Type III polyketide synthases (PKSs), represented by a plant chalcone synthase (CHS), are the condensing enzymes that catalyze the synthesis of aromatic polyketides in plants, fungi, and bacteria. The bacterial genome projects predicted that probable type III PKS genes are distributed in a wide variety of gram-positive and -negative bacteria. The gram-positive model microorganism Bacillus subtilis contained the bcsA-ypbQ operon, which appeared to encode a type III PKS and a methyltransferase, respectively. Here, we report the characterization of bcsA (renamed bpsA, for Bacillus pyrone synthase, on the basis of its function) and ypbQ, which are involved in the biosynthesis of aliphatic polyketides. In vivo analysis demonstrated that BpsA was a type III PKS catalyzing the synthesis of triketide pyrones from long-chain fatty acyl-coenzyme A (CoA) thioesters as starter substrates and malonyl-CoA as an extender substrate, and YpbQ was a methyltransferase acting on the triketide pyrones to yield alklypyrone methyl ethers. YpbQ thus was named BpsB because of its functional relatedness to BpsA. In vitro analysis with histidine-tagged BpsA revealed that it used broad starter substrates and produced not only triketide pyrones but also tetraketide pyrones and alkylresorcinols. Although the aliphatic polyketides were expected to localize in the membrane and play some role in modulating the rigidity and properties of the membrane, no detectable phenotypic changes were observed for a B. subtilis mutant containing a whole deletion of the bpsA-bpsB operon.

Bacillus subtilis is one of the best-characterized gram-positive bacteria. BcsA, which stands for bacterial chalcone synthase, was annotated as a homologue of type III PKS in S. griseus (4). Since then, the genome projects of various bacteria have revealed that type III PKSs are widely distributed in a variety of bacteria. The bacterial genome projects predicted that probable type III PKS genes are distributed in a wide variety of gram-positive and -negative bacteria (see Fig. S1 in the supplemental material). However, most of these type III PKSs have not been characterized.

Received 26 March 2009/Accepted 19 May 2009

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Published ahead of print on 22 May 2009.

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† Supplemental material for this article may be found at http://jb.asm.org/
BpsB by in vivo and in vitro experiments. The in vivo experiments revealed that the overexpression of bpsA in B. subtilis led to the production of triketide pyrones, and the co-overexpression of bpsA and bpsB led to the production of triketide pyrone methyl ethers. In the in vitro analysis showed that BpsA produced triketide pyrones and a small amount of tetraketide pyrones and tetraketide resorcinols from long-chain fatty acyl CoA thioesters as starter substrates and malonyl-CoA as an extender substrate. Therefore, BpsA is a type III PKS that is responsible for the synthesis of alkylpyrones, and BpsB is a methyltransferase that acts on the alkylpyrones to yield alkylpyrone methyl ethers. BpsB is the first enzyme found to methylate alkylpyrones. Furthermore, we attempted to analyze the biological function of the aliphatic polyketides by disrupting the bpsA and bpsB genes, but no distinct phenotypic changes were detected under laboratory conditions.

MATERIALS AND METHODS
Chemicals. [2-14C]malonyl-CoA was purchased from American Radio labeled Chemicals (St. Louis, MO). The nonlabeled CoA esters 13-methyltetradecanoyl acid, 12-methyltetradecanoyl acid, 14-methylhexadecanoic acid, and 14-methylhexadecanoic acid were purchased from Sigma. N-acetylaspartic acid (NAC) was supplied by Aldrich. 13-Methyltetradecanoyl-NAC, 12-methyltetradecanoyl-NAC, 14-methylpentadecanoyl-NAC, 15-methylhexadecanoyl-NAC, and 14-methylhexadecanoyl-NAC were chemically synthesized as described by Oguro et al. (17).

