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Expression and Characterization of Trehalose Biosynthetic Modules in the Adjacent Locus of the Salbostatin Gene Cluster

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Abstract The pseudodisaccharide salbostatin, which consists of valienamine linked to 2-amino-1,5-anhydro-2-deoxyglucitol, is a strong trehalase inhibitor. From our *Streptomyces albus* ATCC 21838 genomic library, we identified thirty-two ORFs in a 37-kb gene cluster. Twenty-one genes are supposed to be a complete set of modules responsible for the salbostatin biosynthesis. Through sequence analysis of the gene cluster, some of the upstream gene products (SalB, SalC, SalD, SalE, and SalF) revealed functional resemblance with trehalose biosynthetic enzymes. On the basis of this rationale, we isolated the five genes (*sal*B, *sal*C, *sal*D, *sal*E, and *sal*F) from the *S. albus* ATCC 21838 and cloned them into the expression vector pWHM3. We demonstrated the noticeable expression and accumulation of trehalose, using only the five upstream biosynthetic gene cluster of salbostatin, in the transformed *Streptomyces lividans* TK24. Finally, 490 mg/l trehalose was produced by fermentation of the transformant with sucrose-depleted R2YE media.

Keywords: Trehalose, salbostatin, *Streptomyces albus*

The free disaccharide trehalose is a common sugar in both prokaryotic and eukaryotic organisms, such as bacteria, fungi, and insects [7, 21]. According to the functional analysis of ORFs from *Mycobacteria* or many other bacteria, it was suggested that trehalose could be generated from glucose-6-phosphate and UDP-glucose (the OtsA-OtsB pathway) [15] or produced from glycogen-like α(1,4)-linked glucose polymers (the TreY-TreZ pathway) [17] and from maltose (the TreS pathway) [8]. On the basis of amino acid sequence similarities, more than 291 sequences of glycosyl hydrolases were classified into 35 families that present a functional framework to understand the mechanisms of the enzymes [11]. Like many other glycosyl hydrolases, trehalase is regarded as crucial in the biosynthesis and degradation of glycoconjugates [3]. The trehalase inhibitors from filamentous *Streptomyces* strains have attracted enormous interest, because the various biological functions of the secondary metabolites are presumably mimicking the transition state of natural substrates [17]. The glycosidase inhibitors could disclose yet unknown biological activities that rise above the mere inhibition of the glycosidase.

Our research group has focused on the overexpression and characterization of the naturally occurring glycosidase inhibitors, particularly the cyclohexane derivatives, such as functionalized aminoglycosides salbostatin. The nonreducing pseudodisaccharide salbostatin (absolute molecular weight 320) was originally isolated from the salinomycin-producing *S. albus* ATCC 21838 [28] and strongly inhibited the activity of trehalase (E.C. 3.2.1.28) because of the structural similarity to the naturally occurring trehalose. Salbostatin is composed of valienamine linked to 2-amino-1,5-anhydro-2-deoxyglucitol. Through sequence analysis of the putative biosynthetic gene cluster from *S. albus* ATCC 21838, we deduced that the two structural motifs of salbostatin could be derived from at least two individual modules. Interestingly, one module that has five gene products (Table 1) that assumed specific functions (SalB, glucokinase; SalC, trehalose-6-phosphate synthase; SalD, transporter; SalE, trehalose-6-phosphate phosphatase; and SalF, ADP-glucose synthase) revealed functional resemblance with trehalose biosynthetic enzymes [1]. Although these genes are not yet described as responsible components for trehalose production, we postulated that trehalose may be synthesized by the five gene products through the OtsA-OtsB-like pathway (Fig. 5). In an attempt to prove that the five genes are probably sufficient number of genes for trehalose biosynthesis, we report here the heterologous production of trehalose in *S. lividans* TK24.

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Table 1. List of the trehalose biosynthetic enzymes and their postulated functions.

