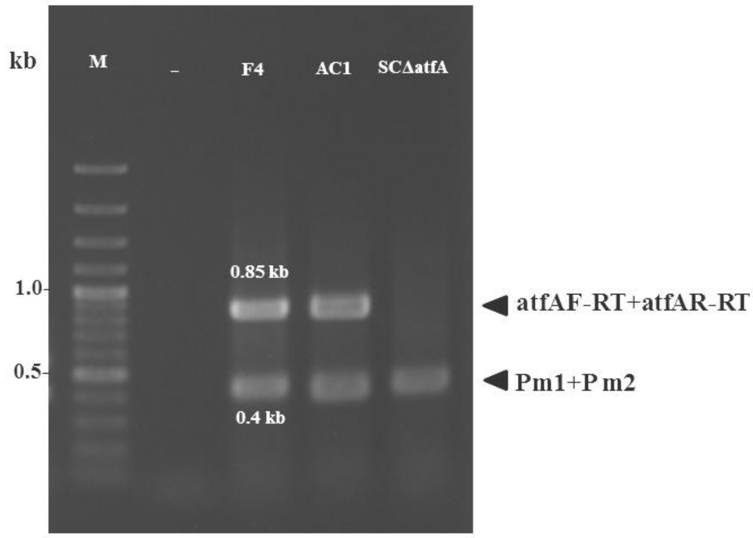


Figure 5.23 Expression of *atfA* gene in *P. marneffei sakA* complemented strain. *P. marneffei* wild type (F4), *atfA* mutant (SCΔatfA) and *atfA* complemented (YSC1) strain cDNA were amplified by primers specific to *atfA* mRNA (*AtfA*F-RT + *AtfA*R-RT) and 18S rRNA (Pm1+Pm2). cDNA was synthesized from RNA extracted from hyphal cells. M represents 100 bp ladder plus. (-) represents negative control and sizes of PCR products are indicated.

5.3.3 Characterization of *sakA* and *atfA* mutants

1) Morphologies and Growth

To investigate whether the *sakA* gene is involved in growth at 25°C, growth curves of the wild type and *sakA* mutant were compared. There was no significant difference between the growth curves of both strains (Figure 5.24).

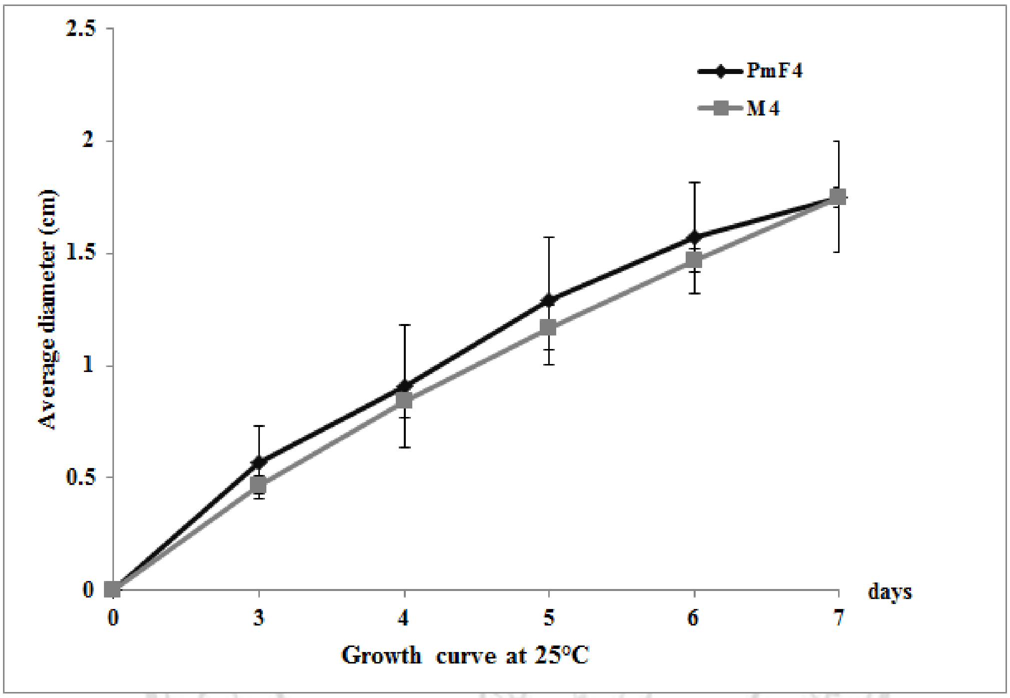


Figure 5.24 Growth curve of *P. marneffei* at 25°C. Colony diameters of five colonies from the wild type (PmF4) and the *sakA* mutant (M4) on PDA at 25°C were measured on day 3 to day 7. Data are from three independent experiments and standard error bars of the mean are shown ($p<0.05$).

To observe colony morphologies, *P. marneffei* wild type, *sakA* mutant and *sakA* complemented strain were grown on PDA and MEA at 25°C. The colonies of the *sakA* mutant were pink-to-red (Figure 5.25A and B) comparing to yellow to green colonies of the wild type and the complemented strains. Under microscope, the fungal structures including matulae and phialide of all strains are not different. However, the mutant (M4) produced less conidia than those of the wild type and the complemented strains (Figure 5.25C).

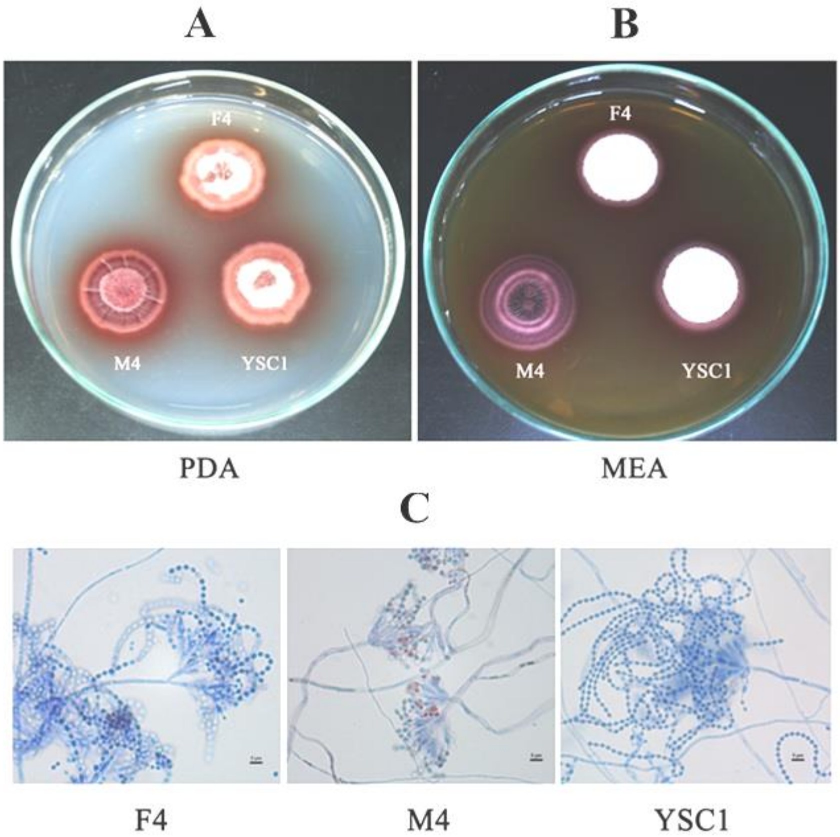


Figure 5.25 Colonies and morphologies of *P. marneffei sakA* mutant at 25°C. The wild type (F4), the *sakA* mutant (M4) and the *sakA* complemented (YSC1) strains were cultured on PDA (A) and MEA (B) plates at 25°C for 6 days. (C) Morphologies of each strain on PDA were observed under microscope using slide culture method. Scale bar represents five micrometers.

