

PksS from *Bacillus subtilis* is a cytochrome P450 involved in bacillaene metabolism

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Abstract

As part of the *pksX* gene cluster of *Bacillus subtilis* strain 168, *pksS* has been preliminarily annotated as a cytochrome P450 homolog that hydroxylates the polyketide product of this cluster, which was recently shown to be involved in the biosynthesis of bacillaene and dihydrobacillaene. Here we report that there is a frame-shift error in the reported sequence for *pksS*, and that we have successfully cloned, overexpressed, and purified the protein encoded by the corrected sequence. By utilizing electronic absorption spectrophotometry, we have observed that the ferrous CO complex of PksS absorbs maximally near 450 nm, which confirms the annotation that this protein is a cytochrome P450. We have also established a cell-free system derived from crude cytosolic *B. subtilis* protein extracts which provides reductase activity essential to sustaining the putative catalytic cycle of PksS. Using LC–MS analysis we have collected data which suggests that the substrate for PksS is dihydrobacillaene.

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Genes from the polyketide biosynthetic *pksX* cluster (Fig. 1) of *Bacillus subtilis* strain 168 were first reported in 1993 [1,2], and preparations of the antibiotic polyketide bacillaene (Fig. 1) were first reported in 1994 [3]. However, the linkage between *pksX* and bacillaene biosynthesis was not confirmed until 2006 [4], and the structure of bacillaene and its reduced derivative dihydrobacillaene had not been solved until recently in 2007 [5]. This information has been elusive because bacillaene and dihydrobacillaene are unstable and difficult to isolate [5], and reports in the literature have indicated that these compounds are part of a complex mixture of several chromatographically distinct isomers and derivatives [3,5,6]. Indeed, the structures of these sensitive compounds were out of reach until workers utilized overlay differential 2D NMR techniques [7] on partially purified mixtures of material obtained from *pksX*⁺ strains and *pksX*[−] mutants [5].

In order to explore the poorly understood role of polyketides in the secondary metabolism of *B. subtilis*, it is important to understand, at the biochemical level, the individual functions of each protein product of the *pksX* cluster. The arrangement of domains within the “Acyl Transferase (AT)-less” [8] polyketide synthases PksJLMNR, (with the requisite *trans*-AT activities likely being furnished by PksDE) and knowledge of the steps catalyzed by the β -branch synthases PksCFGHI, and AcpK [9,10], has led to the suggestion that the first polyketide product of the *pksX* cluster is bacillaene [5]. This assignment has not been proven biochemically, and a *de novo* interpretation of the sequence of modules and domains within these synthases does not directly explain the source of dihydrobacillaene, nor indicate whether this and other potential derivatives of bacillaene are the products of (1) module or domain skipping in the modular synthases, (2) post-polyketide synthase enzymes, or (3) nonenzymatic degradation pathways. Each of these questions represents a gap in our knowledge of polyketide metabolism in *B. subtilis*.

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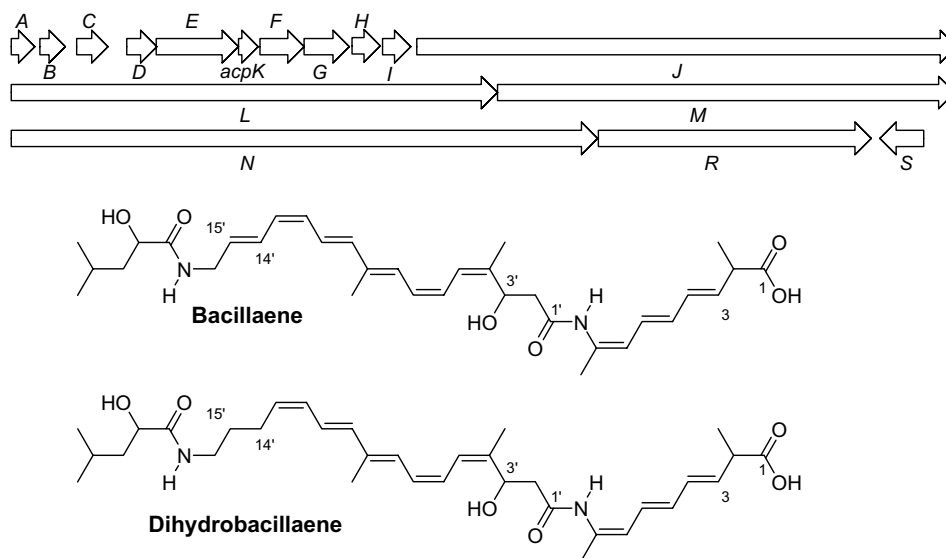


Fig. 1. The *pksX* gene cluster of *Bacillus subtilis* strain 168, and the structures of bacillaene and dihydrobacillaene. Functional assignments of *pksX* genes are given in the text.