Protein	AA	Proposed function (Accession No.)	Matching sequences (%)
SalB	306	Sugar kinase (EF394361)	*Streptomyces coelicolor* A3(2) (64)
SalC	490	Trehalose-6-phosphate synthase (EF394362)	*Streptomyces hygroscopicus* subsp. *jinggangensis* (56)
SalD	384	Major facilitator superfamily (MFS) transporter (EF394363)	*Rhodobacter sphaeroides* 2.4.1 (47)
SalE	293	Trehalose-6-phosphate phosphatase (EF394364)	*Thermobifida fusca* YX (43)
SalF	389	ADP-glucose synthase (EF394365)	*Streptomyces coelicolor* A3(2) (46)

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

S. albus KCTC 9015 (equivalent to *S. albus* ATCC 21838) was obtained from the Korean Collection for Type Cultures (KCTC) and maintained on R2YE agar and cultured in R2YE liquid medium [6]. When needed, 50 μg/ml ampicillin and thiostrepton was added to the medium. The strain *S. lividans* TK24 [30] was employed for the heterologous expression of the trehalose biosynthetic gene cluster (*sal*B, *sal*C, *sal*D, *sal*E, and *sal*F). The 7.5-kb fragments containing the five genes were digested with SacI and ligated into pUC18. This clone was digested with EcoRI and HindIII and subcloned into the high-copy numbered *E. coli-Streptomyces* shuttle vector pWHM3 [30] and designated as pWBF (Fig. 2A). The protocols of Pospiech and Neumann [22] and of Hopwood *et al.* [12] were employed for chromosomal DNA isolation and for *Streptomyces* maintenance, spore collection, and transformation. The techniques for all manipulations and the transformation of recombinant DNA molecules and their analysis by restriction enzyme were carried out according to standard protocols [24]. MegaBACE 1000 and DYEnamic ET Dye Terminator Kits (MegaBACE) were employed for sequencing.

Construction and Screening of Genomic Library

The genomic DNA of *S. albus* KCTC 9015 was partially digested with Sau3AI and the retrieved fragments (~40-kb) were ligated into a SuperCos I vector kit (Stratagene) for generation of a genomic library. The *in vitro* packaging was achieved using a λ-packaging kit (Stratagene), and *E. coli* XL1-Blue MRF was transfected with the packaged phages. A library of cosmid clones was checked by PCR amplification. Based on the internal region of *acb*C, the following primers were employed to screen the salbostatin biosynthetic gene cluster. The enzyme AcbC catalyzes the first bioconversion of the valienamine moiety of acabose, and also salbostatin having a valienamine sugar moiety,