At 37°C, conidia of the wild type and the *sakA* mutant were inoculated on SDA plates and incubated at 37°C for 4 weeks. The *sakA* mutant revealed yeast-like colony with irregular form of colony edge compared with round form colony edge of the wild type (Figure 5.26A). To determine yeast cell morphogenesis *in vitro*, conidia of the wild type, the *sakA* mutant and the *sakA* complemented strains were inoculated into three culture broth media including 1% peptone, BHI and SDB and incubated at 37°C for 7 days. In 1% peptone which is the medium used to induce conidia to germinate as fission yeast cells as found in clinical specimen (Tongchusak *et al*., 2004), conidia from all strains could produce yeast cells within 2 days (Figure 5.26B, left column). For BHI and SDB which are complex media used to generate yeast form in *P. marneffei*, the wild type and the *sakA* complemented strains produced polarized arthroconidia hyphae and arthroconidia within 4 days and fission yeasts were found after 7 days of incubation (Figure 5.26B, middle and right column). On the other hand, a high amount of septate hyphae with few fission yeasts were observed after the *sakA* mutant was incubated in BHI (Figure 5.26B, middle column), whereas in SDB, conidia of the *sakA* mutant were aggregated and some of them germinated to septate hyphae after 7 days of incubation (Figure 5.26B, right column).

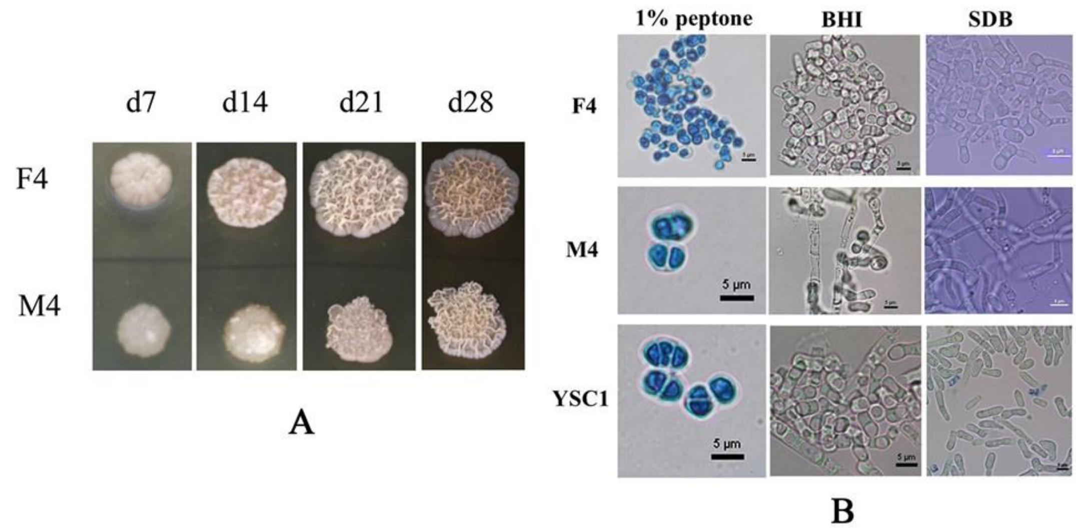


Figure 5.26 Colonies and morphologies of *P. marneffei sakA* mutant at 37°C. (A) growth of conidia from both the *P. marneffei* wild type (F4) and the *sakA* mutant (M4) inoculated on SDA and incubated for 4 weeks at 37°C. (B) germination of conidia from F4, M4 and *sakA* complemented strain (YSC1) inoculated into 1% peptone, BHI and SDB and incubated in shaking incubator at 37°C, 150 rpm for 2 days (peptone) and 7 days (BHI and SDB). *P. marneffei* strains cultured in 1% peptone were stained with lacto phenol cotton blue. Scale bar represents five micrometers.

For yeast production inside macrophages, THP1 and J774 macrophages were infected with conidia from the *sakA* mutant, the wild type and the complemented strains. After 72 hours of post infection, ungerminated conidia and hyphal cells were observed inside macrophages infected with conidia from the *sakA* mutant strain compared with those macrophages infected with the wild type and *sakA* complemented strains that carried fungal yeast cells (Figure 5.27).

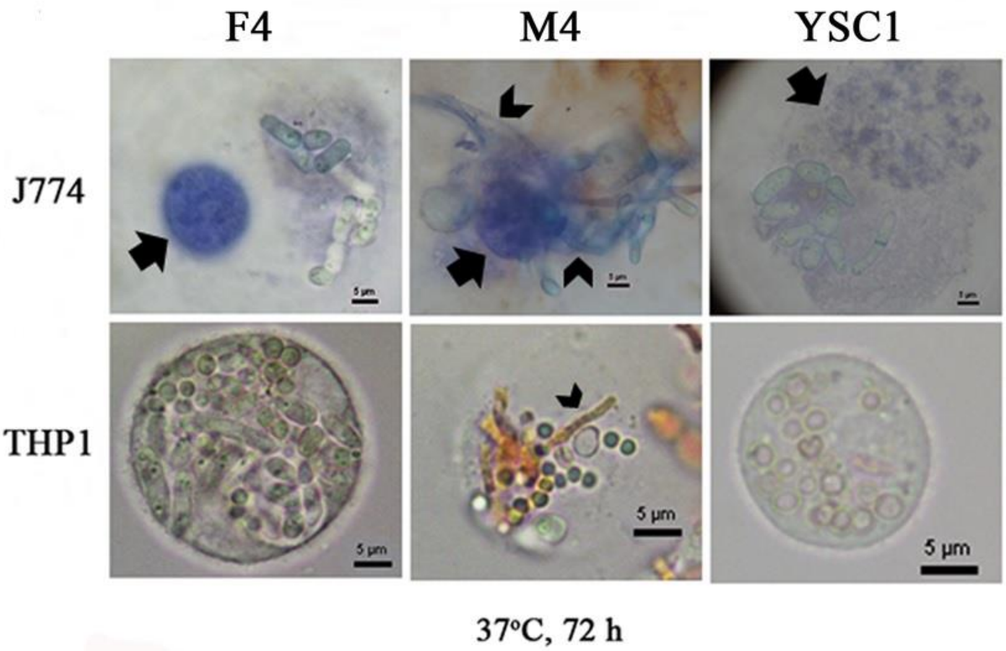


Figure 5.27 Yeast cell transition of *P. marneffei sakA* mutant inside macrophage at 37°C. J774 and THP1 macrophages were infected with conidia from *P. marneffei* wild type (F4), the *sakA* mutant (M4) and complemented (YSC1) strains. After 72 hour post-infection, *P. marneffei* inside macrophages were observed under microscope. Infected J774 cells were stained with lactophenol cotton blue. Nucleus (arrow) and fungal hyphae (arrow head) are indicated. Scale bar represents 5 micrometers.