For the possibility of post-polyketide synthase manipulation, we can first consider the remaining genes from the *pksX* cluster. These are *pksAB*, which appear to encode a transcriptional regulator and zinc-dependent hydrolase, respectively; and *pksS*, which has been assigned by significant sequence similarity as a cytochrome P450 homolog that hydroxylates the polyketide product (i.e., bacillaene or dihydrobacillaene) of the *pksX* cluster [11–13]. As such, *pksS* is expected to be a useful tool for developing a complete picture of post-polyketide synthase bacillaene metabolism. Here we describe the necessary first steps towards uncovering the function of *pksS*.

Materials and methods

Bacterial strains, culture conditions, and DNA. *Bacillus subtilis* strain 168 (*trpC2*) (BS168) was obtained from the Bacillus Genetic Stock Center at Ohio State University (BGSC code = 1A1). *B. subtilis* ATCC strain 39374 was obtained from the American Type Culture Collection (Manassas, VA). *Escherichia coli* M15[pREP4] and the pQE60 plasmid were obtained from Qiagen, Inc. *E. coli* strains were grown in standard Luria–Bertani (LB) medium, and BS168 was grown in well-aerated LB medium. ATCC strain 39374 was grown in well-aerated Landy medium for the production of bacillaenes [14]. Genomic DNA from BS168 was purified using a Wizard Genomic DNA Purification Kit from Promega, according to the manufacturer's instructions. All plasmid DNA was purified using the Qiaprep Spin Miniprep Kit (Qiagen, Inc.).

Cloning and overexpression of *pksS*. Standard methods were used for restriction endonuclease digestions, ligation, and chemical bacterial transformation [15]. PCR amplification of complete *pksS* from BS168 genomic DNA was accomplished using Phusion polymerase (New England Biolabs) and the primers 5'-CG AGG ACC ATG GAA ATG GAA AAA TTG-3' and 5'-GGG GCG TTT TGA AGA TCT TTT TGA AAG TGA AAC AGG-3'. The PCR product was purified with a Qiagen PCR Cleanup Kit, cut with *Nco*I and *Bgl*II, and ligated into the *Nco*I and *Bgl*II sites of pQE60 with T4 DNA ligase to yield *pksS*-pQE60. This vector was constructed such that the native stop codon of *pksS* was replaced in frame by a plasmid-encoded C-terminal His₆-Tag sequence. All plasmid-clones

were verified by dye-terminated sequencing performed by SeqWright, Inc. (Houston, TX).

One *pksS*-pQE60/M15[pREP4] transformant was grown overnight in 5 mL of LB supplemented with ampicillin (50 µg/mL) and kanamycin (30 µg/mL). A portion of the starter culture (2 mL) was then added to 1 L of LB containing 1% glucose, ampicillin (50 µg/mL), kanamycin (30 µg/mL), and was shaken at 37 °C until an OD₅₉₅ of 0.5–0.6 was reached. IPTG was added (0.6 mM final concentration) and the culture was then allowed to shake for 3 h at 37 °C. The cells were harvested by centrifugation (7500g for 30 min) and the pellet was stored at –80 °C until purification.

Purification of the PksS protein. All steps in this section were conducted at 4 °C, unless otherwise noted. The pelleted cells obtained from 1 L of overexpression culture were resuspended in 20 mL binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9) and lysed by sonication on ice for 3 min. The lysate was clarified by centrifugation (11,500g for 30 min), followed by syringe-filtration through a 0.45 µm membrane. The clarified filtrate was applied to a nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography column containing 2 mL of packed column bed. After loading the entire crude extract, 20 mL of binding buffer was passed through the column. Twelve milliliters of wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9) was added to the column, followed by 12 mL of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9), which eluted PksS from the column. To exchange the buffer, the protein was quickly passed through a Sephadex G25 gel filtration column, using 50 mM potassium phosphate buffer (pH 8.0) as eluent. Sterile glycerol was added to give a final concentration of 10%, and the protein was stored at –80 °C. The purity and size of the PksS protein were estimated by 12% Tris–glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [16].

