A [3Fe-4S] cluster and tRNA-dependent aminoacyltransferase BlsK in the biosynthesis of Blasticidin S

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B a cd S a e d ce de a b c. b , e ac c e c a, a d e c a ed e ed a e, LDBS a d LBS, a e bee f d e , d e. Le c a a bee ed, be a e ef- e ce ec a d b a cd S b , e , a d e c a f d, be a, f e e, a f β-a f e a e de c a. H e e, e e b e e a d , ca ed ec a f e c a fe e a, be e cda ed. He e, e e , e , e a, , e e c a fe e f- , e ed a e LDBS b a cd b , e . A e- ca e , BlsK, a bee c a ac e ed b e e, c a d , b ce ca e e e , . T e eca a e , e e c a fe f e c -, a fe RNA (e c -, RNA), e β-a , e a e de c a f DBS. F e, BlsK a f d, c a a e f c e, a e ea a f ac, . T e f d d e a e a e f a e f e, a, ca a- e a a ac -, RNA (aa-, RNA)–de e de, a deb d f a- , a a, a d c, b , e, c a, a .

tRNA-dependent enzymes | natural product | iron–sulfur cluster | leucyl transfer reaction

Amide bond formation in natural product biosynthesis can be catalyzed by nonribosomal peptide synthetases (NRPSs) and ATP-grasp ligases including the ATP-dependent activation of amino acid substrates. The carboxyl group is activated by phosphorylation and adenylation, respectively, in ATP-grasp ligase and NRPS catalyzed reactions (1). Recently, a new family of enzymes has been found to catalyze amide bond formation using aminoacyl-transfer RNA (aa-tRNA) as an activated cosubstrate. aa-tRNA plays a profound role in cells connecting the messenger RNA (mRNA) and protein synthesis at the ribosome in primary metabolism (2). Interestingly, recent studies have revealed that aa-tRNA can also be involved in natural product biosynthesis (3). aa-tRNA–dependent enzymes involved in natural product biosynthesis mainly form three groups: amide bond-forming ligases homologous with FemX peptidyl-transferases from cell wall biosynthesis (4), synthase enzymes including the cyclodipeptide synthase family (5, 6), and dehydratase enzymes in RiPP (ribosomally synthesized and posttranslationally modified peptide) synthesis (7).

Blasticidin S (BS), a representative of the amino hexose pyrimidine nucleoside antibiotics, was first isolated from *S ep om ce g i eoch omogene* (8). The structure of BS features a C2′, C3′-dehydrated pyrose ring that is connected with cytosine at C1′ and β-arginine at C4′ (9). BS shows a broad spectrum of biological activities and had been widely used as a fungicide to protect rice from blast diseases (10, 11). Presently, BS is commonly used as an efficient selection antibiotic for transformed mammalian cells that express appropriate resistance genes (12, 13).

The structural features and commercial value of BS stimulated interest in its biosynthetic studies. Early feeding experiments and characterization of related metabolites determined that glucose, cytosine, L-arginine, and methionine are the basic precursors for BS biosynthesis (14). The biosynthesis gene cluster was reported in 2003 and enabled a proposal for the BS biosynthetic pathway (Fig. 1) (15). However, difficulty carrying out gene knockout experiments in the native producer, *S. g i eoch omogene* , hindered the further characterization of each biosynthetic step. More recently, the BS biosynthetic cluster was engrafted into the chromosome of *S ep om ce li idan* to generate the genetically stable mutant strain WJ2, which is able to produce BS and thereby facilitating in vivo studies of the function of BS biosynthetic genes (13). Gene deletions in WJ2 determined the essential genes for BS biosynthesis including *bl D-bl L* that are transcribed in one direction and the divergently transcribed *bl M*. The first committed step of BS biosynthesis is the hydrolysis of cytidine monophosphate by BlsM to produce free cytosine, which is then condensed with UDP-glucuronic acid by BlsD to form cytosyl-glucuronic acid (CGA) (15, 16). The process from CGA to cytosinine, a putative intermediate for BS biosynthesis, remains to be determined. A radical SAM protein

S f ca ce

T a fe RNA (, RNA) , a fe a a e ac a ed a ac d , , e e d f , e f e de c a , a f- ca , e a e a b . I , a , , RNA a e a bee , f c, de f , a a . I e , e e , e c a ac e a f a e f a ac -, RNA (aa-, RNA)–de e de , e e, B K, f *Streptomyces griseochromogenes*. B K ca a e a aa-, RNA–de e de , e c , a fe eac b a , c d S b , e . B K c , a a [3Fe-4S] c e , a e a f , f c, a d ece- de ed e de , f ed , RNA-de e de , , a fe a e. Beca e B K d e a , a f c, a e , , a e e e a , e f aa-, RNA–de e de , e- e. c , a b ad ca , f b , RNA- de e de , a d f –c , a e e .

