ORIGINAL ARTICLE

Production of Ansamycin Polyketide Precursors in *Escherichia coli*

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Dedicated to the memory of Professor Kenneth L. Rinehart Jr., for his pioneering studies in natural product chemistry, including structure elucidation of the ansamycin antibiotic geldanamycin.

Abstract   For the heterologous production of ansamycin polyketides such as rifamycin and geldanamycin in *Escherichia coli*, a number of unusual but important tools must be engineered into the bacterium. Here we demonstrate efficient production of the starter unit 3-amino-5-hydroxybenzoic acid (AHBA) and the methoxymalonyl extender unit in *E. coli*. Previous work has demonstrated the production of the ansamycin starter unit AHBA in *E. coli* in low yield. It was shown that the low yield is primarily due to acetylation of AHBA into *N*-acetyl-AHBA. Three methods for minimizing this side reaction were evaluated. First, a putative *N*-arylamine-acetyltransferase (NAT) was deleted from the *E. coli* chromosome, although this did not alter *N*-acetyl-AHBA production. Next, *E. coli* grown in media devoid of glucose yielded a nearly equal mixture of AHBA and *N*-acetyl-AHBA. Lastly, the NAT inhibitor glycyrrhizic acid was shown to further inhibit the acetylation reaction. The entire set of genes for synthesizing the methoxymalonyl extender unit was transferred from the geldanamycin producer *Streptomyces hygroscopicus* into *E. coli*. The pathway specific ACP isolated from the resulting recombinant strain was found to predominantly occur as methyoxymalonyl-ACP. Together, these findings set the stage for engineered biosynthesis of ansamycin polyketides in *E. coli*.

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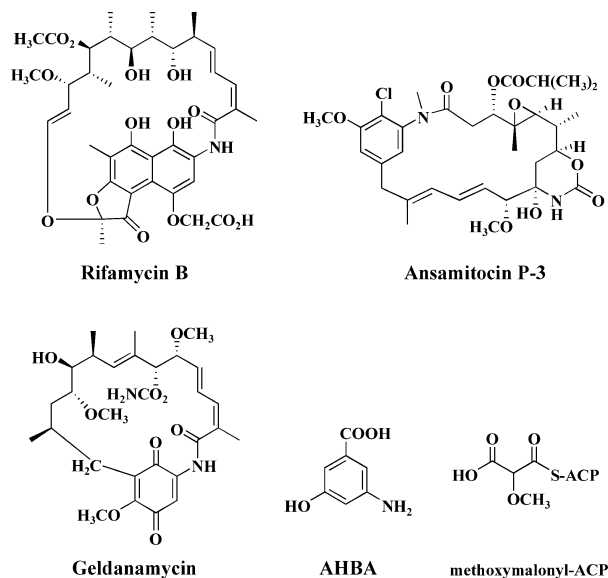
Introduction

Polyketide natural products play an important role in the treatment of a variety of human diseases as well as in animal health and agriculture. Many natural producers of polyketides, typically bacteria and fungi, exhibit undesirable characteristics such as slow growth, low productivity, or poorly understood genetics. As a result, there is considerable interest in making these compounds in heterologous hosts such as *Streptomyces coelicolor* and *Escherichia coli* [1]. *E. coli* has gained attention as a possible heterologous host due to its well defined genetics, rapid growth characteristics, and the existence of high-yielding secondary metabolite fermentation protocols. Previously, an *E. coli* system was engineered for the production of the polyketide 6-deoxyerythronolide B (6-dEB) [2]. Biosynthesis of 6-dEB required synthesis of the non-native extender unit methylmalonyl-CoA in *E. coli*. Other classes of polyketides require unnatural starter and extender units such as 3-amino-5-hydroxybenzoic acid (AHBA) and methoxymalonyl-ACP, respectively. One class of polyketides that requires these polyketide building blocks is the ansamycins.

The ansamycins contain an aromatic ring bridged by a polyketide chain terminating in an amide linkage [3]. Some examples of ansamycins are the antibiotic rifamycin and the anti-tumor compounds geldanamycin and ansamitocin

Fig. 1 Structure of three ansamycin polyketides and the starter unit AHBA.