Spectrophotometric analysis of PksS. A 2 mL sample of purified PksS protein in 50 mM potassium phosphate, pH 8.0 was treated with approximately 5 mg of sodium dithionite. The sample was divided for placement in the sample and reference cells of a Varian Cary 100 Bio UV–visible spectrophotometer. The visible spectrum was recorded between 400–500 nm, after which the 1 mL sample cell was sparged with carbon monoxide for approximately 1 min. With a reduced PksS sample (having no CO) remaining in the reference cell, the spectrum of the CO-complexed reduced PksS was recorded between 400 and 500 nm.

Preparation of cell-free protein extract as source of PksS reductase. An overnight starter culture (5 mL) of BS168 was used to inoculate 500 mL of

LB media, and was shaken at 37 °C for 2 days. The culture was pelleted by centrifugation (8500g for 30 min), resuspended in 10 mL of 50 mM phosphate buffer, pH 8.0, and lysed on ice with lysozyme and sonication. The lysate was cleared by centrifugation (11,500g for 30 min), the supernatant was filtered through a 0.45 µm membrane, and the filtrate was used without further modification in the PksS reductase assay given below.

Assay for PksS reductase activity. First, a PksS solution was placed in a quartz cuvette, and sealed with a rubber septum. The system was placed under a controlled atmosphere in a Schlenk apparatus via needle. To remove all oxygen gas (which would interfere with the reduction of PksS) the solution was subjected to sparging by argon followed by evacuation under high-vacuum. The sparging/evacuation process was repeated two more times. After the deoxygenation of the sample, CO-difference spectra were taken as above, except sodium dithionite was not used, CO was bubbled into the sealed cuvette via needle and proper venting, and NADH and reductase solutions were successively added by syringe.

Analysis of ATCC strain 39374 small molecule extracts treated with PksS system. An overnight starter culture of ATCC 39374 was grown in LB media, of which 100 µL was used to inoculate 50 mL of Landy medium. This culture was shaken at 37 °C for 72 h at 220 rpm. Samples of the raw culture (0.5 mL), including the medium, were extracted with 1 mL of HPLC-grade methanol, vortexed vigorously, and centrifuged at top speed for 2 min. The supernatant was separated from the pellet, and the methanol was removed by centrifuged evacuation in a Speedvac apparatus. The resultant residue was redissolved in 0.5 mL of 50 mM potassium phosphate buffer, pH 8.0. Reactions of a total volume of 600 µL contained 4 µM PksS, 80 µM of either NADH/NADPH, 60 µL of BS168 crude protein extract as a source of PksS reductase (as above from a 10 mL crude extract obtained from 500 mL culture), and the 0.5 mL of bacillaene methanolic extract (from above). Each reaction was incubated at room temperature for 1 h and then filtered using a 0.45 µm membrane. The samples were then analyzed by LC–MS on a LCQ-Advantage ion trap mass spectrometer in positive mode, which was coupled to a HP1100 HPLC using a 5 mM Prevail C-18 column (Alltech). The gradient elution profile was as follows: Begin at 95:5 1% acetic acid:acetonitrile, with the acetonitrile increasing to 95% over a 45 min linear gradient at 0.2 mL/min flow rate.

Results and discussion

Using the published sequence data for strain 168 *pksS* as a guide [11], we conducted several trials of PCR cloning and sequencing, which revealed that *pksS* in actuality lacks guanosine 1111 (with the first nucleotide of the *pksS* start codon being numbered as 1). Removal of this false G from the reported sequence shifts the original stop codon (which started at 1129 bp) out of frame, and the next available in-frame stop codon indicated that the amino-acid coding portion of the *pksS* open reading frame is 1221 bp instead of the previously assigned 1128 bp. The PksS protein should thus have 408 amino acids instead of the previously predicted 376 amino acids. The expected *pksS* protein product can be compared to the *pksS* ortholog *baeS* (GenBank Accession No. CAG23962) from *B. amyloliquefaciens* FZB42 [4]. The *bae* cluster of *B. amyloliquefaciens*, which also encodes the biosynthesis of the bacillaenes, is a nearly exact replica of the *pksX* cluster of *B. subtilis*. The *baeS* gene is located on the complementary strand at the end of the *bae* cluster, in the same orientation with respect to the overall cluster, as with *pksS* in *pksX* shown in Fig. 1. A pairwise global alignment of the original *pksS* protein sequence and the *baeS* protein sequence using the BLOSUM62 similarity matrix [17] resulted in an alignment

