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Two Threonine Residues Required for Role of AfsKav in Controlling Morphogenesis and Avermectin Production in *Streptomyces avermitilis*

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Abstract AfsKav is a eukaryotic-type serine/threonine protein kinase, required for sporulation and avermectin production in *Streptomyces avermitilis*. In terms of their ability to complement SJW4001 (Δ*afsK-av*), *afsK-av* mutants T165A and T168A were not functional, whereas mutants T165D and T168D retained their ability, indicating that Thr-165 and Thr-168 are the phosphorylation sites required for the role of AfsKav. Expression of the *S*-adenosylmethione synthetase gene promoted avermectin production in the wild-type *S. avermitilis*, yet not in the mutant harboring T168D or T165D, demonstrating that tandem phosphorylation on Thr-165 and Thr-168 in AfsKav is the mechanism modulating avermectin production in response to *S*-adenosylmethione accumulation in *S. avermitilis*.

Keywords: *Streptomyces avermitilis*, AfsKav, phosphorylation residue, site-directed mutagenesis, avermectin production

Protein kinases provide an efficient means of reversibly modulating the biochemical properties of proteins, thereby regulating various cellular events and rendering the cell adaptable to metabolic or environmental changes. Eukaryotic-type serine/threonine (Hanks' type) protein kinases (STPKs) have recently emerged as the bacterial components involved in the regulation of differentiation processes [8, 14, 16, 24], and mycobacterial STPKs have attracted attention as the primary subject of special bacterial STPK studies focused on identifying new targets for an antituberculosis drug. The sequence analysis of the *Mycobacterium tuberculosis* genome reveals eleven STPKs [5], yet the predicted number of STPKs in the genome of *Streptomyces*, a renowned producer of antibiotic metabolites, outnumbers that in *Mycobacterium tuberculosis*, with forty-four and thirty-two STPKs predicted in *S. coelicolor* [1] and *S. avermitilis* [15], respectively. This abundance of STPKs in *Streptomyces* may reflect the diversity of environments that this organism can cope with. As early as 1994, AfsK was identified in *S. coelicolor* due to its ability to phosphorylate AfsR, a transcriptional regulatory protein for actinorhodin biosynthesis [13]. The AfsK/AfsR system controls actinorhodin biosynthesis in *S. coelicolor* without any apparent contribution to morphological differentiation, whereas an ortholog system is involved in the control of morphological differentiation in *S. griseus* [19, 20]. In *S. avermitilis*, the avermectin producer, the *afsK* ortholog (*afsK-av*) displays a global regulatory role and controls sporulation, melanogenesis, and avermectin production [17]. Accordingly, the present study performed site-directed mutagenesis and *in vivo* characterization studies to identify the phosphorylation sites that are critical for the *in vivo* function of AfsKav.

The (auto)phosphorylation residues have already been characterized *in vitro* in various bacterial STPK studies: PknB in *M. tuberculosis* [3, 22], PknB, PknD, PknE, and PknF in *M. tuberculosis* [7], PrkC in *Bacillus subtilis* [12], and AfsK in *S. coelicolor* [18]. Moreover, all these STPKs have been found to be autophosphorylated by one or more threonine residues in the activation loop. In terms of phosphorylating a myelin-basic protein, a surrogate substrate, two threonine residues (Thr-171 and Thr-173) are critical for the kinase activity of PknB [7], whereas four threonine residues (Thr-162, Thr-163, Thr-165, and Thr-167) contribute to the activity of PrkC [12]. In the case of AfsK in *S. coelicolor*, Thr-168 has been defined as the only critical autophosphorylation site for phosphorylating AfsR, the specific target of AfsK [18]. The multiple alignment of AfsKav with AfsK, PknB, and PrkC revealed a candidacy of four residues (Ser-163, Thr-165, Thr-168, and Thr-170) for phosphorylation sites, and they are the subjects of the present study (Fig. 1; Thr-168 in AfsKav aligns with Thr-168, Thr-165, and Thr-171 in AfsK, PrkC, and PknB, respectively).