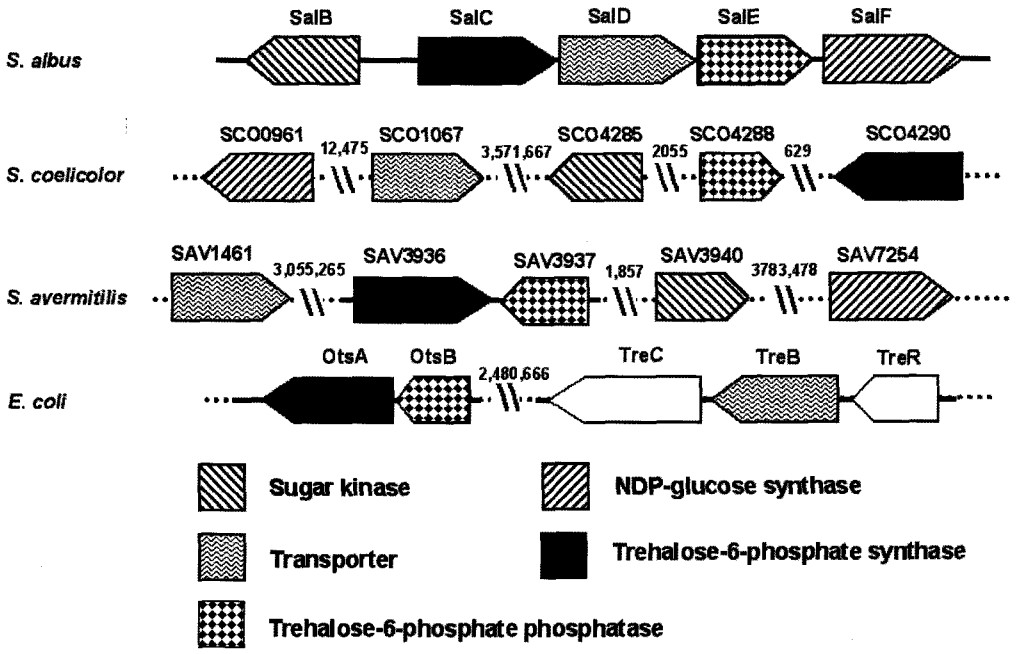

Fig. 1. Map location and presumed functions from the sequence homolog of the *sal*B, *sal*C, *sal*D, *sal*E, and *sal*F genes in the putative trehalose biosynthetic gene cluster from *S. albus* with other known genes of different organisms.
The genes *tre*B and *tre*C encode transporter and trehalose-6-P hydrolase, respectively, and *tre*R represents the gene of the repressor for trehalose biosynthesis. Each number indicates the gap size in bp between the ORFs.

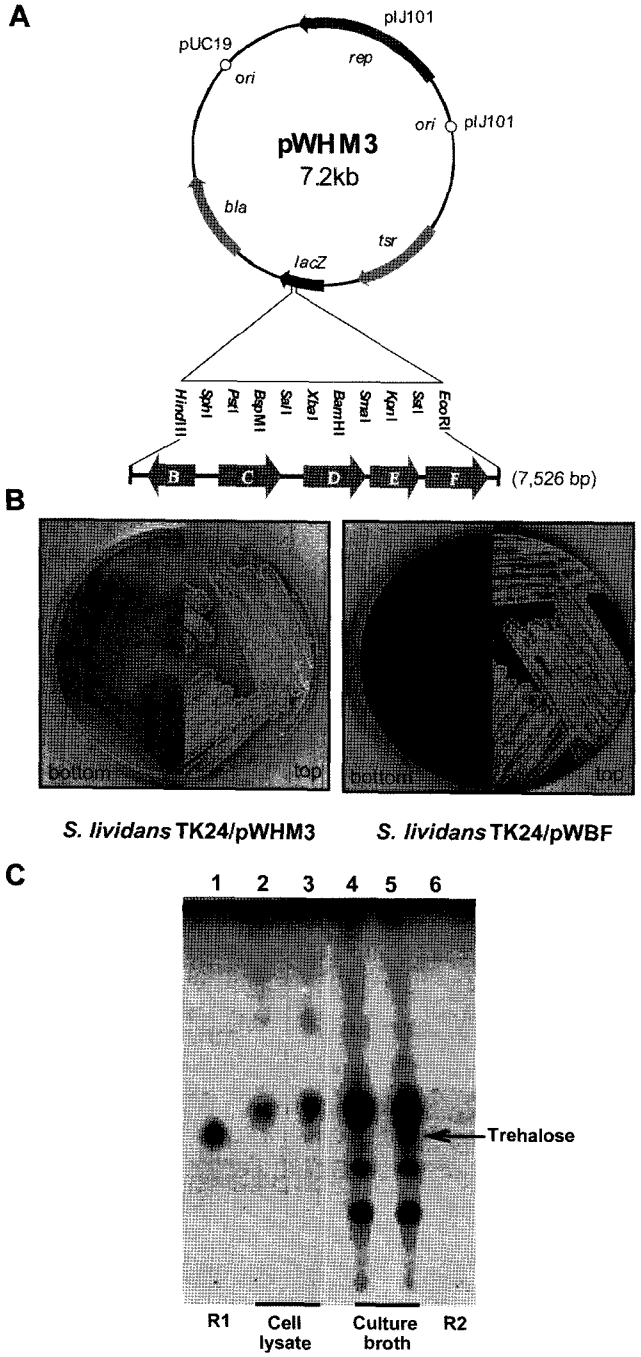

Fig. 2. **A.** Restriction map of expression vector pWHM3 and pWBF containing the trehalose biosynthetic gene cluster. **B.** Comparison of morphology and pigmentation between the *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF. The photos of the plates were taken 5 days after incubation on R2YE. **C.** Thin-layer chromatography analysis of trehalose.
Lane 1 (R1), trehalose; lane 2, *S. lividans* TK24/pWHM3 (cell lysate); lane 3, *S. lividans* TK24/pWBF (cell lysate); lane 4, *S. lividans* TK24/pWHM3 (culture broth); lane 5, *S. lividans* TK24/pWBF (culture broth); lane 6 (R2), mannitol.

and therefore, 2-epi-5-epi-valiolone synthase was regarded as a pivotal biosynthase for salbostatin. The forward primer was 5'-GGSGGSGGSGTSCTSATGGACGTSGCSGG-3' and the reverse primer was 5'-GCCATGTCSACGCASACSG-CCTCSCCGTG-3', with S representing C or G.