Bacterial strains, plasmids, and media. Escherichia coli JM109 and BL21(DE3), plasmids pUC19 and pColdI, restriction enzymes, and other DNA-modifying enzymes used for DNA manipulation were purchased from Takara Biochemicals (St. Louis, MO). The nonlabeled CoA esters 13-methyltetradecanoic acid, 12-methyltetradecanoic acid, and 0.01% antifoam silicone (vol/vol) (Wako, Osaka, Japan) and grown at 37°C. After 2 h, 5 mg/ml of xylose was added to the culture, and the culture was continued for a further 24 h. After cultivation, the cells obtained by centrifugation were extracted by the same method as that used for the analytical scale. The extraction was washed with brine, dried with Na2SO4, and evaporated to dryness. The crude materials were dissolved in a small amount of chloroform and flash-chromatographed on silica gel using chloroform-methanol (80:20, vol/vol) as an eluent. The eluate was evaporated and dissolved in methanol for reverse-phase preparative HPLC. The monohydroxy triketide pyrones (3d to 3i) were purified by reverse-phase preparative HPLC using a Pegasil C18 column (10 by 250 mm; Shimadzu), which was eluted with a linear gradient of 70 to 100% CH3CN in water (each containing 0.1% acetic acid) at a flow rate of 3.0 ml/min. The collected fractions were lyophilized to give 3b plus 3i (1.8 mg), 3f plus 3g (0.3 mg), and 3d plus 3e (3.5 mg) as white solids.

Structure determination. The structures of 3b, 3i, 3d, and 3e were determined by proton and carbon nuclear magnetic resonance (NMR) spectroscopy, with the aid of heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation (HMBC), and LC-APCIMS, LC-APCIMS/MS, and high-resolution mass spectrometry (HR-MS) analyses. The structures of 3f and 3g were determined by proton NMR, LC-APCIMS, LC-APCIMS/MS, and HR-MS analyses. HR-MS was measured on a JEOL (Tokyo, Japan) AccuTOF T-100 equipped with electrospray ionization (in positive ion mode). The NMR and MS data are summarized in the supplemental material.

Production and purification of BpsA. For the production of histidine-tagged BpsA, E. coli BL21(DE3) harboring pColdI-bpsA was inoculated into LB medium containing 100 µg/ml ampicillin and incubated at 37°C. After the optical density at 600 nm had reached 0.4, the cells were kept at 15°C for 30 min. The culture was continued for a further 24 h in the presence of 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and resuspended in 10 mM Tris-HCl (pH 8.0) and 145 mM NaCl and were disrupted by sonication. A crude cell lysate was prepared by the removal of cell debris by centrifugation at 10,000 × g for 20 min. BpsA was purified by using a nickel-nitritolitratric acid spin column (Qiagen, Valencia, CA) according to the manual from the manufacturer, except for adding 10% glycerol to each buffer. The purified histidine-tagged protein was dialyzed against 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol.

In vitro BpsA reaction. The standard reaction mixture contained 100 µM [2-14C]malonyl-CoA, 100 µM starter CoA or starter NAC, 100 mM Tris-HCl (pH 7.5), and 41.4 µg of BpsA in a total volume of 100 µl. Reactions were incubated at 30°C for 30 min before being quenched with 20 µl of 6 M HCl. The products were extracted with ethyl acetate, and the organic layer was evaporated to dryness. The residual material was dissolved in 15 µl of methanol for thin-layer chromatography (TLC) and LC-APCIMS analyses. Silica gel 60 WF254 TLC plates (Merck, Darmstadt, Germany) were developed in benzene-acetonitrile (68:32:1, vol/vol). The CoA-thiolated compounds were detected by using a BAS-MS imaging plate (Fuji Film, Tokyo, Japan). The products from the in vitro BpsA reaction were identified by comparing their LC-MS and MS/MS spectra to those obtained from the authentic standards that had been prepared by Arsc and Type III PKSs from A. vinelandii (5).
Kinetic parameters were determined as follows. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 100 μM malonyl-CoA, and 10.4 μg of BpsA in a total volume of 200 μl. The concentration of 15-methylhexadecanoyl-NAC was varied between 0.1 to 5 μM. We confirmed that the formation of a triketide pyrone as the product was linear during the assay, which implies that the analysis was carried out under steady-state conditions. After the reaction mixture had been preincubated at 30°C for 5 min, the reactions were initiated by adding the substrates. The reactions were continued for 60 s. The reactions were stopped with 20 μl of 0.5 M HCl and the material in the mixture was extracted with ethyl acetate. The conditions for analytical HPLC are described above. Steady-state parameters were determined by fitting the curve to \( v = V_{\text{max}}[S][K_s + [S]] \), where \( V_{\text{max}} \) is the maximum rate of metabolism, \( v \) is the initial velocity of formation of 4-hydroxy-6-(\( n \)-methylpentadecyl)-2-pyrene (2e) by BpsA, and \( S \) is the concentration of 15-methylhexadecanoyl-NAC.