It has been previously demonstrated that a plasmid copy of *afsK-av* (pJWS4004) readily complements the phenotypic

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                          * *   * *
AfsKc     155-DFGIASGV--SNTRLTMTNVAVGTPAYMSPE
AfsKav    155-DFGIASGV--SNTRLTMTNVAVGTPAYMSPE
AfsKg     155-DFGIASGV--SNTRLTMTNVAVGTPAYMSPE
PknB      156-DFGIARAIADSGNSVTQTAAVIGTAQYLSPE
PrkC      152-DFGIATAL--SSTTITHTNSVLGSVHYLSPE
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Fig. 1. Alignment of activation loop regions in certain bacterial STPKs [18]. The conserved residues are shaded, and the residues selected for mutation are marked above.
The alignment was performed using MultAlign software [6]: AfsKc, AfsK (SCO4423) from *S. coelicolor*; AfsKav, AfsK ortholog from *S. avermitilis* (SAV3816); AfsKg (GenBank locus P54742), AfsK ortholog from *S. griseus*; PknB (Rv0014c) from *M. tuberculosis*; PrkC (BSU15770) from *B. subtilis*.

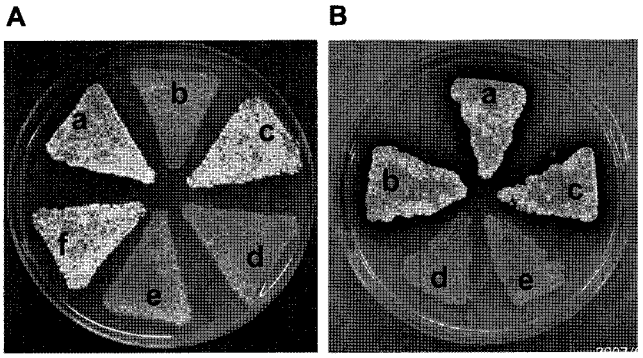

Fig. 2. Complementation of SJW4001 (*afsK-av*::*neo*) with wild-type copy or mutated copies of *afsK-av*. *S. avermitilis* and its derivative strains were grown in R2YE agar media with 50 μg/ml of thiostrepton for 5 days at 28°C.
A. (a) Wild-type strain/pWHM3, (b) SJW4001/pWHM3, (c) SJW4001/pJWS4004 (S163A), (d) SJW4001/pJWS4004 (T165A), (e) SJW4001/pJWS4004 (T168A), and (f) SJW4001/pJWS4004 (T170A). **B.** (a) Wild-type strain/pWHM3, (b) SJW4001/pJWS4004 (T165D), (c) SJW4001/pJWS4004 (T168D), (d) SJW4001/pJWS4004 (T165D/T168A), and (e) SJW4001/pJWS4004 (T165A/T168D).

defects resulting from the loss of *afsK-av* in SJW4001 (*afsK-av*::*neo*) [17]. Therefore, the four potential phosphorylation residues in AfsKav (Ser-163, Thr-165, Thr-168, and Thr-170) were separately switched to Ala using a standard site-directed mutagenesis procedure, and the resulting *afsK-av* fragments subcloned into pWHM3, a *Streptomyces-E. coli* shuttle plasmid [21]. The oligonucleotide primers used for the site-directed mutagenesis are listed in Table 1. The authenticity of all the PCR products was confirmed by nucleotide sequencing. The insert of pJWS4004, the full-length *afsK-av* starting 303-nt upstream from the translational start codon, was used as the template for the site-directed mutagenesis. The resulting pJWS4004 derivatives were introduced into SJW4001 and tested for their ability to revert the phenotypic defects of SJW4001. As shown in Fig. 2A, whereas mutants S163A and T170A restored the ability to sporulate in SJW4001 (*afsK-av*::*neo*), mutants T165A and T168A failed to do so. Avermectin production was also restored by mutants S163A and T170A, yet not by mutants T165A and T168A (data not shown). The production of avermectin was evaluated by an HPLC analysis of the organic extract of an R2YE agar culture, as previously documented [17]. It should also be noted that this Ala replacement mutagenesis was also performed with the AfsKav catalytic domain (300 residues) region in pJWS4008, which was previously validated for its ability to complement SJW4001 [17]. Mutants T165A and T168A also abolished the ability of the AfsKav catalytic domain to complement

Table 1. Oligonucleotide primers used in this study.