For *atfA* genes, conidia of the wild type and the mutant strains were inoculated on PDA and SDA and the plates were incubated at 25°C and 37°C, respectively, to investigate colony morphologies. The result showed that *atfA* mutant strain had colony morphology similar to those of the wild type strain at both temperatures (Figure 5.28 and 5.29A). In addition, yeast cell production of the *atfA* mutant was also undistinguishable from the wild type strain (Figure 5.29B).

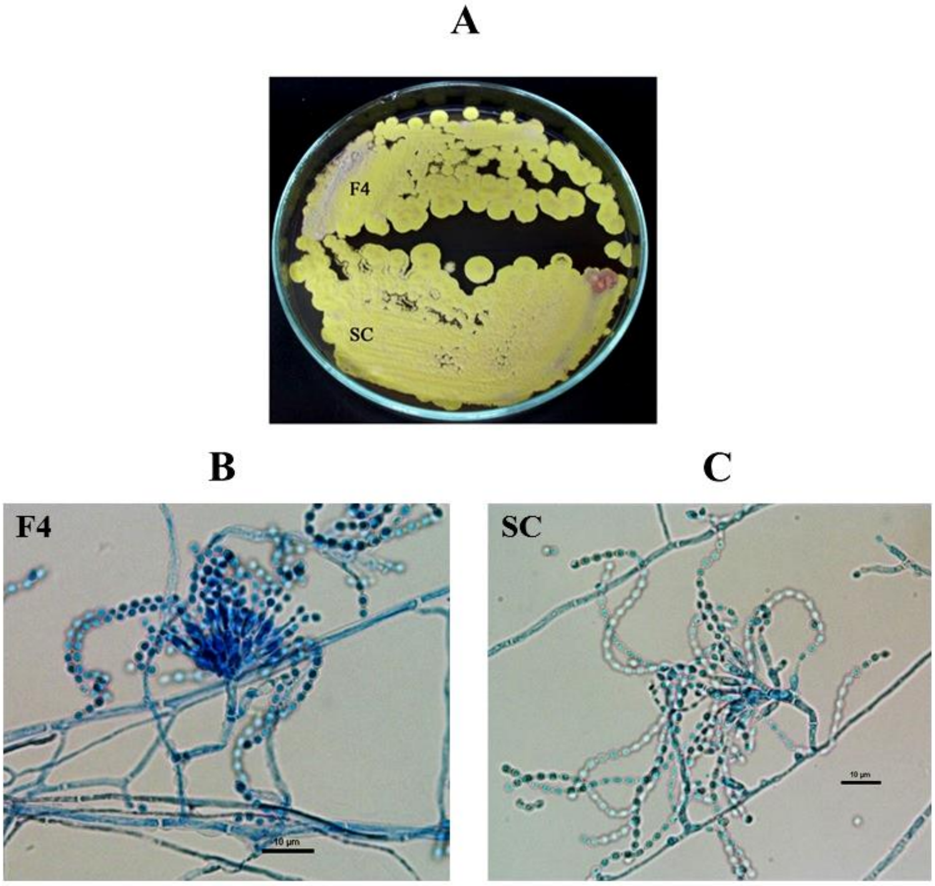


Figure 5.28 Colonies and morphologies of *P. marneffei atfA* mutant at 25°C. (A) The wild type (F4) and the *atfA* mutant (SC) strains were inoculated on PDA plates at 25°C for 7 days. (B and C) Morphologies of each strain on PDA were observed under microscope using slide culture method. Scale bar represents ten micrometers.

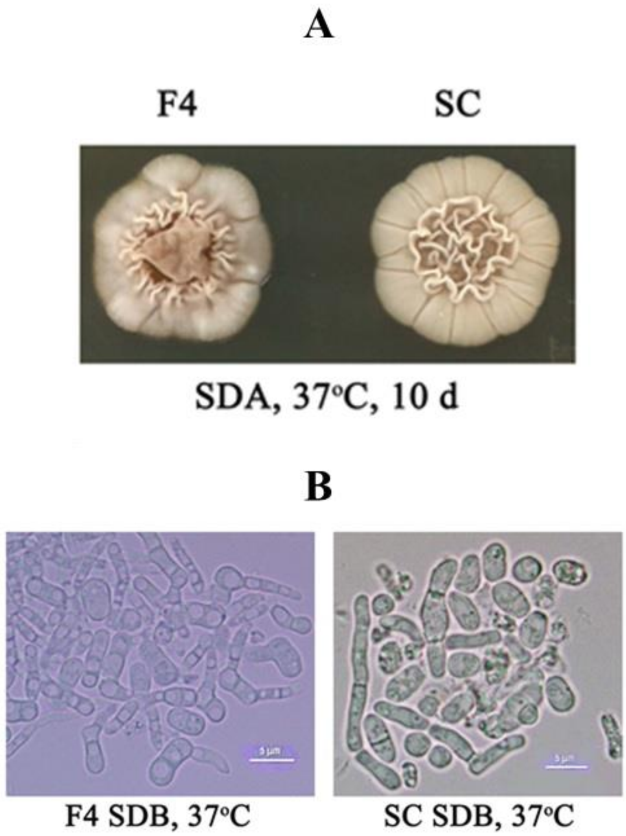


Figure 5.29 Yeast colony and morphology of *P. marneffei atfA* mutant at 37°C *in vitro*. (A) Colonies of wild type (F4) and *atfA* mutant (SC) on SDA incubated at 37°C for ten days. (B) Conidia isolated from both strains were inoculated into SDB and were incubated at 37°C for days. Scale bar represents five micrometers.

2) **Asexual development**

From colony morphology on culture media and under microscope, the conidia produced by the *sakA* mutant were less than the wild type and the complemented strains. To enumerate the number of conidia generated by each strain, conidia from all strains were collected and inoculated on PDA and MEA and incubated at 25°C. The results demonstrated that a number of conidia produced by the *sakA* mutant strains on both culture media were significantly less than those generated by the wild type and the complemented strains (Figure 5.30).