Thin-Layer Chromatography

The method of thin-layer chromatography (TLC) for detecting salbostatin was carried out with modification [5]. The samples were analyzed on a silica gel 60 F254 plate (Merck) to determine the presence of sugars and polyols. Each sample was dissolved in 300 µl of 100% ethanol, warmed at 80°C for 10 min, bath-sonicated for 15 min, and centrifuged at 5,000 ×*g* for 3 min, after which the supernatant was collected. This extraction procedure was repeated twice. The supernatants were pooled and frozen at −70°C for 1h, freeze-dried for 24 h, and the sample was resuspended with 10 µl of ethanol (50%). Each sample (5 µl) was loaded on a TLC. Authentic trehalose, mannitol, and glucose were used as references. A solvent containing ethylacetate:acetic acid:methanol:water=50:15:15:25 (v/v) was used for the separation. The results were visualized after development with a deionized water solution containing 5% hydrogen sulfate, 5% acetic acid, and 5% anisaldehyde, followed by heating at 120°C for 10 min.

Medium Selection for the Optimal Growth Condition

Five different media, minimal media, R2YE [10], ISP2 and ISP4 media (methods of International *Streptomyces* Project) [26], and YEME (yeast extract-malt extract) [12], were evaluated to select the most appropriate medium for the growth of *S. lividans* TK24, *S. lividans* TK24/pWHM3, and *S. lividans* TK24/pWBF. The cells, in 50 ml of media, were cultured at 28°C on a shaking incubator (Woobeom Bio Techno, SH 850S) at 200 rpm. Samples (500 µl each) were taken at various time points and the cell growth rate evaluated at 600 nm by turbidometric analysis. The experiments were performed twice independently.

Assay of Trehalose Biosynthesis

Trehalose, produced by *S. lividans* TK24 and transformants, was confirmed by two assay methods. Firstly, trehalose was measured by analyzing the release of free glucose following incubation of 10-µl samples with 0.037 units (pH 6.0) of trehalase from porcine kidney (T8778, Sigma) at 37°C for 1 h. A glucose detection kit (ASAN PHARM, Asan set glucose AM 201-K) containing glucose oxidase, peroxidase, and an oxidizable substrate was employed. Color development was quantified using a spectrometer at a wavelength of 500 nm. Secondly, high-performance liquid chromatography (HPLC) was applied for the analysis of trehalose. The strains were cultured in 500 ml of R2YE medium at 220 rpm for 7 days at 28°C. The culture broth was centrifuged at 12,000 rpm for 5 min. The 10-µl extracted supernatant was loaded onto an AMINEX HPX-87H column (BIO-RAD) and eluted with HPLC grade distilled

water at the flow rate of 0.6 ml/min. The eluate was monitored with a SEDERE SEDEX 75 ELSD detector.

RESULTS

Sequencing Analysis of the Target DNA Fragments from *S. albus*

From the genomic DNA library of *S. albus* ATCC 21838, one clone was selected to contain the *acb*C homolog by PCR, as described in Materials and Methods, and thus its complete genomic sequencing data was generated. Thirty-two ORFs in a 37-kb gene cluster were identified through sequence analysis. The twenty-one genes out of thirty-two ORFs were designated as *sal*A to *sal*U (from 5' to 3') in alphabetical order. The conserved sequences that contained *sal*Q were deduced to be responsible for the salbostatin biosynthesis, because bioconversion of sedoheptulose-7-phosphate into 2-epi-5-epi-valiolone by SalQ (2-epi-5-epi-valiolone synthase) is thought to be the first step for the valienamine sugar moiety of salbostatin.