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The authors declare no competing interest.

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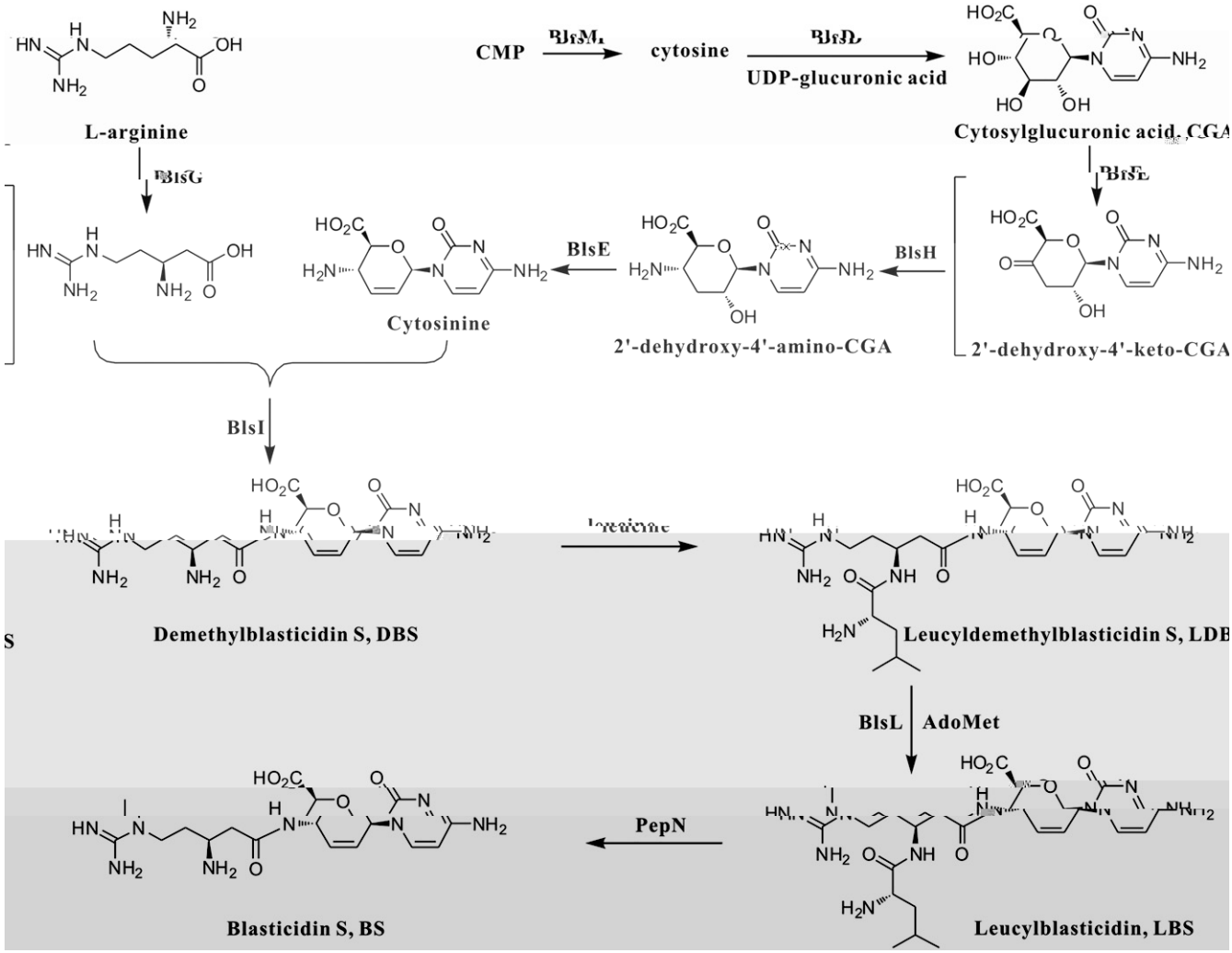


Fig. 1. The proposed biosynthetic pathway of BS. CMP: Cytidine 5-monophosphate; AdoMet: S-Adenosylmethionine.