**Construction of mutant strain bpsA-bpsBD::cat.** Oligonucleotide primers were used to amplify the bpsA fragment (bpsAUF, 5′-CTCTGCAAGGCTCTGTGT GTT-3′; bpsAUR, 5′-CTATGGCCGGCCATCCTTGGCATAC-3′) and the bpsBD fragment (bpsBDF, 5′-GCTCCAGATCTAGCGATTGAAAATCAT TCTAACC-3′; bpsBDR, 5′-TCTCTAGGGTCAATTGG-3′). The chloramphenicol-resistant gene of pCBB31 (9) was amplified by PCR using primers catF (5′-GCCGCAATGATCCCTTAT-3′) and catR (5′-GATCTGGACGT GTAATAAAAACCC-3′). The fragments obtained were used simultaneously as the template for PCR amplification using primers bpsAUF and bpsBDR. The resulting fragment was used to transform Bacillus subtilis 168, and chloramphenicol-resistant transformants were selected. Correct integration was confirmed by PCR amplification.

**Tests of phenotypic changes of mutant bpsA-bpsBD::cat.** Sensitivity to cell wall-targeting antibiotics of the wild-type strain B. subtilis 168 and mutant bpsA-bpsBD::cat was tested by growing the strains on LB agar medium at 30°C for 18 h. The antibiotics used were penicillin G at 1 ng/ml, 0.05, or 0.1 μg/ml, vancomycin at 0.2, 0.5, or 1 μg/ml, and cephalosporin C at 5, 7, or 10 μg/ml. The heat stress survival of the wild-type and bpsA-bpsBD::cat strains was examined in LB liquid medium; the cells were grown at 37°C until the optical density at 600 nm reached 0.2, preadapted to heat by transferring them to a shaking water bath set at 46°C for 60 min with gentle shaking, and challenged at 54°C for 1, 2, 3, and 5 h or at 56°C for 10 min. After the heat shock treatment, portions of the cells were diluted and plated on LB agar.

**RESULTS AND DISCUSSION**

**Organization and distribution of bpsA-bpsB operons in a wide variety of bacteria.** The gene organization of the 1.6-kb bpsA and bpsB genes is shown in Fig. 1. The bpsA and bpsB genes were thought to form an operon, because the stop codon of bpsA was located 73 nucleotides upstream of the start codon of bpsB and because not only in B. subtilis but also in many bacteria, bpsA and bpsB genes almost always are neighbors on the chromosomes (see Fig. S1 in the supplemental material). pbuX, encoding a protein homologous to a xanthine permease, which is located 73 nucleotides upstream of bpsA, appeared not to be functionally linked to the bpsA-bpsB operon.

BpsA, consisting of 365 amino acid residues, shared 35% amino acid sequence identity with SrsA in S. griseus, a type III PKS that catalyzes C-methylated alkylresorcinol synthesis from long-chain fatty acyl-CoAs (6). In addition, a Cys-His-Asn catalytic triad, which is crucial for the decarboxylative condensation activity of all type III PKSs, was conserved (2). The amino acid alignment of BpsA with other type III PKSs, including the catalytic triads, is shown in Fig. S2 in the supplemental material.