Oligonucleotides	Sequences[b]
S163A, sense	5'-GGCGTT**G**CG AACACGCGTCTGACC-3'
S163A, antisense	5'-CGTGTT CG**C** AACGCCCGACGCGAT-3'
T165A, sense	5'-TCGAAC**G**CG CGTCTGACCATGACG-3'
T165A, antisense	5'-CAGACG CG**C** GTTCGAAACGCCCGA-3'
T168A, sense	5'-CGTCTG**G**CC ATGACGAACGTCGCC-3'
T168A, antisense	5'-CGTCATGG**C**CAGACGCGTGTTCGA-3'
T170A, sense	5'-ACCATG**G**CG AACGTCGCCGTCGGC-3'
T170A, antisense	5'-GACGTT CG**C** CATGGTCAGACGCGT-3'
T165D, 1st, sense[a]	5'-TCGAAC**GAG** CGTCTGACCATGACG-3'
T165D,1st, antisense[a]	5'-CAGACG C**TC** GTTCGAAACGCCCGA-3'
T165D, 2nd, sense[a]	5'-TCGAACGA**C**CGTCTGACCATGACG-3'
T165D, 2nd, antisense[a]	5'-CAGACG**GTC** GTTCGAAACGCCCGA-3'
T168D, sense	5'-CGTCTG**GA**CATGACGAACGTCGCC-3'
T168D, antisense	5'-CGTCATG**TC**CAGACGCGTGTTCGA-3'
T165A (T168D) sense	5'-TCGAAC**G**CG CGTCTGGACATGACG-3'
T165A (T168D) antisense	5'-CAGACG CG**C** GTTCGAAACGCCCGA-3'

[a]T165D mutation was achieved by two rounds of site-directed mutagenesis.
[b]Cognate triplet codons are underlined and mutated nucleotide positions are boxed.

SJW4001 in sporulation and avemectin production, whereas mutants S163A and T170A retained this complementing ability (data not shown).

Therefore, these results indicate that Ser-163 and Thr-170 have no relevant contribution to the function of AfsKav, implying that Thr-165 and Thr-168 are the critical phosphorylation residues for the role of AfsKav. Thus, for further confirmation that Thr-165 and Thr-168 are phosphorylation sites, both residues were switched to Asp, which mimics the phosphorylated residue structure, and it was found that mutants T165D and T168D restored the ability to sporulate in SJW4001 (Fig. 2B), thereby substantiating that these two Thr residues are the phosphorylation sites determining AfsKav activity. Tomono *et al.* [18] recently identified Thr-168 as the sole autophosphorylation residue in the AfsK catalytic domain (AfsKΔC), as AfsKΔC obtained from *E. coli* expression displayed phosphorylation towards Ser-71, Ser-128, Thr-168, and Thr-170, among which Thr-168 was identified as the autophosphorylation site contributing to the kinase activity [18]. It has also been asserted that AfsKΔC (T168D) is a constitutively active kinase. The amino acid sequence of AfsK (*S. coelicolor* origin) is nearly identical to that of AfsKav in the N-terminal catalytic domain (five residue differences in the 300 N-terminal residues), allowing the assumption that Thr-165 may be a transient phophorylation site, thereby escaping detection as phosphorylated *in vitro*. It has also been assumed that T165A is inactive, as transient phosphorylation on Thr-165 is required for phosphorylation on Thr-168. If this is the case, mutant T165A/T168D should be active, whereas T165D/T168A is not. To test this hypothesis, double replacement mutants of T165A/T168D and T165D/T168A were prepared from mutant copies of T168D and T165D, respectively. However, in the complementation test, both mutants failed to restore the sporulating ability, indicating that a tandem phosphorylation modification was required to induce AfsKav activity to support the sporulation of *S. avermitilis* (Fig. 2B). Moreover, there was no avermectin production in SJW4001 with T165A/T168D or T165D/T168A. Therefore, it would seem that the phosphorylation residue profiles were not comparable between AfsK and AfsKav, despite the high homology in their catalytic domain sequences.

The role of STPKs in signal transduction is mediated by signal perception, which induces (auto)phosphorylation, and delivery of the perceived message based on phosphorylating other proteins. Thus, when replacing Thr with Asp, the phosphor-amino acid mimic, mutants T165D and T168D (including a double replacement mutant of T165D/T168D) would be supposed to be inactive in the perception process, while still active in phosphorylating a target protein, such as the AfsR ortholog in *S. avermitilis*. It was proposed that the signal perception itself remained intact in mutants T165D and T168D, yet the signal perception was incapable of