BlsE and an aminotransferase BlsH are possibly involved in this transformation (13, 15). The β-arginine moiety of BS is derived from L-arginine through a radical-mediated reaction catalyzed by BlsG, a 2,3-arginine aminomutase (15, 17). It remains unknown whether BlsI, a putative ligase, is involved in the formation of DBS (demethylblasticidin S) by coupling of the carboxyl group of β-arginine and the amido group at C4′ of cytosinine since mutant WJ2Δ*bl I* only accumulates the very early intermediate CGA. It is worth noting that DBS cannot be directly methylated to BS by the *N*-methyltransferase BlsL, which was confirmed by in vitro biochemical characterization (18).

Addition of leucine to DBS at the β-amino group of the arginine side chain forms leucyldemethylblasticidin S (LDBS) which is then methylated by BlsL to form the penultimate product leucylblasticidin S (LBS) (18). Finally, PepN catalyzes the maturation of BS biosynthesis via the hydrolysis of the leucyl group of LBS (19). The cell toxicity of LDBS and LBS are much lower than DBS and BS. Therefore, it is proposed that the circuitous modifications of BS and DBS with a leucyl group function as the self-resistance mechanism during BS biosynthesis (16). Attempts to form LDBS from DBS in a *S. g i eoch omogene* cell-free system were not successful. A plausible reason for the failure of LDBS formation was that even if LDBS could be formed, it would be readily hydrolyzed back to DBS by the conserved aminopeptidase PepN in the cell-free system (16, 19).

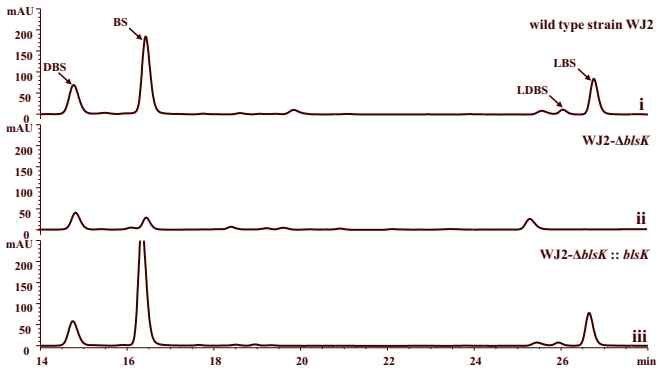
The intermediacy of LBS and LDBS hints at the necessity of an extra ligase other than BlsI in BS biosynthesis (15). In this report, we disclose the mechanism of the cryptic leucylation involved in BS biosynthesis. By combining in vivo gene inactivation and in vitro biochemical assays, we demonstrate that BlsK catalyzes the tRNA-dependent transfer of a leucyl group to the DBS β-arginine moiety by directing leucyl-tRNA^{Leu} to the BS biosynthetic pathway. More interestingly, BlsK is determined to be an iron–sulfur enzyme that does not show similarity with any known iron–sulfur enzymes. BlsK contains a [3Fe-4S] cluster that is critical for its activity. An iron–sulfur protein was shown to be involved in the amide bond formation. These results pave the way to fully understand the self-resistance and biosynthesis of BS.

Results

BlsK Is Necessary for the BS Biosynthesis. Two leucylated intermediates, LDBS and LBS, have been found to be involved in BS biosynthesis. To date, the mechanism of the peptide bond formation between leucine and the β-arginine amine is still unknown. The most common strategy used to form peptide bonds in secondary metabolism is through the NRPS system (20). According to the reported BS biosynthetic cluster, there is no NRPS-like enzyme involved in BS formation, indicating that a different mechanism may be involved (21). It was suggested that BlsK is the most likely candidate for the attachment of a leucyl group in BS biosynthesis

(15). The recently published genome sequence of *S. g i eoch o-mogene* ATCC 14511 indicates that the *bl K* sequence is 1,875 bp instead of the previously reported 1,740 bp (21). When queried in the National Center for Biotechnology Information (NCBI) database, BlsK shares no close homology with any proteins of known function, and structural prediction by HHpred also did not identify any homologous proteins with known structures (22). Cone et al. reported that BlsK shows modest sequence similarity with lysyl-tRNA synthetase and might be involved in leucyl transfer in BS biosynthesis (15). To explore the function of *bl K* in the BS biosynthetic gene cluster, *bl K* was deleted in *S. li idan* WJ2. High performance liquid chromatography (HPLC) analysis of the fermentation culture of the Δ*bl K* mutant strain showed that the production of BS was dramatically decreased indicating that the BS biosynthesis was significantly affected (Fig. 2). Furthermore, intermediates containing the leucyl group, LDBS and LBS, which accumulated in wild-type WJ2, could not be detected in the Δ*bl K* mutant. To exclude a possible polar effect, the mutant strain was complemented with the integrated shuttle plasmid pIB139 containing *bl K* under the control of the *e mE* promoter. The production of BS was restored to a similar level as the wild-type strain and the two leucyl intermediates, LDBS and LBS, were again observed (Fig. 2). Collectively, these data demonstrate that BlsK is involved in BS biosynthesis, and the disappearance of the leucylated intermediates suggests BlsK might function in the attachment of the leucyl group.