BpsB, consisting of 168 amino acid residues, shared 38% amino acid sequence identity to SrsB in S. griseus, a methyltransferase that catalyzes the methylation of alkylresorcinol. bpsB homologues are present in various bacterial species, such as Mycobacterium, and form an operon with a type III PKS gene in most cases.

**In vivo analysis of bpsA and bpsB.** To clarify the functions of BpsA and BpsB, we constructed two plasmids for overexpression, pHO1 carrying bpsA and pHO2 carrying bpsA and bpsB were constructed by using pWH1530 and were introduced by transformation into B. subtilis 168. These genes were under the control of the xylose-inducible xyA promoter. After the B. subtilis cells had been grown in the presence of xylose, cell extracts were prepared and analyzed by HPLC. Under the conditions employed, B. subtilis harboring only the empty pWH1530 vector produced no detectable aliphatic polyketides (Fig. 2). On the other hand, B. subtilis strains overexpressing either bpsA or bpsA and bpsB produced several lipids, giving multiple peaks on HPLC (Fig. 2).

We analyzed the lipids 2d to 2i (Fig. 2) by LC-APCIMS and LC-APCIMS/MS analyses. The mass spectra of 2h, 2i, 2f, 2g, 2d, and 2e showed molecular ions at m/z 307, 307, 321, 321, 335, and 335, respectively, in a negative ion mode. On the basis of the [M−H]− ions of these compounds, 2d to 2i were predicted to be alkylpyrones. Moreover, the MS/MS spectra of 2h, 2i, 2f, 2g, 2d, and 2e showed fragments of m/z 263, 263, 277, 277, 277, 279, and 291, corresponding to [M−CO₂−H]− ions, respectively, which indicated the presence of an α-pyrones ring (20). Thus, 2d to 2i were predicted to be triketide pyrones. Because major fatty acids contained in B. subtilis cells are 12-methyltridecanoic acid (iso-C₁₄; 4%), 13-methyltetradecanoic acid (iso-C₁₅; 14%), 12-methyltetradecanoic acid (anteiso-C₁₅; 33%), n-hexadecanoic acid (n-C₁₆; 6%), 14-methylpentadecanoic acid (iso-C₁₆; 11%), 15-methylhexadecanoic acid (iso-C₁₇; 15%), and 14-methylhexadecanoic acid (anteiso-C₁₇; 10%) (10), the alkyl chains of the triketide pyrones 2d to 2i presumably were derived from these long-chain fatty acids. We then attempted to identify these compounds by comparing their retention times on HPLC. MS, and MS/MS spectra to those obtained from the triketide pyrones that had been prepared by ArsC, a type III PKS from A. vinelandii. ArsC produces alkylpyrones by using long-chain fatty acyl CoA thioesters as starter substrates and malonyl-CoA as the extender substrate (5). The triketide pyrones that were prepared by using branched-chain fatty acyl NAC thioesters (NAC is a mimic of CoA) or straight-chain fatty acyl CoA thioesters as starter substrates yielded the fragment patterns of [M−H]− and [M−CO₂−H]− ions, as determined by LC-APCIMS and LC-APCIMS/MS analyses (see structural data in the supplemental material). By comparing the triketide pyrones produced by ArsC, we showed that B. subtilis harboring pHO1 produced 4-hydroxy-6-(11′-methyltridecyl)-2-pyrene (2h), 4-hydroxy-6-(12′-methyltridecyl)-2-pyrene (2i), 4-hydroxy-6-
(13'-methyltetradecyl)-2-pyrene (2f), 4-hydroxy-6-pentadecyl-2-pyrene (2g), 4-hydroxy-6-(13'-methylpentadecyl)-2-pyrene (2d), and 4-hydroxy-6-(14'-methylpentadecyl)-2-pyrene (2e) (see structural data in the supplemental material).