The extender unit methoxymalonyl-CoA is used to make ansamitocin P-3 and geldanamycin.

P-3 (Fig. 1). The biosynthesis of the ansamycins starts with the synthesis of AHBA, which is then loaded onto a type I polyketide synthase (PKS) where several rounds of elongation occur. The final polyketide chain is then cyclized to yield a macrocyclic lactam. Methylmalonyl-CoA and malonyl-CoA are used in chain elongation during the course of synthesis of all three aforementioned ansamycins [4~6]. In addition, geldanamycin and ansamitocin PKSs also use methoxymalonyl-ACP as an extender unit [7~9]. Thus, engineered biosynthesis of these ansamycins in *E. coli* would require that this heterologous host be able to produce these unusual building blocks.

Previous work has demonstrated low levels of AHBA production in *E. coli* [10] although there are no reports of methoxymalonyl-ACP production in this bacterium. In this work, we demonstrate that the expression of a hybrid rifamycin and ansamitocin AHBA gene cluster in *E. coli* leads to production of *N*-acetyl-AHBA under minimal media fermentation conditions with only low production of AHBA. The use of complex media, such as LB broth, results in a higher level of AHBA being produced. We also show that by expressing the *fkbG-K* gene cluster from *S. hygroscopicus* in *E. coli*, methoxymalonyl-ACP could be generated. These two accomplishments create an *E. coli* platform that can be used for the production of ansamycin polyketides.

Results and Discussion

AHBA Production

Previously, a pET28 *E. coli* expression vector containing the *rifH,N,M,K* and *asm23,47* genes (pKW255) produced 3 mg/liter of isolated AHBA in minimal media with glucose as the sole carbon source [10]. To determine the cause of the low productivity, further analysis of the fermentation broth for side product formation was undertaken. *N*-Acetyl-AHBA was found to be the dominant product in these minimal media fermentations. From these fermentations, very little AHBA could be detected using HPLC analysis. To create a robust platform for making ansamycin polyketides in *E. coli*, methods for shifting the balance of *N*-acetyl-AHBA to AHBA are needed.

Three approaches were evaluated to inhibit the formation of *N*-acetyl-AHBA. First, a putative *N*-arylamine acetyl transferase (NAT), *nhoA* [11] was deleted from the *E. coli* genome based on the hypothesis that this enzyme was responsible for transferring an acetyl group from acetyl-CoA to AHBA. This gene was successfully deleted from the *E. coli* chromosome to generate MAR1. Shake flask fermentations of *E. coli* MAR1/pKW255 in minimal media revealed that *N*-acetyl-AHBA was still the major metabolite, suggesting that NhoA is not solely responsible for AHBA acetylation. Additionally, NhoA may not have even been responsible for any acetylation. Next, alternative fermentation media were tested to see if one favored AHBA production. When Luria-Bertani (LB) medium was tested, AHBA and *N*-acetyl-AHBA were found at concentrations of 55 mg/liter and 75 mg/liter, respectively, after a 24 hours shake flask fermentation with BAPI. Lastly, a known inhibitor of NAT activity, glycyrrhizic acid [12], was added to an LB shake flask fermentation at a concentration of 10 mM. After 24 hours no *N*-acetyl-AHBA was observed, and AHBA was found at 10 mg/ml in BAPI strains. Lower concentrations of glycyrrhizic acid failed to inhibit the acetylation reaction. While the use of glycyrrhizic acid yielded no *N*-acetyl-AHBA side product formation, the use of this compound in ansamycin fermentations could prove to be impractical, as ansamycin cyclases are themselves NATs [13], and may be inhibited by glyccyrrhizic acid. If so, media formulations that leave out glucose, such as LB, would be the best alternative.