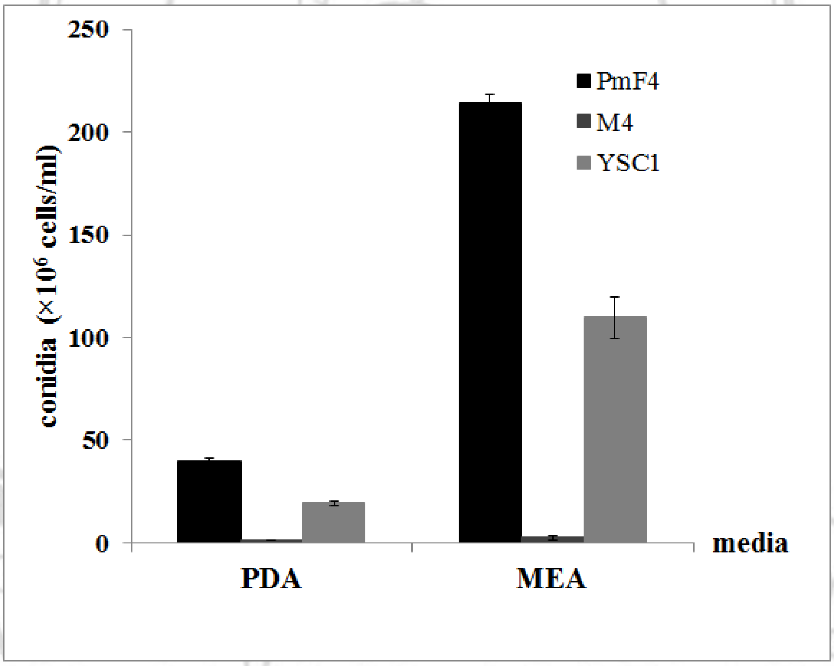


Figure 5.30 A number of conidia produced by the *sakA* mutant comparing to the wild type and the complemented strains. Three thousands of conidia isolated from all strains were inoculated and cultured on PDA and MEA at 25°C for 7 days and conidia were counted using a hemacytometer. Data are from three independent experiments and standard error bars of the mean are shown ($p<0.0001$).

3) Susceptibility to stresses of conidia

To determine the function of the *sakA* and *atfA* genes on stress response, the growth of wild type, mutant and complemented strains on media supplemented with or without different stressors were evaluated.

3.1) Oxidative stress

For the *sakA* gene, at 25°C, the results showed that the *sakA* mutant strain was more slightly sensitive to 1.5 mM H_2O_2 (Figure 5.31B) and 2.5 mM *t*-BOOH (Figure 5.31C) which are inorganic and organic peroxides than the wild type and the complemented strains. At 37°C, growth of the *sakA* mutant was also reduced on media supplemented with 0.5 mM H_2O_2 and 0.5 mM *t*-BOOH (Figure 5.31F and G) when compared to the wild type and the complemented strains. Nevertheless, comparing to wild type and complemented strains, growth of the *sakA* mutant did not decrease on media supplemented with redox-cycling compound, menadione at both temperatures (Figure 5.31D and H).

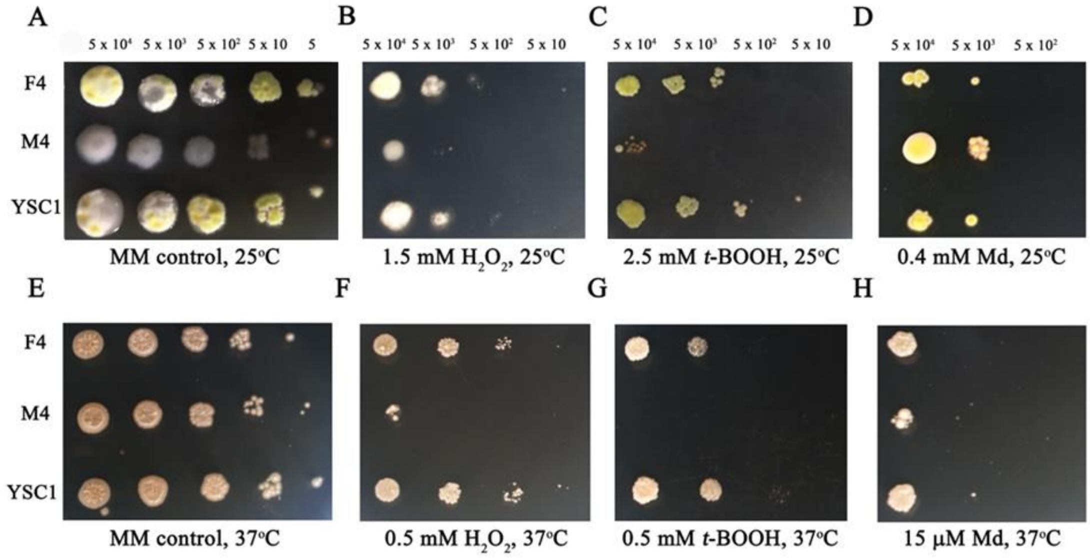


Figure 5.31 Susceptibility of *P. marneffei sakA* mutant to oxidative stress. Growths of *P. marneffei* wild type (F4), the *sakA* mutant (M4) and *sakA* complemented (YSC1) strains at 25°C and 37°C on MM agar supplemented with 1.5 and 0.5 mM H_2O_2 (B, F), 2.5 and 0.5 mM *t*-BOOH (C, G) and 0.4 mM and 15 µM menadione (D, H) incubated for 5 days were shown. Five microliters of cell dilutions (5×10^4 to 5 cells) were inoculated on MM agar containing each compound. (A) and (E) represent MM control plates at 25°C and 37°C, respectively.

For *atfA* gene, at 25°C, *atfA* mutant strain was more slightly sensitive to 2 mM *t*-BOOH (Figure 5.32B) comparing to the wild type and complemented strains. However, growths of all strains were undistinguished under stresses from both 2 mM H_2O_2 and 0.25 mM menadione (Figure 5.32C and D). At 37°C, a slightly higher susceptibility to *t*-BOOH (0.5 mM) was observed in the mutant (Figure 5.32F) and no difference among the mutant, wild type, and complemented strains under one mM H_2O_2 and 25 µM menadione stresses (Figure 5.32G, H).