We next analyzed the products of *B. subtilis* harboring pHO2 by HPLC and LC-APCIMS analyses (Fig. 2). The mass spectra of the products had molecular ions at 323 (3h and 3i), 337 (3f), 337 (3g), and 351 (3d and 3e). On the basis of [M+H]+ ions of these compounds, 3d to 3i were predicted to be methyl ether compounds of alkylpyrones 2d to 2i. To determine the structures of lipids 3d to 3i by NMR and HR-MS analyses, the lipids were separated into 3h and 3i, 3f and 3g, and 3d and 3e by HPLC equipped with a preparative C4 column. Their structures were determined as 4-methoxy-6-(11'-methyltridecyl)-2-pyrene (3h), 4-methoxy-6-(12'-methyltridecyl)-2-pyrene (3i), 4-methoxy-6-(13'-methylpentadecyl)-2-pyrene (3d), and 4-methoxy-6-(14'-methylpentadecyl)-2-pyrene (3e) by NMR and HR-MS analyses (see structural data in the supplemental material). The structures of 3f and 3g were determined as 4-methoxy-6-(13'-methyltetradecyl)-2-pyrene and 4-methoxy-6-pentadecyl-2-pyrene by proton NMR and HR-MS analyses, respectively (see structural data in the supplemental material).

HMBC analysis showed the linkage of the methoxy group to the carbon at position 4 (Fig. 3), which clearly indicated that these alkylpyrone methyl ethers were derived from α-pyrones. The pyrone methyl ether moieties of compounds 3f and 3g were identified by comparing the proton NMR spectra to those of 3h, 3d, 3i, and 3e, showing that 3f and 3g possessed the same pyrone methyl ether moiety. These results were consistent with those for the in vivo products of BpsA. Therefore, BpsA was predicted to be a type III PKS responsible for triketide pyrone production.
synthesis from branched and straight long-chain fatty acyl CoA thioesters as starter substrates and malonyl-CoA as an extender substrate, and BpsB was a methyltransferase acting on alkylpyrones (Fig. 4).

In vitro analysis of recombinant BpsA protein. From the in vivo experiments described above, BpsA was predicted to be a type III PKS responsible for triketide pyrone synthesis. To reconstruct this reaction in vitro, we placed \textit{bpsA} under the control of the \textit{cspA} promoter on the pColdI vector to produce BpsA as a fusion with a His tag at its N terminus. The histidine-tagged BpsA protein, purified with a nickel-nitrilotriacetic acid spin column, gave a single protein band at a position of 43 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 5A). From in vivo analysis, the major product of BpsA was 4-hydroxy-6-(14\text{\textsuperscript{H}})-methylpentadecyl)-2-pyrone (2\text{e}). Compound 2\text{e} was expected to be synthesized from 15-methylhexadecanoyl-CoA or 15-methylhexadecanoyl-ACP as a starter substrate and malonyl-CoA as an extender substrate. Neither 15-methylhexadecanoyl-CoA nor 15-methylhexadecanoyl-ACP was commercially available, therefore we synthesized NAC, a mimic of CoA and ACP, to check the presumed in vitro synthesis. As expected, the incubation of BpsA with 15-methylhexadecanoyl-NAC and malonyl-CoA resulted in the production of 4-hydroxy-6-(14\text{\textsuperscript{H}})-methylpentadecyl)-2-pyrone (2\text{e}) (data not shown). In addition to this major product, BpsA produced a small amount of an alkylresorcinol (data not shown).

We determined the temperature and pH dependence of BpsA for triketide pyrone synthesis using 15-methylhexadecanoyl-NAC as a starter substrate. BpsA showed a temperature optimum at 30°C and a pH optimum at 7.5. The kinetic properties of BpsA were determined under the optimum reaction conditions. The $K_m$ and $k_{cat}$ values for 15-methylhexadecanoyl-NAC were calculated to be 0.98 $\mu$M and 4.90 $\times$ 10$^{-3}$ s$^{-1}$, respectively (Fig. 5B).