Methoxymalonyl-ACP Production

The five *fkbG-K* genes from *S. hygroscopicus* are believed to be responsible for the production of methoxymalonyl-ACP [9]. Previous work has suggested that an unknown glycolytic intermediate is first loaded onto the acyl carrier

protein (ACP) FkbJ. This intermediate then presumably undergoes a series of oxidations and an *O*-methyl transfer to yield methoxymalonyl-ACP [14]. These five genes had been previously transferred to *S. fradiae* where midecamycin A was heterologously produced [15].

To determine if methoxymalonyl-ACP could be made in *E. coli* using the *fkbG-K* operon, the five genes were first cloned into a pET28 expression vector. *FkbJ* was also cloned into a pET28 expression vector independently. Next, the *fkb* operon was expressed in the *E. coli* strain BAP1 [2], which contains the *sfp* phosphopantheniene transferase gene required for post-translational modification of ACPs. At least four of the five proteins were observed on a SDS-PAGE gel after Ni-NTA column purification (Fig. 2). FkbH and FkbI have similar molecular masses, 39 kDa and 38 kDa, respectively, and could not be resolved on a SDS-PAGE gel after Ni-NTA purification. However, when subjected to HiTrap Q anion exchange separation, the two proteins eluted at different times (data not shown). If *E. coli* is able to synthesize the unknown precursor for methylmalonyl-thioester biosynthesis, it may be possible to detect methoxymalonyl-FkbJ. To determine if this was indeed the case, FkbJ was further purified from the other four Fkb proteins, as described in the Experimental Methods section. The protein was then digested with

trypsin, and submitted for LC/MS analysis. The expected mass of the trypsin fragment containing the active site serine and the phosphopantheneine arm is 2785 g/mol. The expected mass of the same fragment with methoxymalonyl attached is 2900 g/mol. At 22.9 minutes two peaks were observed with masses of 1393.5 and 1404.5 g/mol, which
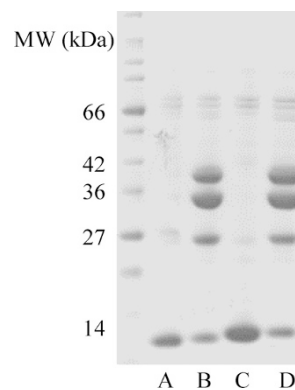

Fig. 2 Purified FkbH-K and GdmJ proteins after Ni-NTA purification.

Protein samples run on a polyacrylamide gel stained with Coomassie Brilliant Blue. Lane A, FkbJ expressed independently; Lane B, FkbG-K (24, 39, 38, 9.5, 29 kDa respectively) expressed simultaneously; lane C, GdmJ expressed independently; lane D, FkbG-I, -K, GdmJ (10 kDa) expressed simultaneously.
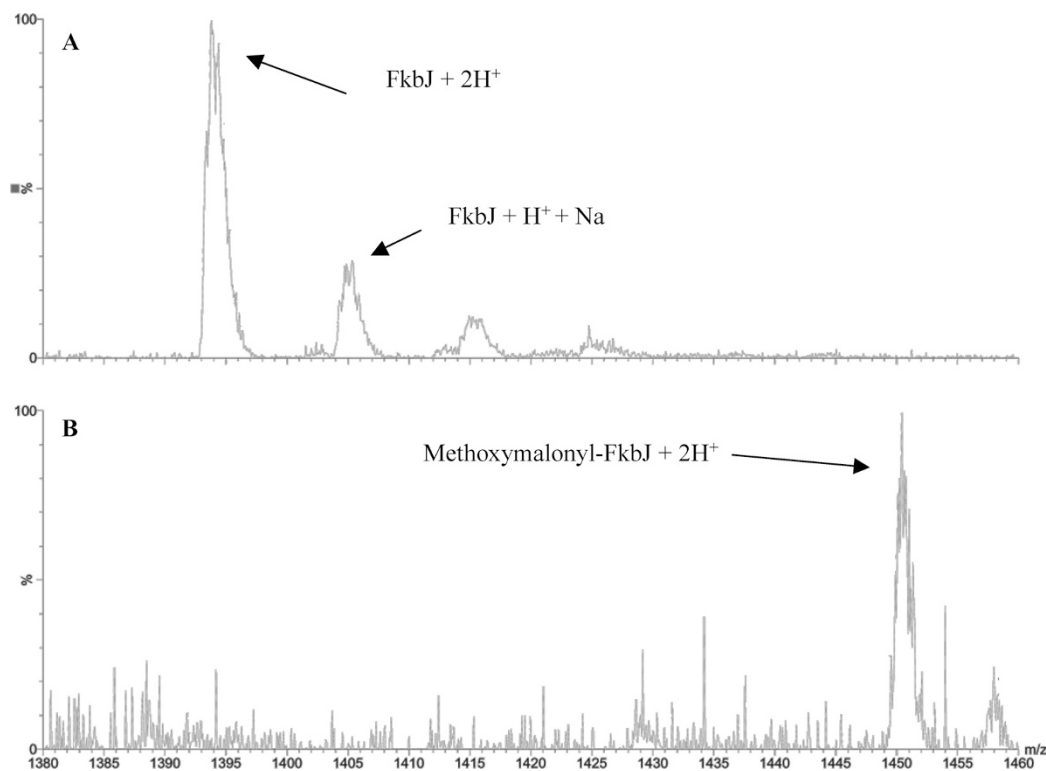

