Short Communication

Transcriptional analysis of genes encoding proteins significantly increased in *Kitasatospora setae* KM-6054^T under submerged culture

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Kitasatospora setae KM-6054^T is the type species of the genus *Kitasatospora* (Ōmura et al., 1982; Zhang et al., 1997). This strain produces setamycin (bafilomycin B1), which possesses antitrichomonal activity (Ōmura et al., 1981b). The complete genome sequence of *K. setae* KM-6054^T has been determined to be a single linear chromosome of 8,783,278 bp and is predicted to encode 7,569 protein-coding genes, including 24 genes or gene clusters associated with the biosynthesis of secondary metabolites (Ichikawa et al., 2010). Although *Kitasatospora* strains resemble the genus *Streptomyces* with respect to the production of long aerial spore chains, the cell wall peptidoglycan composition and 16S rDNA sequences, among several other features, clearly differ between these genera. In addition, one of the distinguishing characteristics of *Kitasatospora* strains is the production of submerged spores in liquid culture. Furthermore, spores of *Kitasatospora* strains contain LL-diaminopimelic acid (DAP) in the cell wall, whereas the mycelial cell wall contains *meso*-DAP (Ōmura et al., 1981a; Takahashi et al., 1983, 1999). In contrast, *Streptomyces* strains contain LL-DAP in both spores and mycelia. We previously reported that submerged spores are not produced by *Kitasatospora* strains cultured in chemically defined minimal medium (MMT), but they are formed in MMT supplemented with a nutritional factor such as yeast extract or casamino acids (CA) (Takahashi et al., 1996). In synchronous submerged culture in MMT supplemented with casamino acids (CA), submerged spores begin to germinate within 1 h and develop into filamentous mycelia, and after 10 h, premature spore chains are formed at the tip of the mycelia (Takahashi et al., 1991). However, the regulation of submerged spore formation in actinomycete strains has rarely been reported. Here, to elucidate the mechanisms of submerged spore formation by *Kitasatospora* strains, proteomic and transcriptional analyses were performed.

To prepare samples for two-dimensional electrophoresis (2-DE) analysis, synchronous culture of *K. setae* KM-6054^T was performed according to our previous reports (Takahashi et al., 1983, 1996). Briefly, submerged spores were inoculated into non-sporulation medium (MMT medium) and sporulation medium (MMT+CA) followed by incubation at 27°C on a reciprocal culture. Cells cultured in MMT medium were harvested at 72 h. Cells cultured in MMT+CA were harvested serially (6, 8, 10, 12, 14, 72 h). MMT medium was composed of (per liter) 10 g of glucose, 5 g of (NH₄)₂SO₄, 0.5 g of K₂HPO₄, 0.1 g of MgSO₄ · 7H₂O, 0.1 g of NaCl, 5.75 g of TES and 0.5 ml of trace element solution. Trace element solution was composed of (per liter) 0.2 g of FeSO₄ · 7H₂O, 0.2 g of MnCl₂ · 4H₂O, 0.2 g of ZnSO₄ · 7H₂O, 0.2 g of CuSO₄ · 7H₂O and 0.2 g of CoSO₄ · 2H₂O. MMT+CA denotes MMT with 1% (w/v) casamino acid. Harvested cells were washed, suspended in cold lysis buffer containing 8 M urea, 2 M thiourea, 1% (w/v) CHAPS, 50 mM DTT, 1% Triton X-100, and protease inhibitor cocktail (Roche, Mannheim, Germany), and then disrupted by sonication. After removal of insoluble particles by centrifugation (20,000 × *g*, 30 min), samples containing 100 µg protein were applied to agar GELS (pH 3–10; Atto, Tokyo, Japan) and then separated by isoelectric focusing. The second separation was performed in 5–20% gradient SDS-polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250 (Fig. 1). Circles in Fig. 1 (b)–(f) indicate protein spots that significantly increased under sporulation conditions. Protein spots were then analyzed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry after in-gel digestion. Peptide matching and protein database searches against the *K. setae* NBRC 14216^T (=KM-6054^T) protein database were performed using the MASCOT program (Matrix Science, Boston, MA). Table 1 shows the spot numbers and ORF

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Fig. 1. 2-D gel separation of proteins extracted from cells cultured under (a) non-sporulation conditions (MMT, 72 h) and (b, c, d, e, f) sporulation conditions (MMT+CA, 6, 8, 10, 12, 14 h).

Circles in (b)–(f) indicate protein spots that significantly increased under sporulation conditions.

numbers of 28 proteins that significantly increased under sporulation conditions. Among these 28 proteins, the functions of 21 proteins were predicted based on amino acid sequence homology.