We directly submitted the newly determined nucleotide sequence data into GenBank with assigned accession numbers (Table 1). The deduced amino acid sequences of SalB/C/D/E/F showed similarities with known functional proteins of other organisms (Table 1). In this work, the sequence data revealed the genes (from *sal*B to *sal*I, ~10-kb) next to the biosynthetic genes of the valienamine sugar moiety of salbostatin, which were regarded as a functionally clustered module for trehalose metabolism. In particular, the five ORFs (from *sal*B to *sal*F, 7.5-kb; *sal*B, sugar kinase/glucokinase; *sal*C, trehalose-6-phosphate synthase; *sal*D, transporter; *sal*E, trehalose-6-phosphate phosphatase; and *sal*F, ADP-glucose synthase) are supposed to be an adequate set of modules for trehalose biosynthesis through one of the three known trehalose biosynthetic pathways [8]. Deduced functions of the sequence homologs from the putative trehalose biosynthetic gene cluster of *S. albus* were compared with other known genes of different organisms (Fig. 1). The involvement of trehalose-6-phosphate synthase (SalC having 56% identity with OtsA) and trehalose-6-phosphate phosphatase (SalE having 43% identity with OtsB) suggests a pathway where SalC converts glucose-6-phosphate and ADP-glucose into trehalose-6-phosphate, and then SalE changes trehalose-6-phosphate to trehalose, respectively (Table 1).

In order to confirm that the five genes are real biosynthetic genes for trehalose, the expression vector pWBF that contains a 7.5-kb HindIII/EcoRI DNA fragment on the high copy number plasmid pWHM3 was constructed (Fig. 2A).

Analysis of Trehalose Biosynthesis

S. lividans TK24 was transformed with pWBF for heterologous expression of trehalose. The transformants (*S. lividans* TK24/pWHM3 as the control and *S. lividans* TK24/pWBF) were grown on solid R2YE medium with 50 μg/ml of thiostrepton for five days. In the strain of *S. lividans* TK24/pWBF, strong blue pigmentation was observed in R2YE medium, with enhanced sporulation (Fig. 2B). The cell growth rate of *S. lividans* TK24/pWBF was relatively higher than that of *S. lividans* TK24/pWHM3 after five days of cultivation (Fig. 4B).

Using silica-gel thin-layer chromatography (TLC), the presence of sugars and polyols were analyzed from the cellular extracts and culture broth of *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF in SFM media. Authentic trehalose (R1, positive control) and mannitol (R2, negative control) were used as references (Fig. 2C). As expected, no spot was detected in the lane of negative control. However, a strongly visible spot matching with the trehalose standard was observed in the lane of culture broth of *S. lividans* TK24/pWBF. A small amount of trehalose was also detected in the cell lysate only from *S. lividans* TK24/pWBF (Fig. 2C).

Growth of *S. lividans* TK24/pWHM3 and of *S. lividans* TK24/pWBF on five different media was measured to see whether the presence of *sal*BF genes had an effect on the biomass under the tested conditions (Fig. 3). Contrasting to the solid agar media, *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF displayed similar growth patterns on R2YE, YEME, ISP4, and MM (minimal medium). R2YE was confirmed as an optimal medium for the best growth of *S. lividans* TK24/pWHM3 and of *S. lividans* TK24/pWBF, whereas the latter was significantly impaired in ISP2 broth. Prior to quantifying the trehalose (using HPLC analysis), in the culture broth of R2YE, a calibration curve was made with a trehalose standard (SIGMA; D(+) trehalose dihydrate, from *Saccharomyces cerevisiae*). The