In Vitro Characterization of BlsK. In order to directly investigate the exact role of BlsK in the biosynthesis of BS, recombinant BlsK was overexpressed as a C-terminal His-tagged fusion protein and purified to homogeneity. Due to the proposed function of BlsK as a leucyl transferase, we first assessed activity in vitro with DBS plus leucine and ATP as potential substrates. ATP was added, because the carboxyl group of leucine must be activated for amide bond formation and the activation is often ATP dependent (20). However, no formation of LDBS was observed after the assay mixture was incubated in 37 °C for half an hour (Fig. 3*A*). Regardless of how assay conditions were changed, such as longer incubation times or varied pH, no LDBS could be detected. To exclude the possibility that the activity was lost during the purification process, *E che ichia coli* BL21 (DE3, Δ*pepN*)/pET44b-*bl K* was cultured, induced with IPTG, and the cell-free extract (CFE) was directly incubated with DBS, leucine, and ATP. Given that *E. coli* PepN is able to excise the leucyl group from LDBS (19), *E. coli* BL21 (DE3, Δ*pepN*) was used to avoid the interference of PepN activity (*SI Appendi* , Fig. S1). Under these conditions, HPLC analysis revealed efficient production of LDBS, which was confirmed by spiking with an authentic LDBS standard and liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis (Fig. 3 *B* and *C*). When the CFE of *E. coli* BL21 (DE3, Δ*pepN*)/pET44b was used as control, no production of LDBS was detected, indicating the formation of LDBS was BlsK dependent (Fig. 3*B*). The above results clearly show that BlsK in the CFE has the ability to catalyze the leucyl transfer, indicating that either the purified BlsK lost activity after purification or it requires some cofactor(s) from the CFE of *E. coli* BL21 (DE3, Δ*pepN*) to carry out its function. To discriminate between these two possibilities, purified BlsK was incubated with DBS, leucine, ATP, and CFE of *E. coli* BL21 (DE3, Δ*pepN*). HPLC analysis indicated the generation of LDBS, although in relatively low efficiency (Fig. 3*B*). Together, these results clearly demonstrate that BlsK is active in vitro, and the CFE of *E. coli* BL21 (DE3, Δ*pepN*) facilitates the transfer of the leucyl group to DBS.



F . 2. HPLC analysis of *blsK* knockout and its complementation experiments in strain WJ2: (*i*) wild-type strain WJ2 as control; (*ii*) *blsK* gene knockout strain WJ2-Δ*blsK* (WXK3); and (*iii*) *blsK* complementation strain WJ2-Δ*blsK*:: *blsK* (WXK4).

BlsK Is a leucyl-tRNA–Dependent Transferase. To gain further insight into the mechanism of BlsK and explore the exact reasons that purified BlsK cannot catalyze the leucyl transfer without CFE, we looked for the active component(s) in *E. coli* BL21 (DE3, Δ*pepN*) CFE necessary for BlsK to transfer the leucyl group. Based on some similarity of BlsK with lysyl-tRNA synthetase, we speculated that tRNA in the CFE might help BlsK carry out its function (15). To test this hypothesis, we first investigated whether tRNA is necessary for the activity of BlsK. RNase A was added to BL21 (DE3, Δ*pepN*) CFE to decompose endogenous tRNA. When the RNase A–treated CFE was incubated with purified BlsK, DBS, leucine, and ATP, no LDBS was produced, suggesting that BlsK catalyzes the leucyl transfer reaction in a tRNA-dependent manner (Fig. 4*A*). To further confirm the role of tRNA, *E. coli* total tRNA was added to the reaction mixtures (purified BlsK, DBS, leucine, and ATP). Under these conditions, LDBS was produced efficiently (Fig. 4*B*). Together these results show that tRNA is necessary for the BlsK-catalyzed leucyl transfer reaction. We propose that leucine is first activated as leucyl-tRNA and then transferred to DBS to form LDBS. However, no exogenous leucyl-tRNA synthetase (LeuRS) was added to the reaction mixture, indicating that BlsK may catalyze the leucyl-tRNA formation and then transfer the leucyl group from leucyl-tRNA to DBS consecutively. In addition, there is the possibility that the endogenous LeuRS of *E. coli* was copurified with BlsK and is responsible for the leucine activation when purified BlsK was used in the in vitro activity assay.