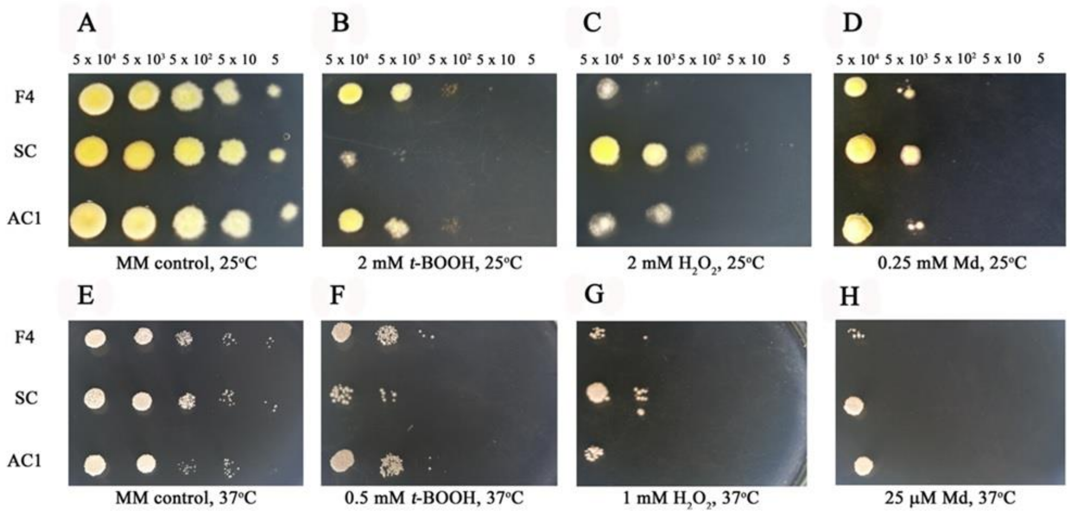


Figure 5.32 Susceptibility of *P. marneffei atfA* mutant to oxidative stresses. Growth of *P. marneffei* wild type (F4), the *atfA* mutant (SC) and *atfA* complemented strain (AC1) at 25°C and 37ºC on MM agar supplemented with 2 and 0.5 mM *t*-BOOH (B and F), 2 and 1 mM H_2O_2 (C and G), and 0.25 mM and 25 µM menadione (D and H). Five microliters of cell dilutions (5×10^4 to 5 cells) were inoculated on MM agar containing each compound. (A) and (E) represent MM control plates at 25°C and 37°C, respectively.

3.2) Osmotic stress

For *sakA* gene, the result of osmotic stress demonstrated that growths of *P. marneffei* wild type and *sakA* mutant on media supplemented with 1 M and 0.5 M sorbitol at 25°C and 37°C were not different (Figure 5.33). For *atfA gene*, the results revealed that growths of *P. marneffei* wild type, *atfA* mutant and *atfA* complemented strains on media supplemented with NaCl or sorbitol were not different at both 25°C and 37°C (Figure 5.34A to F).

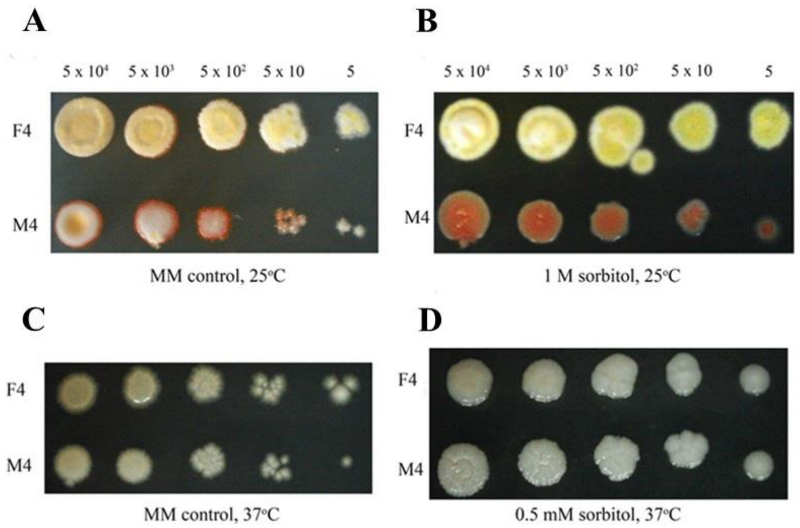


Figure 5.33 Susceptibility of *P. marneffei sakA* mutant to osmotic stresses . Five microliters of cell dilutions (5×10^4 to 5 cells) of the wild type (F4) and the *sakA* mutant (M4) strains were inoculated on MM agar supplemented with 0.5 and 1 M sorbitol (B and D). (A) and (C) represent MM control plates at 25°C and 37°C, respectively.

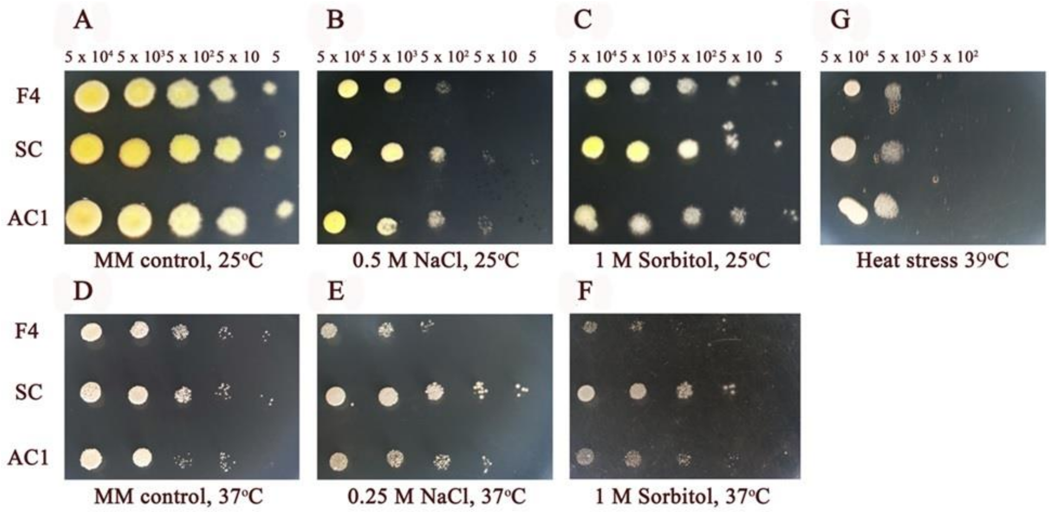


Figure 5.34 Susceptibility of *P. marneffei atfA* mutant to osmotic and heat stresses. Five microliters of cell dilutions (5×10^4 to 5 cells) of wild type (F4), *atfA* mutant (SC) and *atfA* complemented (AC1) strains were inoculated on MM agar supplemented with 0.5 and 0.25 M NaCl (B and E) and 1 M sorbitol (C and F). (G) MM agar was incubated at 39°C. (A) and (D) represent MM control plates at 25°C and 37°C, respectively.

3.3) Chitin deposition and cell wall stress

To investigate the function of *P. marneffei sakA* in cell wall integrity, wild type, *sakA* mutant and *sakA* complemented strains were grown for 4 days at 25°C and were stained with calcofluor white (an anionic dye that binds to nascent chitin chain) to visualize cell wall and chitin deposition. The results demonstrated that all strains showed normal branching and no cellular swellings. However, there were abnormal chitin deposits along the hyphae of the *sakA* mutant comparing to those of the wild type and the complemented strains (Figure 5.35A). The susceptibility to cell wall stresses was tested. Conidia from the wild type, the *sakA* mutant and the complemented strains were inoculated on media supplemented with sodium dodesyl sulphate (SDS) which is able to destabilize cell wall (Tomazett *et al*., 2011) and calcofluor white (CFW) which binds to chitin and inhibits cell wall synthesis. The *sakA* mutant had a higher sensitivity to SDS at both 25°C and 37°C (Figure 5.35D to E). However, growths of all strains were similar under CFW stress at 25°C (Figure 5.35F) and the *sakA* mutant was more slightly sensitive to this compound at 37°C (Figure 5.35H).