Generally, type III PKSs show broad starter substrate specificity (2). We determined whether BpsA accepts acyl-CoA esters possessing C\textsubscript{2} to C\textsubscript{22} straight-chain alkyl moieties. BpsA accepted the C\textsubscript{4} to C\textsubscript{22} CoA esters and produced the corresponding triketide pyrones (Fig. 6). Moreover, this radio-TLC analysis revealed that BpsA produced not only triketide pyrones but also tetraketide pyrones and alkylresorcinols from the C\textsubscript{8} to C\textsubscript{20} esters (Fig. 6). BpsA produced 6\text{a}, 6\text{b}, 6\text{c}, and 6\text{g} from the C\textsubscript{16} to C\textsubscript{22} esters (Fig. 6), but we could not determine...
their structures because of the very small amounts of the compounds produced. By carefully examining the HPLC analysis of the ethyl acetate extract of *B. subtilis* harboring pHO1, several phenolic lipids were observed by LC-APCIMS in addition to compounds 2d to 2i, but the structures of these compounds could not be identified because the amounts were too small.

Analysis of the possible biological function of aliphatic polyketides. Recently, we revealed that the bacterial phenolic lipids that are synthesized by type III PKSs have some important roles in the membrane/cell wall (5, 6). The amphiphilic phenolic lipids alkylresorcinols and alkylpyrones are readily incorporated into phospholipid bilayers and biological membrane, thereby causing considerable changes in their structure and properties (12). In fact, the alkylresorcinols are fractionated mainly in the membrane/cell wall fraction (6). In *A. vinelandii*, alkylresorcinols and alkylpyrones, major components in the cyst membrane that are produced by the type III PKSs, ArsB and ArsC, are essential for cyst formation (5). The phenolic lipids synthesized by the Srs enzymes in *S. griseus* confer resistance to β-lactam antibiotics (6). Therefore, we speculate that aliphatic polyketides play some roles in the membrane in *B. subtilis*.

We constructed a mutant in which both *bpsA* and *bpsB* were deleted (mutant *bpsA-bpsB::cat*) to observe the possible phenotypic changes. Mutant *bpsA-bpsB::cat* grew normally in LB liquid and on agar medium, showing that the aliphatic polyketides had no detectable effects on growth. First, we compared the metabolic profiles of the lipid fraction of the wild-type and mutant *bpsA-bpsB::cat* strains by LC-APCIMS analysis. Although we carefully examined the data, both strains produced no detectable amounts of triketide pyrone derivatives.

The biological role of phenolic lipids observed for *A. vinelandii* and *S. griseus* prompted us to examine the biological roles of BpsA and BpsB. We examined their sensitivity to β-lactam antibiotics. However, no difference in sensitivity to the antibiotics of the mutant *bpsA-bpsB::cat* was observed between the wild-type and mutant strains (data not shown). We next examined the survival activity against a heat stress, because the transcription of *bpsA* was increased by a heat shock, as determined by microarray analysis (BSORF *Bacillus subtilis* genome database [http://bacillus.genome.jp/]). In *B. subtilis*, the sigB-dependent general stress genes and heat shock proteins, such as the GroESL and DnaK chaperone machines, and the proteins of the Clp families of chaperones and proteases
contribute to coping with heat stresses (8, 13, 14, 16, 21, 22). The preadaptation of \textit{B. subtilis} by exposure to 48°C induces the heat-specific protection systems mentioned above (8, 22). After the wild-type and mutant \textit{bpsA-bpsB::cat} strains had been preadapted by exposure to 48°C for 1 h, they were challenged by heat treatment at 54 or 56°C, and their survival rates were quantified. However, the survival rates of mutant \textit{bpsA-bpsB::cat} and the wild-type strain were the same (data not shown). In this study, we could not observe any other phenotypic differences between the wild-type strain and the mutant \textit{bpsA-bpsB::cat}. In addition, we could not observe the triketide pyrone derivatives by HPLC analysis of the ethyl acetate extract prepared from the wild-type cell exposed to 48°C, although the transcription of \textit{bpsA} increased upon a heat shock by microarray analysis (BSORF Bacillus subtilis genome database). Further analysis of the biological function of alkylpyrones and alkylpyrone methyl ethers in \textit{B. subtilis} is now in progress.