To investigate whether BlsK has leucyl-tRNA synthase activity, purified LeuRS was added to the previous reaction system (purified BlsK, DBS, leucine, *E. coli* total tRNAs, and ATP). The production of LDBS was increased by about 10-fold, indicating the important role of LeuRS in the leucyl transfer process (Fig. 5 and *SI Appendi* , Fig. S2). No LDBS was produced when BlsK was not added in the control experiment (*SI Appendi* , Fig. S2). Thus, we suspect that BlsK purified by affinity chromatography is likely to contain trace amounts of LeuRS, which is responsible for the activation of leucine in the original in vitro BlsK activity assay lacking exogenous addition of LeuRS. In order to confirm this speculation, an antibody against *E. coli* LeuRS was generated and coupled to protein A beads then used to remove any potential contaminated LeuRS from affinity and size-exclusion chromatography–purified BlsK. This further-purified BlsK was then assayed for LDBS production without the addition of LeuRS. Indeed, BlsK itself cannot transfer leucine to DBS in the presence DBS, leucine, *E. coli* total tRNAs, and ATP (Fig. 5). When LeuRS was added to the reaction mixture, the formation of LDBS was restored (Fig. 5). Hence, our results strongly suggest that BlsK catalyzes the transfer of leucine to DBS via leucyl-tRNA, whose formation is catalyzed by LeuRS in primary metabolic pathway. BlsK itself does not have leucyl-tRNA synthase activity.

To further prove that BlsK can directly use leucyl-tRNA^{Leu} as a substrate, we generated tRNA^{Leu} by in vitro transcription, which

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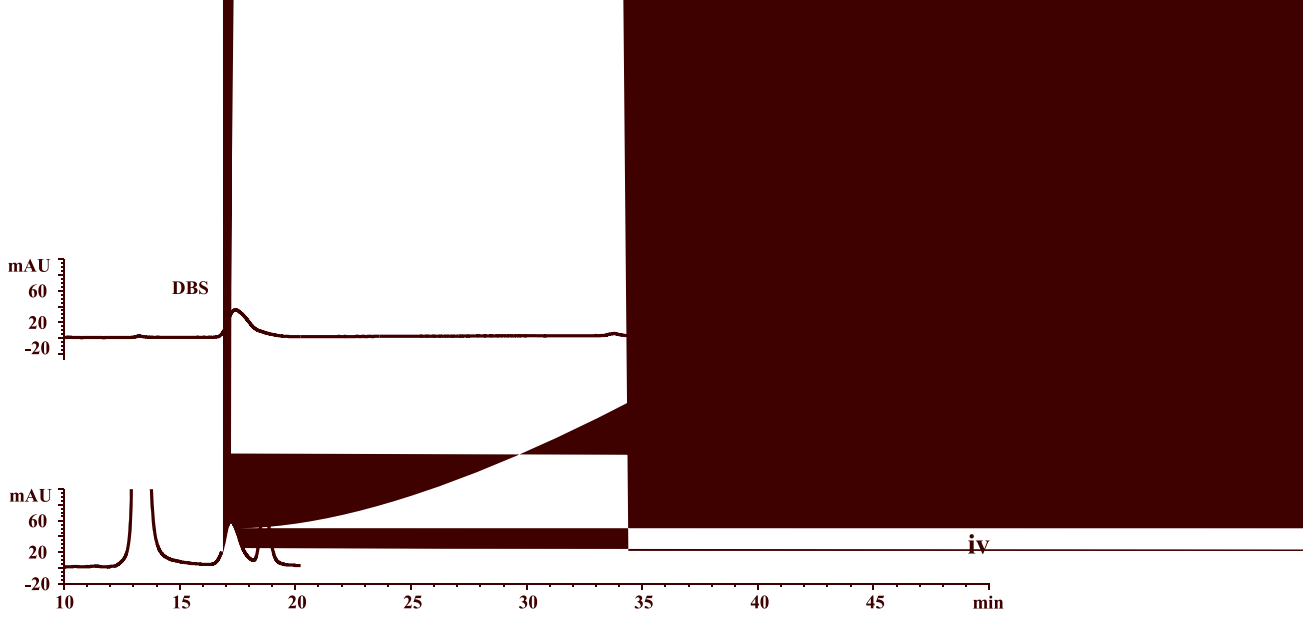