For *atfA* gene, the wild type, the *atfA* mutant and the complemented strains were grown on PDA at 25°C and the cells were stained with CFW after four and seven days of incubation to visualize cell wall and chitin deposition. The results demonstrated that all strains showed normal chitin deposition along their hyphae (Figure 5.36A). In addition, the response of conidia from the *atfA* mutant to cell wall disrupted agent CFW was similar to those of wild type and *atfA* complemented strains (Figure 5.36C and F). Nevertheless, conidia from the mutant had a higher sensitivity to SDS at both 25°C and 37°C when compared to the wild type and complemented strains (Figure 5.36D and G).

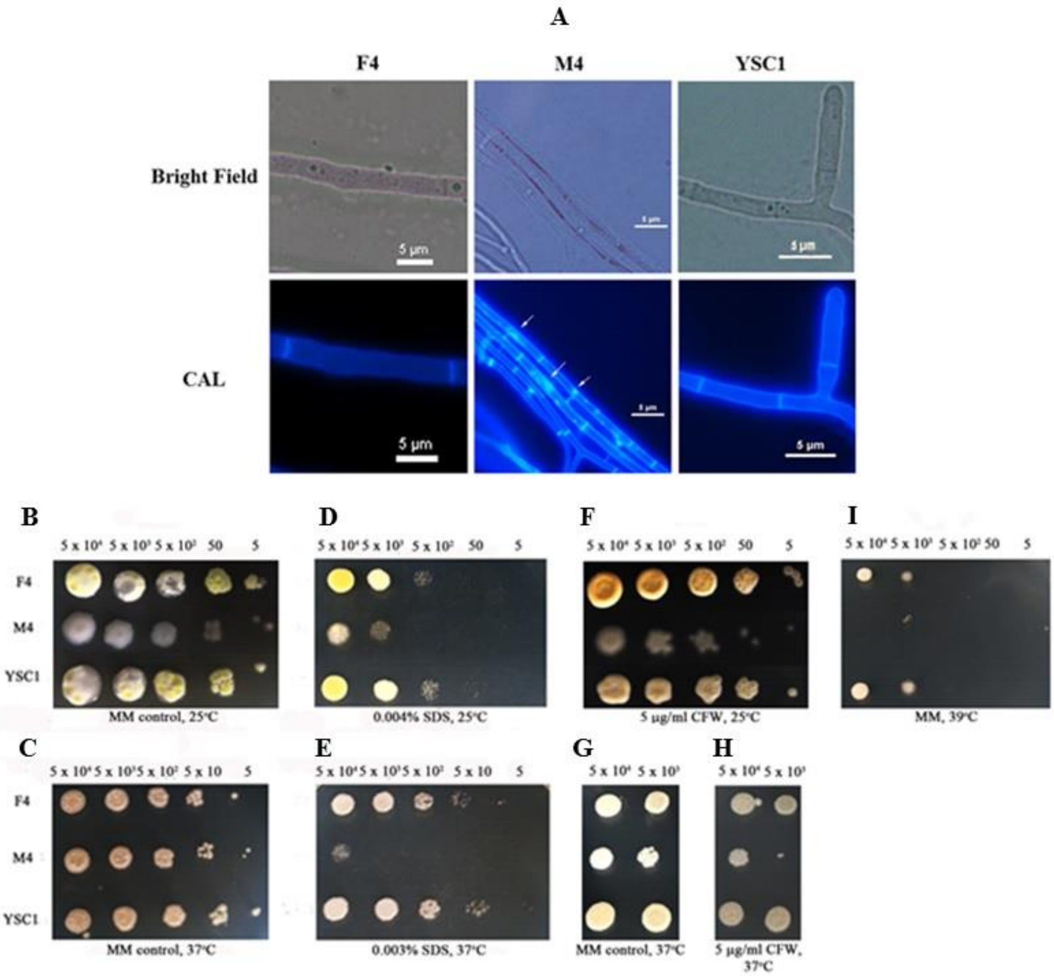


Figure 5.35 Chitin deposition and cell wall stress of *P. marneffei sakA* mutant. (A) *P. marneffei* wild type (F4), *sakA* mutant (M4) and *sakA* complemented (YSC1) strains were grown for 4 day at 25°C on PDA and stained with calcofluor white (CAL) to visualize cell wall and septa. Unusual chitin depositions along the hyphae of the *sakA* mutant are indicated by white arrows. (B to H) susceptibility of *P. marneffei* to cell wall and heat stresses. Five microliters of conidial dilutions (5 × 10^4 to 5 cells) of wild type (F4), *sakA* mutant (M4) and *sakA* complemented (YSC1) strains were inoculated on media supplemented with 0.004% SDS (D), 0.003% SDS (E), and 5 µg/ml CAL (F and G) and incubated at 25°C or 37°C for 5 days. (B) and (C) represent MM control plates. (I) conidia of all strains were inoculated on MM agar and incubated at 39°C for 5 days. Scale bar represents five micrometers.