Fig. 3 LC/MS spectrum of FkbJ containing the phosphopantheniene arm.

(A) FkbJ expressed independently after digestion with trypsin. (B) FkbJ expressed with FkbG-I, -K after digested with trypsin.

correspond to the FkbJ+2H$^+$ and FkbJ+H$^+$+Na$^+$ peaks, respectively (Fig. 3). At 18.5 minutes a peak was observed with a mass of 1451.4 g/mol which corresponds to methoxymalonyl-FkbJ+2H$^+$. Based on the peak area ratios, approximately 20% of FkbJ was loaded with methoxymalonyl extender units. When FkbJ was expressed without the other Fkb proteins and subjected to similar analysis, only the FkbJ+2H$^+$ peak was observed. The methoxymalonyl-FkbJ+2H$^+$ peak was absent.

The ability of FkbJ to interact with PKS modules from heterologous synthases has not been fully explored. Although it has been shown that FkbJ can productively interact with the midecamycin A polyketide proteins, it is unknown if FkbJ will interact with ansamycin polyketide proteins. For example, GdmJ, the homolog of FkbJ found in the geldanamycin pathway, has approximately 50% sequence identity with FkbJ. This difference may prevent FkbJ from effectively transferring a methoxymalonyl extender unit to the geldanamycin PKS. To circumvent this potential problem, the corresponding methoxymalonyl-

ACP from the geldanamycin system, *GdmJ*, was cloned with *FkbGHIK* into the same pET28 expression vector. All five proteins were co-expressed (see Fig. 2) and GdmJ was purified, digested with chymotrypsin, and submitted for LC/MS analysis. The expected mass of the chymotrypsin fragment containing the active site serine and the phosphopantheneine arm is 1195 g/mol. The expected mass of the same fragment with methoxymalonyl attached is 1311 g/mol. At 13.6 minutes two peaks were observed with masses of 1196.3 and 1218.4 g/mol that corresponds to the GdmJ+H$^+$ and GdmJ+Na$^+$ respectively. At 16.6 minutes a major peak was observed with mass of 1312.3 g/mol that corresponds to methoxymalonyl-GdmJ+H$^+$ (Figs. 4 and 5). Approximately 60% of GdmJ was loaded with methoxymalonyl extender units, based on the peak area ratios. When GdmJ was expressed without the other Fkb proteins and subjected to the same analysis, only the GdmJ+H$^+$ peak was observed. The methoxymalonyl-GdmJ H$^+$ peak was absent.