was then aminoacylated by LeuRS to obtain the leucyl-tRNA^{Leu}. The results showed that when leucyl-tRNA^{Leu} was added to reaction mixtures containing BlsK and DBS, LDBS was generated, and the amount of LDBS produced was proportional to the amount of added leucyl-tRNA^{Leu} (*SI Appendi* , Fig. S3). This clearly indicates BlsK is a leucyltransferase that steals the activated leucyl-tRNA^{Leu} formed by LeuRS and then transfers the leucyl group to DBS to generate LDBS. At the same time, we found that the amount of LDBS produced is comparable using either mature *E. coli* tRNAs or the tRNA obtained from in vitro transcription as cosubstrates (*SI Appendi* , Fig. S3), indicating that posttranscriptional modification does not affect recognition of tRNA by BlsK.

BlsK Contains a [3Fe-4S] Cluster. Purified BlsK is brownish in color, with a broad absorption band at around 420 nm in the ultraviolet-visible (UV-visible) spectrum (Fig. 6*A*). Addition of dithiothreitol (DTT)

darkened the color of BlsK slightly and increased the UV-visible absorption, while addition of sodium dithionite bleached the brownish color of BlsK and abolished the absorption around 420 nm (*SI Appendi* , Fig. S4). Although the sequence of BlsK does not show similarity with any known iron–sulfur protein, its color and features within the UV-visible spectrum are characteristic of iron–sulfur proteins. To confirm the presence of iron–sulfur cluster and characterize the cluster, chemical composition analysis and electron paramagnetic resonance (EPR) were used. Considering most iron–sulfur clusters in iron–sulfur proteins are sensitive to oxygen and not stable under aerobic conditions, BlsK was purified and further reconstituted under anoxic conditions and chemical analysis was used to measure the contents of iron and sulfur in the protein. This revealed 3.5 ± 0.4 Fe and 4.1 ± 0.2 S atoms per molecule of BlsK, indicating the existence of iron–sulfur cluster. For a more accurate analysis of the iron and sulfur content, inductively coupled plasma mass spectrometry (ICP-MS) analysis was employed and demonstrated that one molecule of BlsK contained 3.2 ± 0.2 Fe and 4.3 ± 0.3 S atoms, suggesting a possible [3Fe-4S] cluster in BlsK. To confirm this hypothesis, EPR analysis was performed. The EPR results showed that BlsK had a signal of $g = 2.01$ ($S = 1/2$) (Fig. 6*B*), which is characteristic of $[3Fe\text{-}4S]^{+}$ clusters. When the reducing agent sodium dithionite was added, the EPR signal disappeared, indicating that $[3Fe\text{-}4S]^{+}$ was reduced to $[3Fe\text{-}4S]^{0}$, which is EPR silent (Fig. 6). The activity of BlsK was not affected with the reduction of $[3Fe\text{-}4S]^{+}$ by dithionite (*SI Appendi* , Fig. S5). Collectively, UV-visible and EPR spectra combined with iron and sulfur contents analysis are most consistent with the presence of [3Fe-4S] cluster in BlsK.

To further characterize the $[3Fe\text{-}4S]^{+}$ cluster of BlsK, we identified the amino acid residues involved in the binding of the [3Fe-4S] cluster. As BlsK does not show similarity with known iron–sulfur proteins, MetalPredator was used to predict the metal-binding sites but gave no information about BlsK. The most common iron–sulfur cluster binding sites are cysteine residues (23). In order to determine which cysteine residues in BlsK may be involved