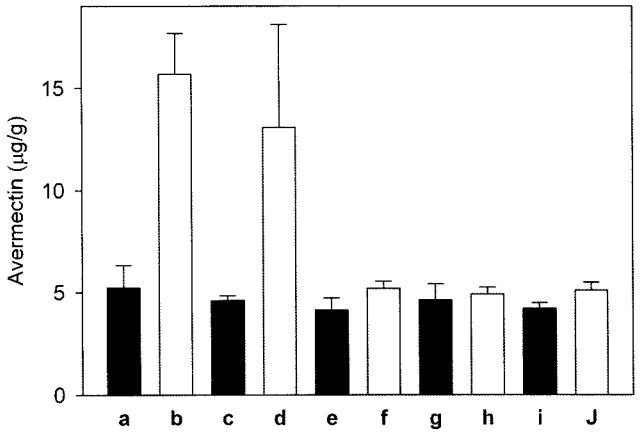

Fig. 3. Avermectin production by SJW4001 (*afsK-av*::*neo*) strains complemented with mutated copies of *afsK-av* in pJWS4004 and the *S*-adenosylmethione synthetase gene *metK* in pSET152.

S. avermitilis and its derivative strains were grown in R2YE agar media with 50 μg/ml of thiostrepton for 5 days at 28°C, and the agar extracted with ethylacetate and methanol for an HPLC analysis. The avermectin yield is presented as the avermectin (μg)/wet cell weight (g). (a) Wild-type strain/pWHM3, (b) Wild-type strain/pWHM3/*metK*, (c) SJW4001/pJWS4004, (d) SJW4001/pJWS4004/*metK*, (e) SJW4001/pJWS4004 (T165D), (f) SJW4001/pJWS4004 (T165D)/*metK*, (g) SJW4001/pJWS4004 (T168D), (h) SJW4001/pJWS4004 (T168D)/*metK*, (i) SJW4001/pJWS4004 (T165D/T168D), and (j) SJW4001/pJWS4004 (T165D/T168D)/*metK*.

conferring a competent phosphorylation modification in these mutants, as one of the critical residues was already fixed phosphorylated. This hypothesis was tested by employing *S*-adenosylmethionine (SAM) synthetase (MetK) as a stimulator for avermectin production. SAM has already been documented as a positive effecter for secondary metabolisms in *S. coelicolor* [4, 11, 25] and the expression of *metK* has induced SAM accumulation and activated a secondary metabolism in *S. lividans* [10]. Moreover, the introduction of multicopy *metK* to *S. avermitilis* has been demonstrated to enhance avermectin production by two- to four-fold compared with the vector-alone control [23]. In the present study, a *metK* expression plasmid was prepared by cloning *S. spectabilis metK* [10] in pSET152, a site-specific integrating plasmid [2] under the control of the 280-bp *ermE* promoter [9]. The introduction of the *metK* gene enhanced the avermectin yield by no less than two-fold in the wild-type strain and SJW4001/pJWS4004 (*afsK-av*), yet not in mutant T165D, T168D, or T165D/T168D (Fig. 3), thereby supporting the hypothesis that the expression of *metK* results in *S*-adenosylmethionine accumulation, which then induces a signal to activate the (auto)phosphorylation of AfsKav on Thr-165 and/or Thr-168 to promote avermectin biosynthesis.

In conclusion, this *in vivo* characterization study of AfsKav successfully identified the phosphorylation residues essential to its regulatory role. Two Thr residues in the activation loop were confirmed to be essential to the role

of AfsKav *in vivo*, thus expanding current knowledge on *Streptomyces* STPKs.