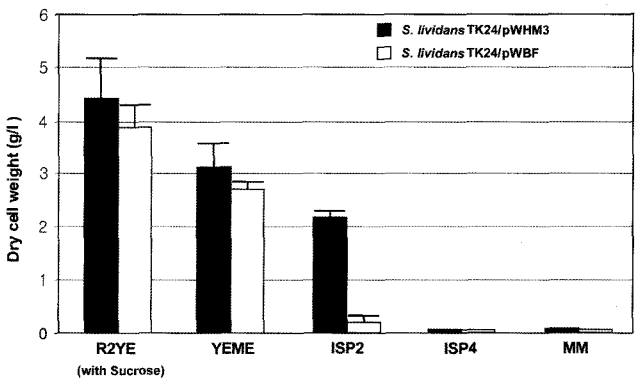

Fig. 3. Effect of media composition on biomass yield of *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF.
Each strain was grown in R2YE medium supplemented with sucrose, YEME, ISP2, ISP4, and minimal medium supplemented with glucose as carbon source. The cell growth was determined by measuring dry cell weight after four days of growth. All data shown are mean values from at least three replicate experiments.

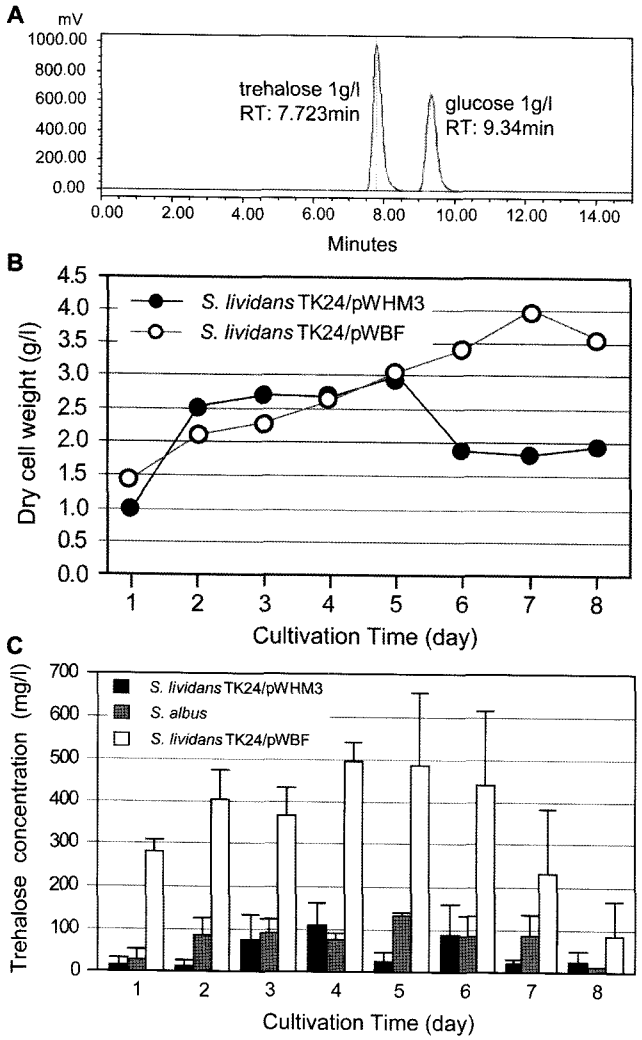

Fig. 4. **A**. Trehalose analysis by HPLC. **B**. Growth curves of trehalose-producing transformants (*S. lividans* TK24/pWBF) and trehalose-nonproducing transformants (*S. lividans* TK24/pWHM3) in R2YE media containing no sucrose. The data shown are the mean values from at least three replicate experiments. **C**. Time course for trehalose production by *S. lividans* TK24/pWHM3, *S. albus*, and *S. lividans* TK24/pWBF.

trehalose in the culture broth was analyzed as described in Materials and Methods, using the authentic trehalose and glucose that were detected at the retention time of 7.7 min and 9.3 min, respectively (Fig. 4A). However, the trehalose and sucrose could not be separated by HPLC is probably due to the similar structure and molecular weight of trehalose and sucrose (data not shown). Thus, we commenced the time course (from day 1 to day 8) in R2YE broth without sucrose (Figs. 4B and 4C). In this case, not only was the cell growth lower but the trehalose production was also decreased compared with the cultures in the R2YE with sucrose.