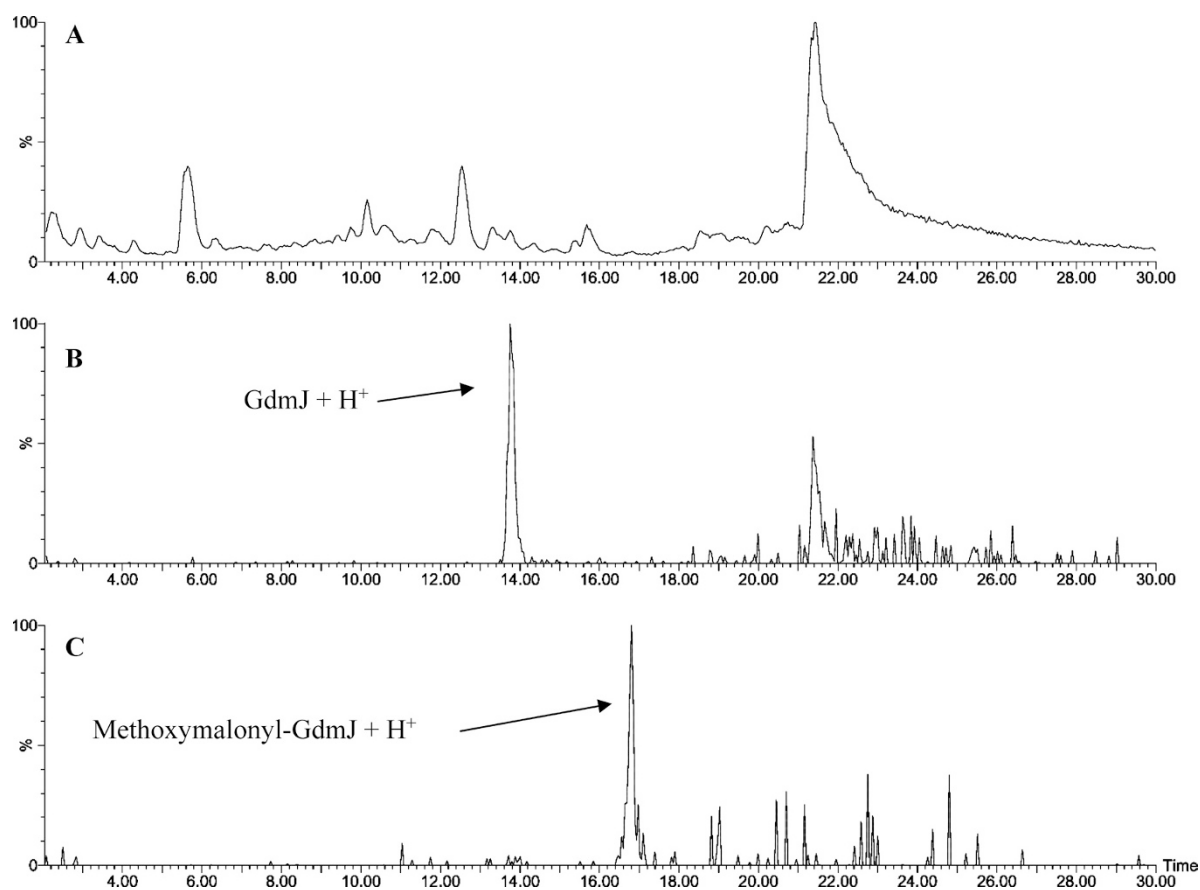The ability to efficiently synthesize AHBA and

Fig. 4 LC/MS traces from 2~30 minutes for GdmJ digestion when co-expressed with FkbG-I, -K.