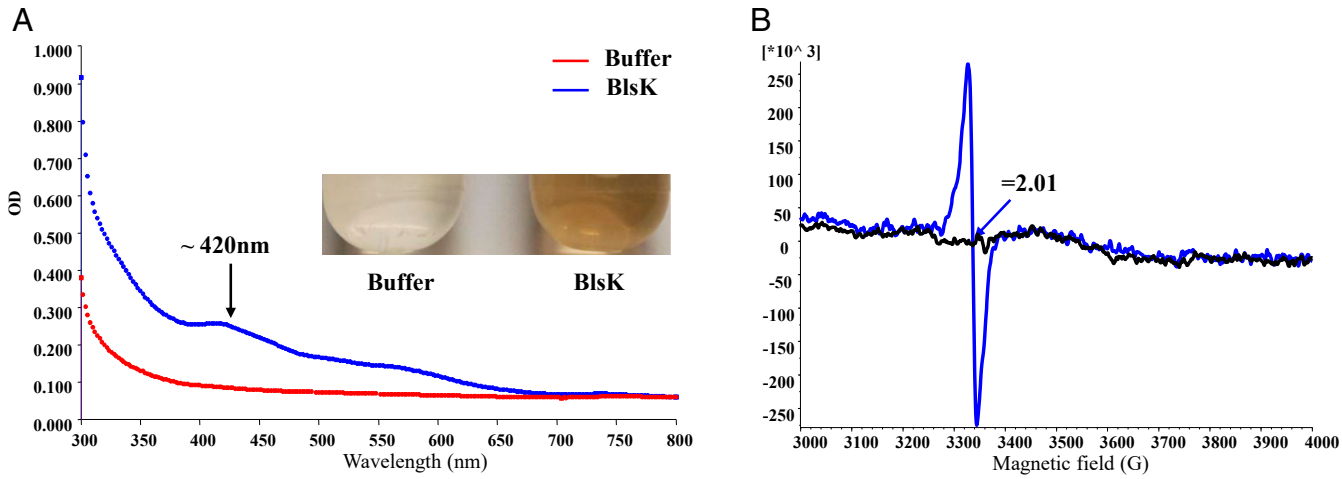

Fig. 6. Spectroscopic characterization of recombinant BlsK. (*A*) Color and UV-visible spectrum analysis of BlsK protein. The blue curve represents BlsK protein and red curve is the buffer control. (*B*) EPR analysis of BlsK with (black) and without (blue) addition of 1 mM sodium dithionite. EPR parameters: microwave frequency, 9.39 GHz; microwave power, 2 mW; modulation amplitude, 3 Gauss; modulation frequency, 100 kHz; and temperature, 10 K. The protein concentration used was 10 mg /mL.

in the coordination of the [3Fe-4S] cluster, we conducted site-directed mutagenesis of cysteine residues in BlsK. All nine cysteine residues in BlsK were mutated to serine residue respectively. Except for BlsK (C482S), all the other BlsK mutants were expressed as soluble proteins and purified similarly with wild-type BlsK. Among these soluble mutants, BlsK (C236S), BlsK (C253S), and BlsK (C259S) lost the brown color, and the peak around 420 nm in the UV-visible spectra disappeared, indicating these three mutants lost the $[3Fe\text{-}4S]^{+}$ cluster (*SI Appendi* , Fig. S6*A*). Concomitant with the disappearance of the characteristic UV-visible absorption band, the EPR spectra of these three BlsK mutants lost peaks centered at $g = 2.01$ (*SI Appendi* , Fig. S6*B*). Instead, a signal for Fe^{3+} at $g = 4.3$ in the EPR spectra appeared, indicating the mutants could chelate the ferric ion and possibly form incomplete iron–sulfur clusters. Therefore, the data indicate the iron–sulfur cluster in BlsK might be coordinated by C236, C253, and C259.

The [3Fe-4S] Cluster Is Essential for the Function of BlsK. It is well known that the most common function of iron–sulfur clusters in proteins is to participate in redox reactions (24). It is surprising to find an iron–sulfur cluster inside BlsK because its catalyzed reaction, amide bond formation, does not include electron transfer. Thus, it is worth further studying the role of the iron–sulfur cluster in BlsK. It is noteworthy that the iron–sulfur cluster in BlsK seems somewhat resistant to oxygen, as the aerobically purified BlsK has the characteristic UV-visible signal of $[3Fe\text{-}4S]^{+}$ and is able to catalyze the transfer of leucyl group to DBS. However, the addition of DTT increased the activity of aerobically purified BlsK (*SI Appendi* , Fig. S7). When BlsK was purified and assayed anaerobically, its activity increased compared with the aerobically purified BlsK (Fig. 7*A*). When the anaerobically purified BlsK was further incubated with ferrous iron, sodium sulfide, and DTT, its activity was increased further (Fig. 7*A*). All these results suggest a possible relationship between the leucyl transfer activity of BlsK and the iron–sulfur cluster.