In the study of cell growth, by measuring dry cell weight, *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF showed different patterns of growth in R2YE

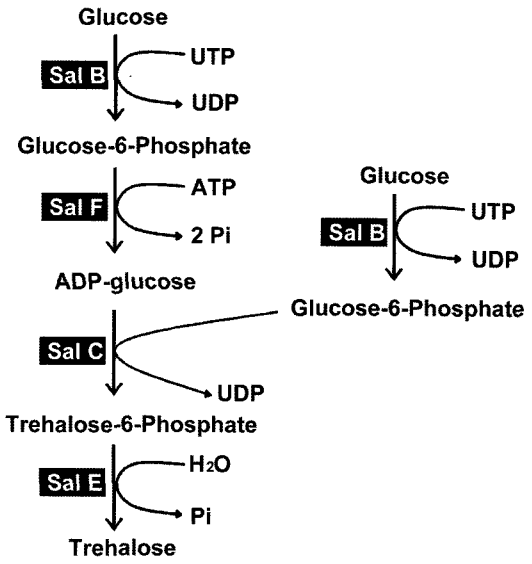

Fig. 5. A hypothetical pathway for trehalose biosynthesis.
SalB catalyzes the synthesis of glucose-6-phosphate from glucose. SalF could generate ADP-glucose by removing phosphate (see Table 1). SalC catalyzes the synthesis of trehalose-6-phosphate. Finally, SalE removes the phosphate from trehalose-6-phosphate, yielding trehalose.

media without sucrose (Fig. 4B). The control strain grew up to 3.0 g/l biomass (5 day), and then decreased to a concentration of 1.9 g/l (day 8). On the other hand, the trehalose-producing strain grew to a maximum of 4.0 g/l (day 7), and then decreased slowly (Fig. 4B). In contrast to the control strain, the retarded decrease of growth of *S. lividans* TK24/pWBF was probably due to the consumption of trehalose for cell growth in sucrose-deprived R2YE media (Fig. 4C). Taken as a whole, our data demonstrate the optimal time point of maximal amount of trehalose, as the concentration of trehalose was subsequently increased to 0.49 g/l, and then decreased. That was probably due to a depletion of glucose in the medium as detected by HPLC (data not shown) and to a consequence of the introduced *sal*B-F genes to *S. lividans* TK24.

DISCUSSION

The properties and applications of trehalose [α-D-glucopyranosyl-(1,1)-α-D-gluco-pyranoside] have been described in detail [9, 10]. However, one of the problems remaining in trehalose study is the ubiquitous occurrence of trehalose with the approximation of genes involved. To refine this, we reduced the number of genes from twenty-one to five, based on a functional resemblance with known trehalose biosynthetic enzymes (*sal*B, sugar kinase/glucokinase; *sal*C; trehalose-6-phosphate synthase; *sal*D, transporter; *sal*E, trehalose-6-phosphate phosphatase; and *sal*F, ADP-glucose synthase). Our postulation that the five

genes could be responsible for the trehalose biosynthesis in *S. albus* ATCC 21838 was confirmed by heterologously producing trehalose in the transformed *S. lividans* TK24.

During the growth on R2YE solid media, the aerial hypha formation of *S. lividans* TK24/pWBF strain occurred considerably earlier compared with that of *S. lividans* TK24/pWHM3. Furthermore, the production of blue pigment (actinorhodin) was induced in *S. lividans* TK24/pWBF. Thus, it should be noted that overproduced trehalose may have an effect on the downstream developmental genes, resulting in the earlier morphogenesis and formation of secondary metabolites such as blue pigmentation (Fig. 2B).

Under various growth conditions including R2YE, YEME, ISP4, and MM broth, *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWBF displayed similar growth patterns. Whereas the growth of both control and *S. lividans* TK24/pWBF was the best on R2YE medium, the introduction of *sal*B-*sal*F genes to *S. lividans* TK24 resulted in the significantly impaired growth of *S. lividans* TK24/pWBF on ISP2 medium (Fig. 3).