Acknowledgments

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References

1. Bentley, S. D., K. F. Charter, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, *et al.* 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417:** 141–147.
2. Bierman, N., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116:** 43–49.
3. Boitel, B., M. Ortiz-Lombardía, R. Durán, F. Pompeo, S. T. Cole, C. Cerveñansky, and P. M. Alzari. 2003. PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phosphor-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **49:** 1493–1508.
4. Chong, Y., J. Young, J. Kim, Y. Lee, K. S. Park, J. H. Cho, H. J. Kwon, J. W. Suh, and Y. Lim. 2006. *S*-Adenosyl-L-methionine analogues to enhance the production of actinorhodin. *J. Microbiol. Biotechnol.* **16:** 1154–1157.
5. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, *et al.* 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genomic sequence. *Nature* **393:** 537–544.
6. Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucl. Acids Res.* **16:** 10881–10890.
7. Durán, R., A. Villarino, M. Bellinzoni, A. Wehenkel, P. Fernandez, B. Boitel, S. T. Cole, P. M. Alzari, and C. Cerveñansky. 2005. Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of *Mycobacterium tuberculosis* Ser/Thr protein kinases. *Biochem. Biophys. Res. Commun.* **333:** 858–867.
8. Kenelly, P. J. 2002. Protein kinases and protein phosphatases in prokaroytes: A genome perspective. *FEMS Microbiol. Lett.* **206:** 1–8.
9. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. *Practical Streptomyces Genetics.* John Innes Centre, Norwich Research Park, England.
10. Kim, D. J., J. H. Huh, Y. Y. Yang, C. M. Kang, I. H. Lee, C. G. Hyun, S. K. Hong, and J. W. Suh. 2003. Accumulation of *S*-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividans* TK23. *J. Bacteriol.* **185:** 592–600.
11. Lee, Y., J. Young, H. J. Kwon, J. W. Suh, J. Kim, Y. Chong, and Y. Lim. 2006. AdoMet derivatives induce the production of actinorhodin in *Streptomyces coelicolor*. *J. Microbiol. Biotechnol.* **16:** 965–968.
12. Mandec, E., A. Stensballe, S. Kjellström, L. Cladière, M. Obuchowski, O. N. Jensen, and S. J. Séror. 2003. Mass spectrometry and site-directed mutagenesis identify several autophosphorylated residues required for the activity of PrkC, a Ser/Thr kinase from *Bacillus subtilis*. *J. Mol. Biol.* **330:** 459–472.
13. Matsumoto, A., S. K. Hong, H. Ishizuka, S. Horinouchi, and T. Beppu. 1994. Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene* **146:** 47–57.
14. Ogawa, H., N. Aoyagi, M. Watanabe, and H. Urabe. 1999. Sequences and evolutionary analyses of eukaryotic-type protein kinases from *Streptomyces coelicolor* A3(2). *Microbiology* **145:** 3343–3352.
15. Omura, S., H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, H. Kikuchi, T. Shiba, Y. Sakaki, and M. Hattori. 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deciphering the ability of producing secondary metabolites. *Proc. Natl. Acad. Sci. USA* **98:** 12215–12220.
16. Petrickova, K. and M. Petricek. 2003. Eukaryotic-type protein kinases in *Streptomyces coelicolor*: Variation on a common theme. *Microbiology* **149:** 1609–1621.
17. Rajkanikar, A., H. J. Kwon, Y. W. Ryu, and J. W. Suh. 2006. Catalytic domain of AfsKav modulates both secondary metabolism and morphologic differentiation in *Streptomyces avermitilis* ATCC 31272. *Curr. Microbiol.* **53:** 204–208.
18. Tomono, A., M. Mashiko, T. Shimazu, H. Inoue, H. Nagasawa, M. Yoshida, Y. Ohnishi, and S. Horinouchi. 2006. Self-activation of serine/threonine kinase AfsK on autophosphorylation at threonine-168. *J. Antibiot.* **59:** 117–123.
19. Umeyama, T., P. C. Lee, K. Ueda, and S. Hourinochi. 1999. An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in *Streptomyces griseus*. *Microbiology* **145:** 2281–2292.
20. Umeyama, T., P. C. Lee, and S. Hourinochi. 2002. Protein serine/threonine kinases in signal transduction for secondary metabolism and morphogenesis in *Streptomyces*. *Appl. Microbiol. Biotechnol.* **59:** 419–425.
21. Vara, J., M. Lewandowska-Skarbek, Y. G. Wang, S. Donadio, and C. R. Hutchinson. 1989. Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway

in *Saccharopolyspora erythea* (*Streptomyces erythreus*). *J. Bacteriol.* **171:** 5872–5881.
22. Young, T. A., B. Delagoutte, J. A. Endrizzi, A. M. Falick, and T. Alber. 2003. Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nat. Struct. Biol.* **10:** 168–174.
23. Yoon, G. S., K. H. Ko, H. W. Kang, J. W. Suh, Y. S. Kim, and Y. W. Ryu. 2006. Characterization of *S*-adenosylmethionine synthetase from *Streptomyces avermitilis* NRRL8165 and its effect on antibiotic production. *Enz. Microb. Technol.* **39:** 466–473.
24. Zhang, C. C. 1996. Bacterial signaling involving eukaryotic-type protein kinases. *Mol. Microbiol.* **20:** 9–15.
25. Zhao, X. Q., Y. Y. Jin, H. J. Kwon, Y. Y. Yang, and J. W. Suh. 2006. *S*-Adenosylmethionine (SAM) regulates antibiotic biosynthesis in *Streptomyces* spp. in a mode independent of its role as a methyl donor. *J. Microbiol. Biotechnol.* **16:** 927–932.