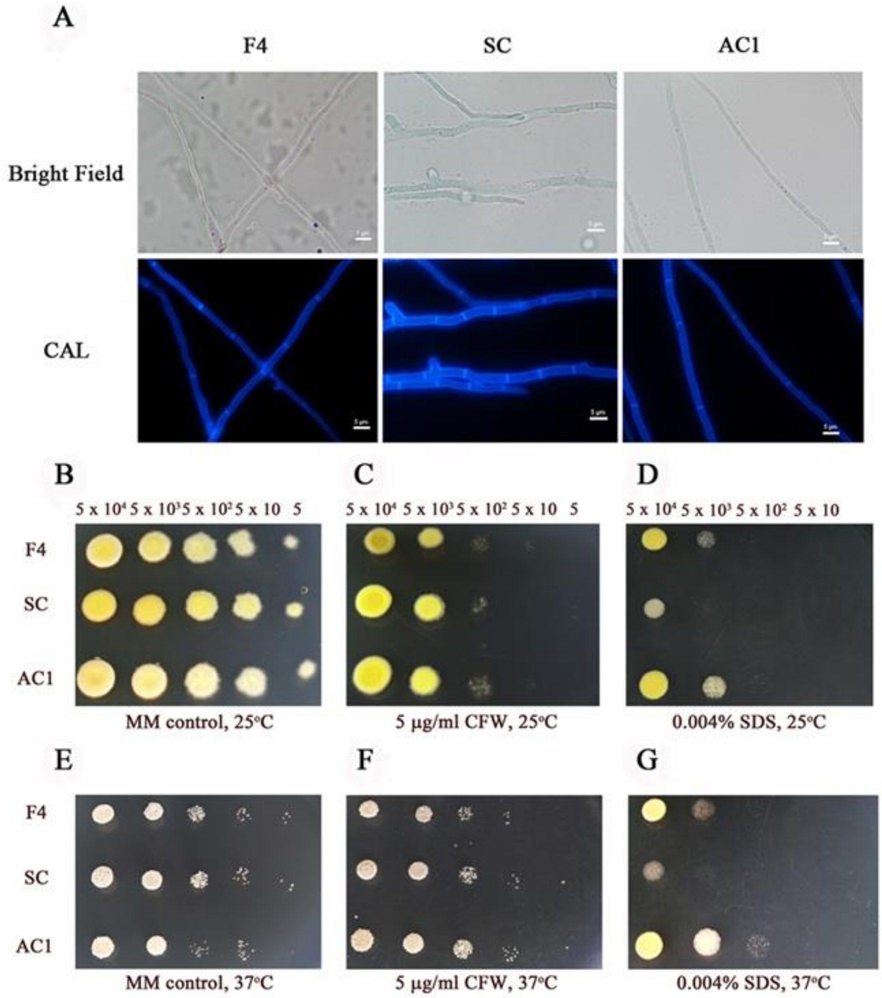


Figure 5.36 Chitin deposition and cell wall stress of *P. marneffei atfA* mutant. (A) *P. marneffei* wild type (F4), *atfA* mutant (SC) and *atfA* complemented (AC1) strains were grown for seven day at 25°C on PDA and stained with CFW day four and day seven to visualize cell wall and septa. Scale bar represents five micrometers. (B to G) five microliters of cell dilutions (5 × 10^4 to 5 cells) of wild type, *atfA* mutant and *atfA* complemented strains were inoculated on media supplemented with 5 µg/ml (C and F) and 0.004% SDS (D and G). (B) and (E) represent MM control plates at 25°C and 37°C, respectively.

3.4) Heat stress

For response to heat stress, conidia of wild type, *sakA* mutant and *sakA* complemented strains were incubated at 39°C. The results revealed that conidia of *sakA* mutant could not grow at this temperature in all concentration comparing with those of wild type and complemented strains (Figure 5.35I).

On the other hand, growth of conidia from *atfA* mutant at 39ºC was similar to the wild type, and *atfA* complemented strains (Figure 5.34G) and viabilities of all strains were not significantly different when the temperature was increased to 42ºC (Figure 5.37A). In addition, the expressions of *sakA* and *atfA* genes in conidia of *P. marneffei* wild type incubated at 42ºC for 10, 20, 30 and 40 minutes were evaluated. The results revealed that the expressions of both genes were increased at every time point (Figure 5.37B). To investigate whether SakA regulates the expression of *atfA* gene under heat stress at 42ºC, conidia of *sakA* mutant were incubated at 42ºC for 20 minutes and the relative expression level of *atfA* gene were identified. The result demonstrated that deletion of *sakA* gene did not affect the increase of *atfA* expression under heat shock stress. On the other hand, the expression of *atfA* in *sakA* mutant was significantly higher than the wild type strain under this kind of stress (Figure 5.37C).

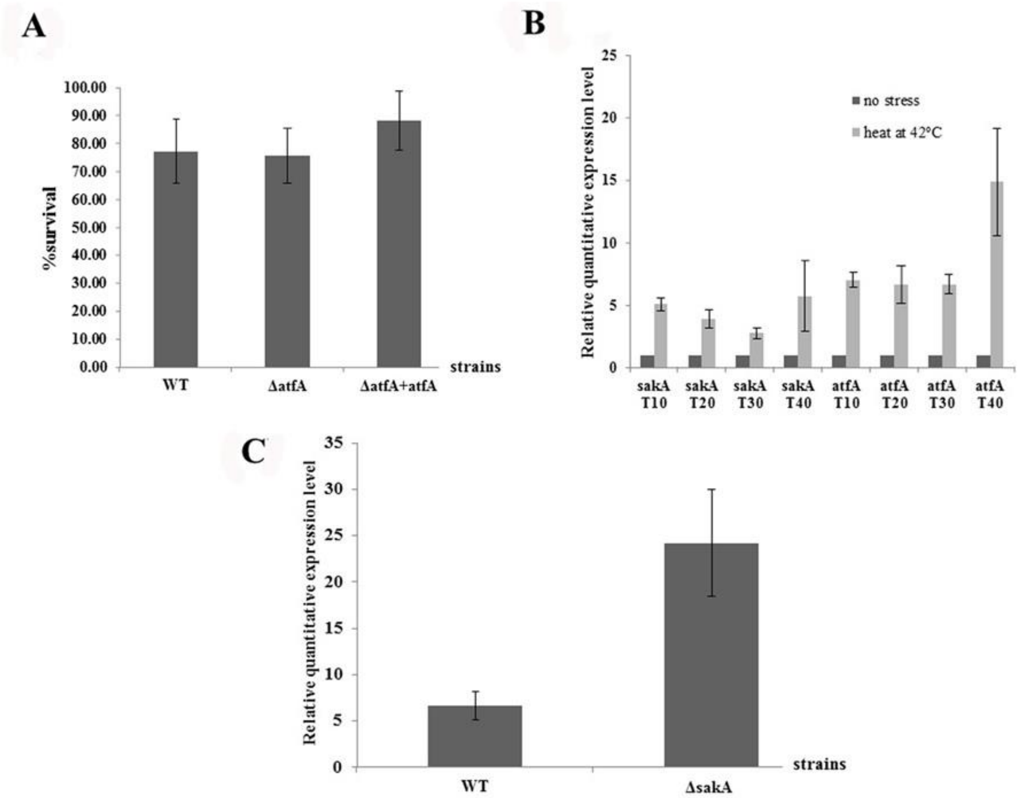


Figure 5.37 Survival and gene expressions of *P. marneffei* under heat stress at 42ºC. (A) Survival of conidia from *P. marneffei* wild type (WT), *atfA* mutant (Δ*atfA*) and complemented strains (Δ*atfA* + *atfA*) after incubating in BHI at 42ºC for one hour. (B) Relative RNA expression of *sakA* and *atfA* genes of conidia from *P. marneffei* wild type determined by real-time PCR. Conidia were incubated at 42ºC for 10, 20, 30 or 40 minutes. Total RNA was extracted from conidia and subjected to real-time PCR. Expression level of heat stress cells is presented as relative value to the expression level from no stress cells which is given a value of 1. GAPDH gene expression level was used to normalize amounts of input RNA. (C) Relative RNA expression level of *atfA* gene of conidia from *P. marneffei* wild type (WT), *sakA* mutant (Δ*sakA*). Conidia were incubated at 42ºC for 20 minutes and total RNA was extracted and subject to real-time PCR. Results were obtained from three independent experiments and standard error bars of the mean bars are shown ($p<0.01$).