Eukaryotic organisms, such as trehalose-producing transgenic tobacco plants or yeast, show improved growth performance under drought, heat, or salt stress [14, 21, 23]. Although we cultured the transformants in various stressed conditions, such as dried solid media, high temperature, and high salt concentration, no difference was observed between the growth of *S. lividans* TK24/pWBF and *S. lividans* TK24/pWHM3 (data not shown). Trehalose biosynthetic genes have been reported in various prokaryotes including bacteria and archaea [13, 18]. In *Streptomyces griseus*, trehalose is accumulated in the spore, and rapidly degraded at the onset of germination, implying its dual functions as a stabilizer for macromolecules in the spore and energy source for germination [19]. Interestingly, our novel results revealed that the trehalose high-producer strain (*S. lividans* TK24/pWBF) had improved capability to grow and develop into spores, better than the trehalose lower producer strain (*S. lividans* TK24/pWHM3) in R2YE solid media (Fig. 2B). In addition, the trehalose high-producer strain stably maintained its cell mass for a longer cultivation time than the trehalose lower producer strain in R2YE broth (Fig. 4). Although our result strongly supports the idea that a higher concentration of trehalose may induce better growth and earlier sporulation in solid media and help to sustain cell viability in liquid media, further precise works are required to prove whether the increased performance for growth and morphogenesis are due to an impact of trehalose biosynthesis against various stresses.

The control strain, *S. lividans* TK24/pWHM3, did not show a total lack of trehalose production. This fact suggests trehalose synthesis probably occurs in *S. lividans* TK24/pWHM3 by its own biosynthetic genes. The *ots*A and *ots*B homologs are found in the genomic sequence of *S. coelicolor*, which is genetically close to *S. lividans*, implying an *ots*A-*ots*B-like pathway may be responsible for the production of trehalose in *S. lividans* TK24/pWHM3 (Fig. 1).

Depending on the deduced functions of *sal*BCDEF, the following biosynthetic pathway for trehalose is predictable; sugar kinase (SalB) catalyzes the synthesis of glucose-6-phosphate from glucose, and ADP-glucose synthase (SalF) generates ADP-glucose by removing phosphate. Trehalose-6-phosphate synthase (SalC) catalyzes the synthesis of trehalose-6-phosphate. Subsequently, trehalose-6-phosphate phosphatase (SalE) removes the phosphate, yielding trehalose. This assumption was deduced from the OtsA-OtsB pathway [15] because of the involvement of trehalose-6-phosphate synthase (SalC having functional similarity with OtsA) and trehalose-6-phosphate phosphatase (SalE having similarity with OtsB), which are predicted to synthesize trehalose-6-phosphate from glucose-6-phosphate and ADP-glucose and then changes trehalose-6-phosphate into trehalose, respectively. Highly purified trehalose-6-phosphate synthase showed that it was active with all of the naturally occurring glucose sugar nucleotides (*i.e.*, ADP-glucose, CDP-glucose, GDP-glucose, TDP-glucose, and UDP-glucose), supporting our assumption [9]. Even though each sequence homolog of *ots*A and *ots*B was separately found in the genetically best described strains, *S. coelicolor* A3(2) and *S. avermitilis*, they are not located in one module as a group (Fig. 1). Conversely, in *S. albus* and *E. coli*, the trehalose-6-phosphate synthase and the trehalose-6-phosphate phosphatase reside linked together as a module. That implies the OtsA-OtsB may be important in the *S. albus* (Fig. 1).

In eukaryotes, the main known role of trehalose is protecting the cell against stress rather than just as storage of carbohydrate [21, 29]. However, trehalose is also used as storage of carbohydrate [2]. Trehalose can be converted back to two glucose molecules by trehalases [2, 16, 20]. On the other hand, in *E. coli*, the catabolism of trehalose occurs by the trehalase (TreA and TreF) or by the trehalose-6-phosphate hydrolase (TreC) to glucose and glucose-6-phosphate [13]. Interestingly, the SalI (trehalose phosphorylase) found downstream of the *sal*F gene was assumed to convert trehalose with inorganic phosphate to glucose and glucose-6-phosphate [25]. This finding that the *sal*I gene encoding a unique trehalase is located between the trehalose biosynthetic module and salbostatin (trehalase inhibitor) biosynthetic module begs our attention to recognize the importance of the precise control for trehalose biosynthesis and degradation in *S. albus*. Thus, unraveling the regulatory cascade for trehalose metabolism will give us some important insights about the present function of an ancient disaccharide, trehalose.

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