Transcriptional analysis of three of the identified protein-encoding genes, *metK*, *ald*, and *bldKB* (shown in gray in Table 1), which are reported to participate in morphological differentiation, was performed by real-time PCR. For the transcription assay, *K. setae* KM-6054^T cells were synchronously cultured in non-sporulation medium (MMT medium) and sporulation medium (YD medium) as described in the 2-DE analysis section. YD medium was composed of (per liter) 10 g of yeast extract and 10 g of dextrose. The time course of submerged spore formation in YD medium was similar to that in MMT+CA. Cells cultured in MMT and YD medium were harvested serially (4, 6, 8, 10, 13, 15, and 24 h). To preserve RNA, the cells were treated with RNAlater[®] (Ambion, Austin, TX) prior to the extraction of total RNA using a RiboPure-Bacteria Kit (Ambion). cDNA was obtained after reverse transcription of 2.5 µg of DNase I-treated total RNA using the OmniScript[®] RT Kit (Qiagen, Hilden, Germany). The time-course expression of the three genes was analyzed by quantitative real-time PCR using a StepOne[™] Real-Time PCR instrument (Applied Biosystems, Warrington, UK) and Power SYBR[®] Green PCR Master Mix (Applied Biosystems). As standards for the quantitative real-time PCR analysis, partial gene sequences of *metK*, *bldKB*, and *ald* were amplified by PCR using gene-specific primers. Figure 2 shows the transcriptional analysis of *metK* (a), *ald* (b), and *bldKB* (c).

The *metK* encodes *S*-adenosylmethionine (SAM) synthase, which catalyzes the biosynthesis of SAM from ATP and L-methionine. Kim et al. (2003) reported that overexpression of the SAM synthase gene in *Streptomyces lividans* TK23 inhibits sporulation and aerial mycelium formation in solid culture, but enhances the production of actinorhodin in both solid and liquid media. Here, the *metK* mRNA levels in *K. setae* KM-6054^T cells in submerged culture were low, and no differences in the time-dependent changes of *metK* mRNA levels were detected between sporulation and non-sporulation conditions (Fig. 2-a). Thus, it appears that MetK protein expression was increased in sporulation, but *metK* transcription was not induced or alternatively, MetK protein degradation was decreased in sporulation conditions. Furthermore, the addition of SAM to non-sporulation medium did not induce submerged spore formation (data not shown). Based on these findings, we concluded that MetK protein does not directly trigger submerged spore formation.

The *ald* gene encodes alanine dehydrogenase, which produces pyruvate, ammonia, NADH, and hydrogen ion from L-alanine, water and NAD⁺. Siranosian et al. (1993) reported that the *ald* of *Bacillus subtilis* 168 is involved in the generation of energy during sporulation. As shown in Fig. 2-b, time-dependent changes of *ald* mRNA levels in *K. setae* KM-6054^T cells were similar under both sporulation and non-sporulation conditions, but the levels of *ald* mRNA under sporulation conditions were approximately 4-fold higher than those under non-sporulation conditions at the time of 4 and 6 h after the inoculation. These results suggest that a large number of *ald* transcripts are needed to drive submerged spore formation. This is the first report to identi-

Fig. 2. Transcriptional analysis of *metK* (a), *ald* (b), and *bldKB* (c).

Experiments were performed in triplicate and the average values are indicated. Error bars represent SD.

○: sporulation conditions, ●: non-sporulation conditions.

fy that the *ald* is involved in inducing actinomycete sporulation.

The *bldKB* encodes an ABC transporter solute-binding protein that is involved in the *bld* signaling cascade in *S. coelicolor* A3(2) (Nodwell et al., 1996). During solid culture of *S. coelicolor* A3(2), *bld* genes control aerial hyphae formation. The ABC transporter BldK, which is composed of BldKA, KB, KC, KD, and KE, is located at the beginning of the *bld* signaling cascade and acts as an importer of differentiation signals (Willey et al., 1993). The levels of *bldKB* mRNA in *K. setae* KM-6054^T cells cultured under sporulation conditions were approximately 10-fold higher than those observed in cells under non-sporulation conditions (Fig. 2-c). The transcription levels of *bldKB* were highest after 8 h of cultivation, and decreased thereafter. Previously, we reported that submerged spore formation in *K. setae* KM-6054^T requires supplementation of the culture medium with nutritional factors, such as some peptides involved in yeast extract or casamino acids, 4–8 h after inoculation (Takahashi et al., 1996). In the present transcriptional analysis, *bldKB* was specifically induced in *K. setae* KM-6054^T cells under sporulation conditions after 4 h of cultivation, which coincided with the timing of the requirement for the nutritional factor needed for spore formation. This finding suggests that *bldKB* also imports a signaling peptide

Table 1. Proteins of *K. setae* KM-6054^T significantly increased under sporulation conditions in submerged culture.