3.5) UV stress

To demonstrate the roles of *sakA* and *atfA* genes under UV stress, *P. marneffei* wild type, *sakA* and *atfA* mutant conidia were exposed to different doses of UV light. The results showed that the survival of all strains after exposure to UV light were not significantly different (Figure 5.38).

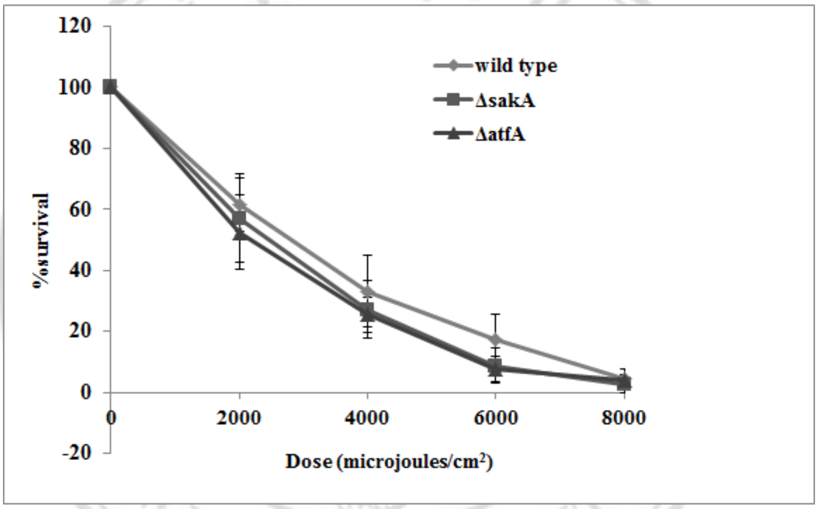


Figure 5.38 Susceptibility of conidia from *P. marneffei* to UV light. Conidia of *P. marneffei* wild type, *sakA* mutant (Δ*sakA*) and *atfA* mutant (Δ*atfA*) were plated in duplicate on SDA and exposed to different UV light radiation at 0, 2000, 4000, 6000 and 8000 microjoules/cm^2. Data are from three independent experiments and standard error bars of the mean bars are shown.

3.6) Susceptibility to oxidative stresses

For the function of *sakA* gene in oxidative stress response in mycelia, the sensitivities to oxidative stressors of mycelia plugs from *P. marneffei* wild type and the *sakA* mutant strains were investigated. The result demonstrated that mycelial growth of *P. marneffei sakA* mutant were indistinguishable from the wild type under both H_2O_2 and *t*-BOOH stresses (Figure 5.39).

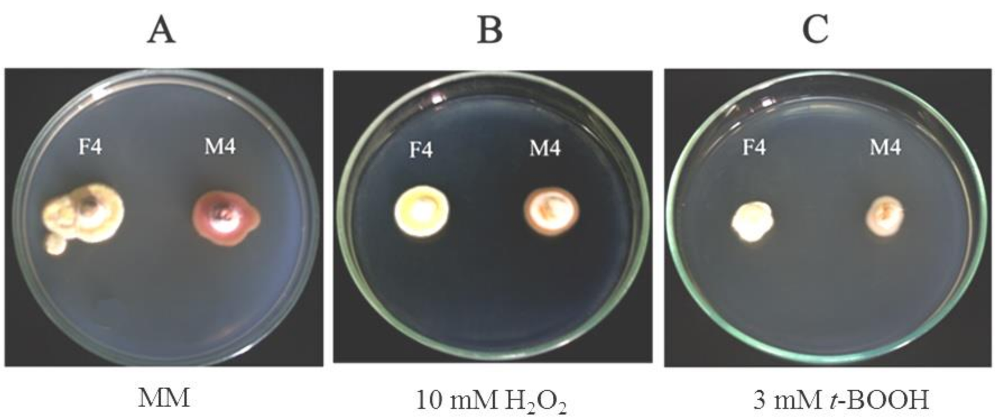


Figure 5.39 Susceptibility of *P. marneffei* mycelia to oxidative stress. Mycelial plugs from the wild type (F4) and the *sakA* mutant (M4) were transferred to MM media supplemented with/without H_2O_2 (B) or *t*-BOOH (C) and were incubated for 5 days at 25°C. (A) represents MM control plates.

4) Intracellular survival inside macrophages

To investigate the role of *sakA* and *atfA* genes for survival of *P. marneffei* inside macrophages, both mouse (J774) and human (THP1) macrophage cell lines were infected with conidia from *P. marneffei* wild type, *sakA* and *atfA* mutants, and *sakA* and *atfA* complemented strains. Twenty four hours post-infection, the survival of conidia from both the *sakA* and *atfA* mutants was significantly decreased in both cell types comparing to the wild type and the complemented strains (Figure 5.40).

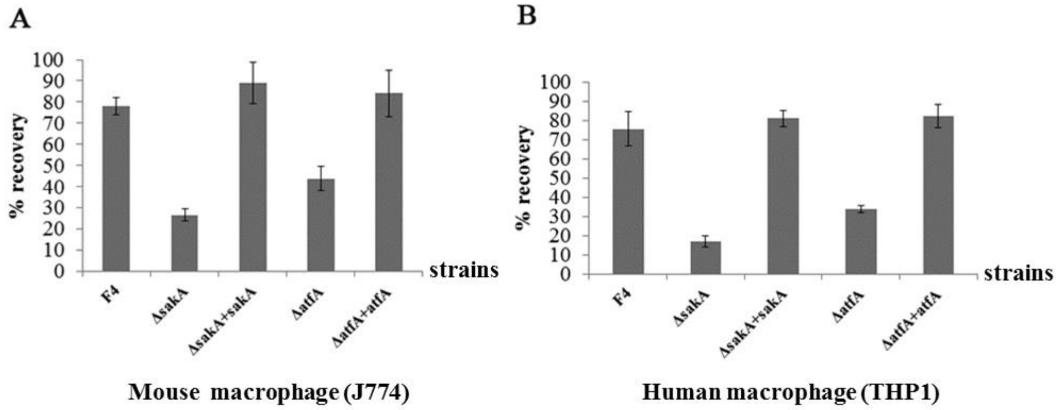


Figure 5.40 Survival of *P. marneffei* inside mouse and human macrophage cell lines. Mouse (A) and human (B) macrophage cell lines were infected with conidia of *P. marneffei* wild type (F4), *sakA* mutant (Δ*sakA*) and *atfA* mutant (Δ*atfA*), and *sakA* (Δ*sakA*+*sakA*) and *atfA* complemented (Δ*atfA*+*atfA*) strains. Percent recovery was calculated from number of colonies recovered after 24 hours post-infection using number of colonies recovered after 2 hours post-infection as the initial inocula. Data are from three independent experiments and standard error bars of the mean bars are shown ($p<0.0001$ for wild type and *sakA* mutant and $p <0.01$ for wild type and *atfA* mutant).