score of 1400 with 68% sequence identity and 78% similarity. The corresponding alignment of the corrected *pksS* protein sequence with *baeS* resulted in a higher alignment score of 1577 with 73% identity and 84% similarity, all indicating a better alignment. The newly assigned stretch of ~30 amino acids at the C-terminus of PksS has a clear well-aligned counterpart at the C-terminus of the independently sequenced *baeS* protein. These factors are suggestive that this additional stretch of C-terminal sequence should be important for the structure and function of PksS. The corrected sequences (DNA and amino acid) are available (GenBank Accession No. EF546698).

We employed PCR to clone the corrected *pksS* sequence into the pQE60 overexpression vector. Sequencing of this clone verified that the insert had the expected sequence, minus the aberrant G. This plasmid was overexpressed in *E. coli* strain M15[pREP4], and the cells were lysed by sonication. Ni–NTA chromatography of the cleared lysate resulted in an intensely orange-colored protein eluent, which was shown by SDS–PAGE (see Fig. 2) to have a >95% pure constituent with a denatured molecular mass of approximately 47 kDa, which is consistent with the calculated mass of the corrected amino acid sequence of PksS. On average, a yield of 0.12 µmol (5.6 mg) of P450 was obtained per liter of culture.

Having successfully isolated the PksS protein, we turned to characterizing this protein with respect to its expected identity as a cytochrome P450 homolog. Diluted samples of the purified protein were treated with sodium dithionite, and exposed to carbon monoxide. Visible spectra were immediately taken, and an intense Soret peak at 448 nm was observed (Fig. 3), demonstrating that PksS indeed satisfies the spectral definition of a cytochrome P450 protein [18].

There are currently two obstacles to a full characterization of PksS. The first is that we do not know the identity of the reductase which maintains the activity of PksS. The two major classes of reductases are the Class I (ferredoxin/ferredoxin reductase) and the Class II (a membrane-bound flavoprotein) [19]. Unfortunately, there are no homologs of Class I or Class II reductase systems proximal to *pksS*, and there are a large abundance of genes in the genome which show similarity to a wide variety of electron transport motifs, including those common to P450 reductases. We

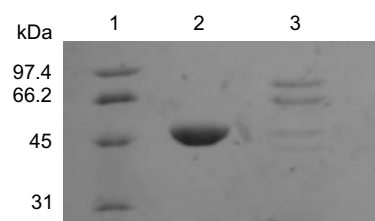


Fig. 2. SDS–PAGE of purified PksS protein. Lane 1: molecular weight markers. Lane 2: PksS obtained after Ni–NTA chromatography. Lane 3: post-induction crude extract of M15[pREP4] which had overexpressed pQE60-pksS.

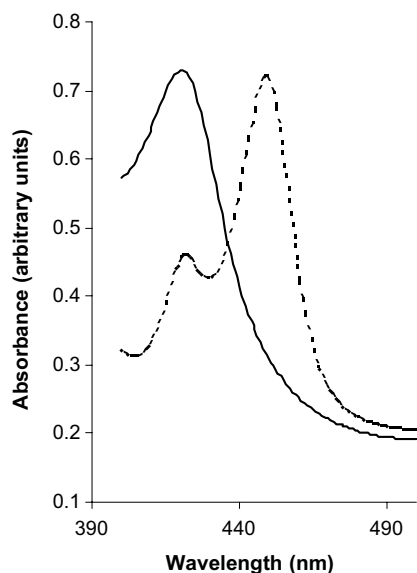


Fig. 3. Visible electronic absorption spectra of PksS. The ferric (solid) and reduced-CO complex (dashed) of the PksS gene product.

have shown that crude protein extracts from BS168 are able to induce formation of the PksS-dependent 448 nm peak, when NADH and CO are present, and only if oxygen was thoroughly removed from the sample. Research is currently underway to isolate and characterize the genetic source(s) for this native PksS reductase system.