(A) Total ion count. Post 30 minutes, no new peaks were observed. (B) Extracted ion count for 1196 g/mol. (C) Extracted ion count for 1312 g/mol.
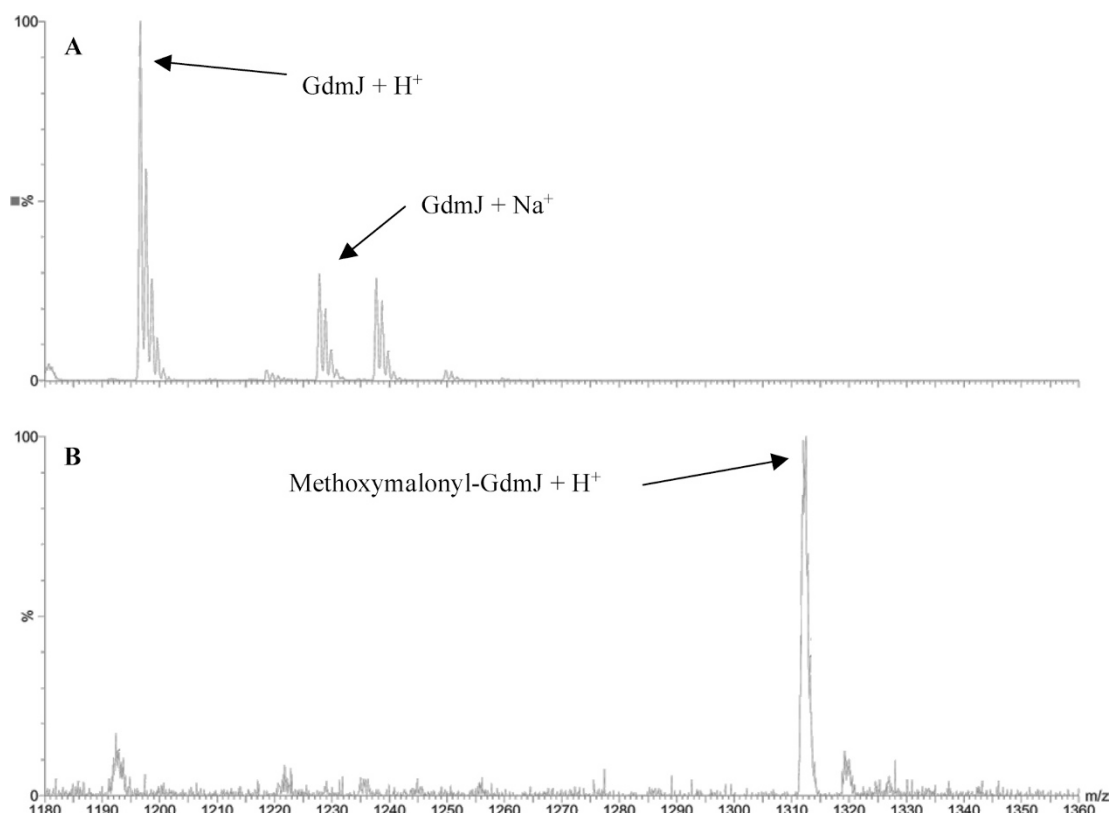
Fig. 5 LC/MS spectrum of GdmJ containing the phosphopantheniene arm.

(A) GdmJ expressed independently after digestion with chymotrypsin. (B) GdmJ expressed with FkbG-I, -K after digestion with chymotrypsin.

methoxymalonyl-ACP in *E. coli* expands the tools available to make polyketides in this bacterium. Previous work introduced necessary polyketide post-translational modifying enzymes and pathways to make the extender unit methylmalonyl-CoA in *E. coli*. The combination of these accomplishments should allow work to continue on expressing ansamycin polyketides in heterologous hosts.

Experimental

AHBA Fermentations and Analysis

pKW255, described previously, was used for all AHBA fermentations. pKW255 was first introduced *via* transformation into both BAP1 and MAR1 (see below for details). AHBA shake-flask fermentations were carried out by inoculating 100 ml of either LB media or M9 minimal media with 0.5 ml of the desired strain of *E. coli* from an overnight culture. Cultures were agitated at 37°C until an OD_{600} of 0.6 was reached. IPTG was added to a final concentration of 200 μM and the culture was agitated at 30°C for 24 hours.