The catalytic properties of BpsA and BpsB. The present in vivo and in vitro experiments have demonstrated clearly that BpsA is the alkylpyrone synthase that catalyzes the synthesis of triketide pyrones from long-chain fatty acyl CoA thioesters and malonyl-CoA. BpsA was found to possess broad substrate specificity and accept branched and nonbranched fatty acyl CoA thioesters with alkyl chain lengths of C₄ to C₂₂. Therefore, BpsA may directly accept the acyl moiety of acyl-ACP that is synthesized by anoyl-CoA and hexanoyl-ACP (7). The de novo products by bacterial \textit{type II} fatty acid synthases are released as ACP thioesters (18), and BpsA with fatty acyl-AcpA, which is an ACP responsible for the direct accept the acyl moiety of acyl-ACP that is synthesized by anoyl-CoA and hexanoyl-ACP (7). Therefore, BpsA may directly accept the acyl moiety of acyl-ACP that is synthesized by the type II fatty acid synthase system. The in vitro reaction of BpsA with fatty acyl-AcpA, which is an ACP responsible for the type II fatty acid synthesis in \textit{B. subtilis}, is our future work.

PKS18, a type III PKS in \textit{Streptomyces coelicolor} A3(2), accepts an acyl moiety from both hexanoyl-CoA and hexanoyl-ACP (7). Therefore, BpsA may directly accept the acyl moiety of acyl-ACP that is synthesized by the type II fatty acid synthase system. The in vitro reaction of BpsA with fatty acyl-AcpA, which is an ACP responsible for the type II fatty acid synthesis in \textit{B. subtilis}, is our future work.

PKS18, a type III PKS in \textit{Mycobacterium tuberculosis}, exhibits a broad starter substrate specificity and incorporates C₄ to C₆ CoA esters to produce tri- and tetraketide pyrones. San-karanarayan et al. (19) discovered a novel substrate binding tunnel in PKS18 that accommodates an alkyl moiety, as observed by the X-ray crystallography of an enzyme-myristic acid complex, and found that the side chains of the amino acids Thr144, Cys205, and Ala209 were crucial in determining the cavity volume. These residues correspond to Thr113, Cys174, and Gln179 of BpsA. We suppose that a similar binding tunnel accommodating various fatty acyl CoA esters as starter substrates exists in BpsA.

BpsB is the first reported enzyme that is responsible for catalyzing the methylation of alkylpyrones. BpsB also may act on alkylresorcinols, because it shows significant similarity to SrsB catalyzing the methylation of alkylresorcinols (6).

Regarding a possible role of the aliphatic polyketides produced by the BpsA-BpsB enzyme system, contrary to our expectation, mutant \textit{bpsA-bpsB::cat} showed no detectable phenotypic changes. A possible biological function of the alkylpyrones in \textit{B. subtilis} therefore remains unknown. The wide distribution of a \textit{bpsA-bpsB} operon in gram-positive and -negative bacteria and the distinct roles of the phenolic lipids in \textit{A. vinelandii} (5) and \textit{S. griseus} (6) suggest important, but thus far overlooked, role of the phenolic lipids probably incorporated in the membrane. There also was no significant difference in the lipids produced between mutant \textit{bpsA-bpsB::cat} and the wild-type strains, and the production of alkylpyrones was not detected in the wild-type strain. The amounts of the pyrones may be under the detection limits, or the triketide pyrone methyl ethers may be modified further by some enzymes existing in \textit{B. subtilis}. Therefore, the compounds synthesized by BpsA and its tailoring enzymes are mysterious. The genome sequence revealed that in the neighborhood of the \textit{bpsA-bpsB} operon, there are no genes that appear to encode enzymes able to modify the alkylpyrones or its methyl ethers. Further study will reveal their biological functions.

ACKNOWLEDGMENTS

G. Akamura was supported by the Japan Society for the Promotion of Science. This work was supported, in part, by a research grant from the New Energy and Industrial Technology Development Organization of Japan and a Grant-in-Aid for Scientific Research on Priority Area Applied Genomics from Monkasho.

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