Category	Spot number	Gene	ORF number	Putative function
Transport	69	<i>bldKB</i>	KSE_48280	Putative peptide ABC transporter substrate-binding protein
	114	<i>atpD</i>	KSE_50190	ATP synthase subunit β
Metabolism	34	<i>ino1</i>	KSE_38980	Putative <i>myo</i> -inositol-1-phosphate synthase
	44	<i>lpd1</i>	KSE_22160	Dihydrolipoyl dehydrogenase
	110	<i>eno</i>	KSE_46750	Enolase
	122	<i>pgk</i>	KSE_55410	Phosphoglycerate kinase
	30	<i>mdh</i>	KSE_30550	Malate dehydrogenase
	10	<i>metK</i>	KSE_13830	S-Adenosylmethionine synthase
	11	<i>glyA1</i>	KSE_50870	Serine hydroxymethyltransferase
	12	<i>ald</i>	KSE_57460	Alanine dehydrogenase
	8	<i>purH</i>	KSE_30590	Bifunctional purine biosynthesis protein PurH
	35	<i>hemC</i>	KSE_23210	Porphobilinogen deaminase HemC
DNA replication	53	<i>dnaN</i>	KSE_39200	DNA polymerase III subunit β
	83	<i>rpsD</i>	KSE_14260	30S ribosomal protein S4
	54	<i>rpsA</i>	KSE_20210	30S ribosomal protein S1
	96	<i>rplB</i>	KSE_31650	50S ribosomal protein L2
	116	<i>tsf</i>	KSE_52580	Elongation factor Ts
Other	86	<i>pepA</i>	KSE_22150	Probable cytosol aminopeptidase
	2	<i>tig</i>	KSE_26290	Trigger factor
	109	<i>groL2</i>	KSE_44110	60 kDa chaperonin 2
	21	<i>sodF</i>	KSE_35460	Superoxide dismutase
Unknown	55		KSE_21120	
	111		KSE_48150	
	3		KSE_55000	
	27		KSE_10360	
	42		KSE_14360	
	56		KSE_20340	
	9		KSE_34600	

that induces submerged spore formation. *S. griseus* strains are also known to form submerged spores in the late stage of cultivation. Akanuma et al. (2011) reported that BldK participates in aerial spore formation in solid cultures of *S. griseus* IFO13350, but is dispensable with respect to submerged spore formation. Although their results are inconsistent with our present findings, it should be noted that the process of submerged spore formation in *K. setae* KM-6054^T appears to differ from that in *S. griseus*. In *S. griseus*, submerged spore formation occurs at late stage of cultivation and submerged spores are randomly produced by fragmentation of the mycelia (Kendrick and Ensign, 1983; Szabo et al., 1961). In *K. setae* KM-6054^T, submerged spores are formed in specific parts of the mycelia during the early stage of cultivation and the mycelia co-exist with the submerged spores until the late stage of culture (Takahashi et al., 1991). In the present study, we suggested that the control mechanism of submerged spore formation of *K. setae* KM-6054^T also differed from

that of *S. griseus* IFO13350, and indicated the possibility that the gene related to morphological differentiation in solid cultures of *S. coelicolor* A3(2) also participates in morphological differentiation in submerged cultures of *K. setae* KM-6054^T. To our knowledge, this is also the first report to suggest that *bldKB* participates in actinomycete submerged spore formation.

The wide region including the *bldK* cluster of *K. setae* KM-6054^T (Ichikawa et al., 2010) has syntenic homology with that of *S. griseus* IFO13350 and *S. coelicolor* A3(2). In the genome of *K. setae* KM-6054^T, two gene clusters encoding peptide ABC transporters exist immediately adjacent to the *bldK* region. These clusters are expected to be peptide transporters. Furthermore, amino acid sequences of solute-binding proteins of these two clusters are similar to that of BldKB (solute-binding protein of the BldK cluster) of *S. griseus*. This protein was confirmed to participate in aerial spore formation in solid culture. Taking into consideration these

facts, we think these two gene clusters are possibly related to aerial spore formation in solid culture.

The present study suggested that *ald* and *bldKB* participate in submerged spore formation in *Kitasatospora setae* KM-6054^T. To confirm the role of these genes, further experiments such as gene disruption are indispensable.

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