AHBA and *N*-acetyl-AHBA fermentation concentrations were determined by injecting 100 μl of clarified broth onto a Phenomenex C18 reverse phase HPLC column with a linear gradient between buffer A (water, 0.5% trifloroacetic acid) and buffer B (acetonitrile, 0.5% trifloroacetic acid) from 0% to 40% over 40 minutes at a flow rate of 1.0 ml/minute. Authentic references were used to determine the retention time of the products and were also used to generate standard curves. AHBA was chemically synthesized as previously described [16]. *N*-Acetyl-AHBA was isolated from shake flask fermentations using methods previously described for isolating rifamycin intermediates [10]. *N*-Acetyl-AHBA; ^{1}H NMR (400 MHz, CD_3OD), $\delta = 7.58$ (t, *J* = 1.6 Hz, 1H), $\delta = 7.42$ (t, *J* = 2.0 Hz, 1H), $\delta = 7.15$ (t, *J* = 1.6 Hz, 1H), $\delta = 2.10$ (s, 3H)); MS (ESI+) calculated for $C_9H_9NO_4$ [M+Na$^+$] 218.2, found 218.3 and MS (ESI−) calculated for [M−H$^+$] 194.1, found 194.1.

Gene Knockout

To delete the putative *E. coli* NAT, *nhoA*, the temperature sensitive plasmid named pMAR46 was created as follows. First, regions approximately 1 kb upstream and downstream

Table 1 Primer sequence used in PCR reactions

Gene or chromosomal region	Forward primer sequence (5′~3′)	Reverse primer sequence (5′~3′)
Upstream of *NhoA*	GGT**AAGCTT**TACTGACATCTTTCAAAAGGAGCG	TAT**GGATCC**GCATGCCAGGCGTTTCCCTTAGTGGTTTTC
Downstream of *NhoA*	GTA**GGATCC**ATCGAATTTTATGTCAGGTTGCCGGATGCGG	TAT**GAGCTC**GGAGAACGTTAATGAAACCGCAGG
NhoA	GAAAACCACTAAGGGAAACGCCTG	
FkbG	GGTTTT**CATATG**GCTAATCAGATAACCCTGTCC	CTAT**GAATTC ACTAGT**TCACCGTTTCCGCAGCAGGGT
FkbH	GCATTT**CATATG**ACCATTGTCAAGTGTCTGGTC	CATT**GAATTC ACTAGT**TCACCCTGCTGCGGAAACGGT
FkbI	GCATTT**CATATG**CCTGAGCGTGACGCGCTGCTG	GCTTT**GAATTC ACTAGT**TCAAGCCGGGAGCGCCAGCGC
FkbJ	GCATTT**CATATG**ACCGCACCCGTCAAGGACGAC	GCAAA**GAATTC ACTAGT**TCAGGCATCGACGGCCCGCAG
FkbK	GCATTT**CATATG**GACCACCAGCTGATCGTGCTC	GCATT**GAATTC ACTAGT**TCATGAGACACCTTCTCGTA
GdmJ	TTTT**CATATG**ACCATCTCATCCGAGCCGGGC	AAAA**GAATTC ACTAGT**TCACGGCTGTGCCCCGCGCAG

Engineered restriction sites are in bold.

of the NAT were amplified by PCR and ligated into PCR-Blunt vectors. To the upstream fragment a *Hin*dIII site and a *Bam*HI site were engineered. To the downstream fragment a *Bam*HI site and a *Sac*I site were added. Table 1 contains sequences of primers used. These fragments were assembled into pUC18 to form pNM4. Next, the cassette was transferred to pMAK705 [17] using *Hin*dIII and *Sac*I, yielding pMAR46.

pMAR46 was introduced *via* transformation into the *E. coli* strain BAP1. Gene replacement *via* double crossover events was engineered as described elsewhere [17]. Transformants were screened with PCR using one primer designed 1 kb after the NAT and the second primer designed at the beginning of the NAT gene. Transformants containing the NAT gave a PCR product of 1.8 kb, while transformants with the desired deletion yielded 1 kb PCR products.