The second challenge to studying this cytochrome P450 is that the identities of its substrate(s) and product(s) are unknown. The annotation from the genome project has merely assigned *pksS* as a putative hydroxylase of the product of the *pksX* cluster. Several compounds and isomers dependent on the *pksX* cluster have been noted, and only bacillaene and dihydrobacillaene have been structurally characterized. It is not clear which of these two (if any) should be assigned as the genetically encoded product of the polyketide synthases, though domain analysis of these synthases led to the proposal that bacillaene is the immediate product. Whereas polyketide-modifying cytochrome P450s are typically hydroxylases [20,21] or epoxidases [22], no epoxy-bacillaene is known; and all of the hydroxyl groups on bacillaene or dihydrobacillaene can reasonably be explained by the domain content of the PksJLMNR synthases.

In order to determine the substrate for PksS, we incubated PksS/reductase with crude methanolic extracts of metabolites from the *B. subtilis* strain ATCC 39374, a wild-type producer of the bacillaene and difficidin polyketides [23–25]. We turned to this strain because we have found that it consistently yielded known polyketide products in a reproducible manner. After removal of the methanol, the ATCC 39374 extracts were treated with purified PksS, crude reductase, NADH, NADPH, and the resulting mixtures were analyzed by LC–MS (Fig. 4). In all samples, with or without any of the added P450 components, there is one major peak (not shown in Fig. 4) with a mass of

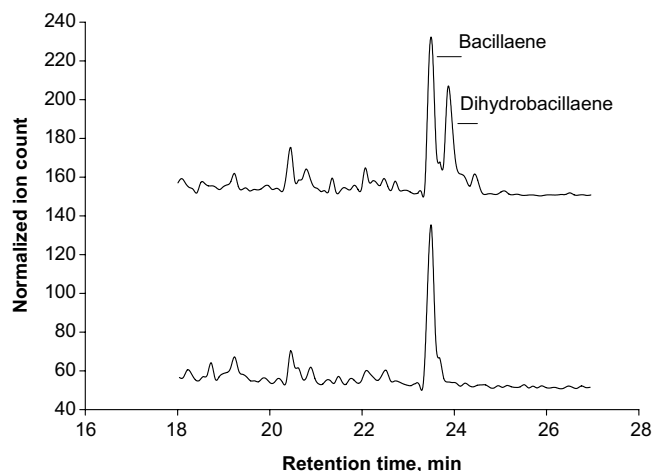


Fig. 4. Partial LC–MS chromatograms of ATCC 39374 extracts. Top trace, LC–MS of untreated ATCC 39374 methanolic extracts; Bottom trace, LC–MS of the same extract treated with PksS, crude reductase, and NADH. Traces are offset for clarity.

599 m/z , which is consistent either with a hydrated bacillaene or hydroxylated dihydrobacillaene [bacillaene + 18 + H^+]. There were two other less-intense peaks, the first with a mass of 581 m/z (consistent with [bacillaene + H^+]) and the second had a mass of 583 m/z (consistent with [dihydrobacillaene + H^+]). When the same extracts were treated with the full complement of PksS and cofactors, the 583 m/z peak corresponding to dihydrobacillaene was eliminated, with no apparent change in the putative hydrated bacillaene ($m/z = 599$) and unhydrated bacillaene ($m/z = 581$) peaks. These results suggest that dihydrobacillaene, and not bacillaene, is the substrate for PksS, though the complexity of the mixture has not allowed us to unambiguously assign the product. We propose two possibilities for this product, that are each consistent with available data.

The first proposal is that bacillaene exits the polyketide synthase as recently postulated, and an unknown reductase converts it to dihydrobacillaene. PksS would then follow to form a hydrated bacillaene (equivalent to hydroxylated dihydrobacillaene), which has never been characterized. Our second suggestion is that dihydrobacillaene exits the polyketide synthase, and PksS hydroxylates it at either C14' or C15', which is ultimately eliminated to form bacillaene. Since the function of the *pksJLMNR* synthases has not been biochemically confirmed, we expect that PksS will be a useful tool for marking structural features which are not originated by the polyketide synthase repertoire. Our efforts in characterizing the product of the PksS reaction will be reported in due course.

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