Construction of *fkbG-K* and *fkbG-I, -K, gdmJ* Expression System

To construct the fkb operons, PCR was first used to clone out each of the *fbkH-K* genes as well as the *gdmJ* gene. An *N*-terminal *Nde*I site and a *C*-terminal *Eco*RI site followed by an *Spe*I site were engineered into each gene. Each gene was cloned into pET28b (Novagen) using *Nde*I and *Eco*RI. Table 1 contains the sequence of primers used. This generated pMAR113 and pMAR128 expression vectors for *fkbJ* and *gdmJ*, respectively. To engineer the desired operons, *Xba*I-*Spe*I cassettes were assembled into a separate pET28b vector sequentially by taking advantage of compatible sticky ends produced by these two restriction

enzymes. Each of the *Xba*I-*Spe*I cassettes included individual ribosome binding sites. This generated pMAR118 and pMAR130 expression vectors for *fkbG-K* and *fkbG-I, -K, gdmJ* respectively.

Expression and Purification of FkbG-K and FbkG-I, -K, GdmJ

E. coli BAP1 [2] was used as a host to express the *fkbGHIJK* and *gdmJ* genes. A single transformant was used to start 5 ml of LB media cultures with kanamycin (50 mg/liter) at 37°C and 250 rpm. The starter culture was grown overnight and used to inoculate 1 liter of LB medium containing the same concentration of kanamycin. The culture was grown at 37°C and 250 rpm until an OD of 0.6~0.8 at which point the cultures were placed on ice for 15 minutes. Next the cultures were induced with 100 μM of isopopropyl-β-D-thiogalactopyranoside and incubated at 18°C and 250 rpm for 20 hours.

All purification procedures were performed on ice or at 4°C. FkbJ and GdmJ were isolated by first spinning the cell culture at 2,500×g for 20 minutes and resuspending the cell pellet in disruption buffer (200 mM sodium phosphate, pH 7.2/200 mM sodium chloride/0.2 mM DTT/1.5 mM benzamine/2 mg/liter pepstatin/2 mg/liter leupeptin/30% glycerol). Resuspended cells were lysed using sonication and then clarified at 40,000×g for 60 minutes. The lysate was then equilibrated with 2 ml of Ni-NTA resin (Qiagen) for 1 hour. The resin was first rinsed with 10 mM imidazole in a pH 7.2 100 mM sodium phosphate buffer, and then the protein was eluted off the resin with 200 mM imidazole in the same buffer. Additional purification was carried out

using anion-exchange chromatography. The protein solution was loaded onto a HiTrap Q 5 ml column (Amersham). A gradient of 0~1 M sodium chloride in 100 mM HEPES (pH 7.2), 2.0 mM DTT, and 10% vol glycerol was run at 3 ml/minute for 15 column volumes. Three ml fractions were collected, and those fractions containing FkbJ or GdmJ were pooled, concentrated, and buffer exchanged into 100 mM sodium phosphate (pH 7.2), 2.0 mM DTT, 20% vol glycerol using an Amcion Ultra protein concentrator (Waters). Purified protein was analyzed on a 4~12% Bis-Tris polyacrylamide gel (Invitrogen) stained with Coomassie brilliant blue stain. This protein purification procedure yielded 10 mg/liter of purified FkbJ or GdmJ.

LC/MS Analysis of FkbJ and GdmJ

FkbJ was digested with trypsin and GdmJ was digested with chymotrypsin as follows. Each digestion was carried out in 50 μl reaction with 100 mM sodium phosphate (pH 7.2), 100 μM FkbJ or GdmJ, and 2.5 μM trypsin or chymostrypsin. The reactions were incubated at 30°C for 60 minutes. The reactions were then quenched with 50 μl of a 10% formic acid solution.

Fifty microliters of each quenched reaction were analyzed using a Surveyor HPLC system (ThermoFinnigan) equipped with a Vydac C18 reverse phase polymer column. Mass spectra were collected on an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometer equipped with an electrospray ion source operating in positive ion mode. The sheath gas was set to 60 (arbitrary units), spray voltage to 4.5 kV, and capillary temperature to 200°C. A linear gradient between buffer A (water, 0.1% formic acid) and buffer B (acetonitrile, 0.1% formic acid) from 5% to 95% over 60 minutes at a flow rate of 0.2 ml/minute was used.

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