To further investigate whether the [3Fe-4S] cluster is essential for activity, EDTA was used to remove iron ions in BlsK. After incubation, BlsK lost its brownish color and became colorless, and the apo-BlsK lost the activity to transfer a leucyl group to DBS, demonstrating the importance of the [3Fe-4S] cluster to the activity of BlsK (Fig. 7*B*). Also, the UV-visible signal for $[3Fe\text{-}4S]^{+}$ disappeared, indicating the loss of the $[3Fe\text{-}4S]^{+}$ cluster (Fig. 7*C*). The apo-protein was then reconstituted with the addition of ferrous iron, sodium sulfide, and DTT in vitro. The reconstituted BlsK regained the brownish color and showed the typical absorption around 420 nm in the UV-visible spectrum seen with the untreated BlsK, indicating the successful reconstitution of the [3Fe-4S] cluster (Fig. 7*C*). In line with the expectations, the activity of the reconstituted BlsK was restored (Fig. 7*B*). In further support of the importance of the [3Fe-4S] cluster, activity assays showed that C236S, C253S, and C259S mutants, which do not contain functional [3Fe-4S] clusters, lost the capacity to catalyze LDBS formation (Fig. 7*D*). Circular dichroism (CD) spectra of these mutant proteins were very similar to that of wild-type BlsK, indicating that their secondary structures were similar (*SI Appendi* , Fig. S8). Thus, the loss of protein activity was due to the incompleteness of the [3Fe-4S] clusters. Viewed together, the above results clearly demonstrate that the [3Fe-4S] cluster is critical for the leucyl transfer activity of BlsK.

Surprisingly, as the activity of BlsK was increased with the in vitro [3Fe-4S] cluster reconstitution, an additional product peak appeared (Fig. 7*A*). When labeled leucine (α-carbon with ^{13}C isotope) was used in the catalytic reaction, the high-resolution mass spectra demonstrated that the molecular weight of the product other than LDBS is 637.3329 ($[M+H]^{+}$), which is 114 mass units greater than that of LDBS (containing one ^{13}C) (the same molecular weight difference between LDBS (containing one ^{13}C) and DBS), indicating attachment of another leucyl group (*SI Appendi* , Fig. S9). Also, the MS/MS result of the product is consistent with the proposed product, leucyl-LDBS (*SI Appendi* , Fig. S9). Moreover, we found that about 10% LDBS was produced using the anaerobic-purified and -reconstituted BlsK without the addition of tRNA compared with that in the presence of tRNA (*SI Appendi* , Fig. S10*A*). To exclude the possibility that a trace amount of tRNA purified with BlsK might interfere with the results, RNase A was added to the reaction system. However, LDBS could still be produced, similar with that in the control group without RNase A (*SI Appendi* , Fig. S10*B*). Also, the possibility that purified BlsK had leucyl group anchored on some residues was ruled out because incubation of only purified BlsK and DBS could not form LDBS (*SI Appendi* , Fig. S11). Leucyl-AMP could be generated by LeuRS when tRNA is not present. The results indicated that in addition to leucyl-tRNA, BlsK may utilize other leucyl-activated species, probably leucyl-AMP, as activated leucyl species although with a strong preference for

leucyl-tRNA^{Leu}. A similar finding was observed in PacB in pacidamycin biosynthesis (25).

Discussion

An interesting feature in BS biosynthesis is the existence of a cryptic leucylation in the overall biosynthetic pathway. A leucyl group is first transferred to the β-amino group of the β-arginine side chain of the intermediate DBS and is then removed in the final biosynthetic step (15, 16). This cryptic enzymatic step has also been seen in several other natural product biosyntheses, such as chlorination during cyclopropyl amino acid biosynthesis and C-terminal methylation in thiostrepton biosynthesis (26, 27). We report here the identification of BlsK as a leucyl transferase in BS biosynthesis. The main purpose of the incorporation of the leucyl group during BS biosynthesis appears to be self-resistance, although it was found that the efficiency of BlsL, the N-methyl transferase in BS biosynthesis, is much higher with LDBS compared with DBS (16, 18). Thus, the function of BlsK is important to the BS biosynthesis. When *bl K* is deleted, BS production is significantly decreased. The small amount of BS that accumulates in the mutant strain may be derived from the direct methylation of DBS to BS. The addition of the leucyl group greatly increases the efficiency of N-methylation in BS biosynthesis catalyzed by BlsL (18). Thus, investigating the role and mechanism of leucylation is important to fully understand the biosynthesis of BS.

With the identification of LBS as a biosynthetic intermediate, BS biosynthesis requires two amino acid (arginine and leucine) activation enzymes. In the BS biosynthetic cluster, there are no NRPS-like genes, and *bl I* encodes an ATP-grasp enzyme, which could be used to activate one amino acid (16, 21). BlsI has been suggested to activate and ligate arginine to the cytosinine skeleton in the early step of BS biosynthesis (16). We could not identify another carboxyl activation enzyme in the BS cluster. Previous research suggested that BlsK could be a possible candidate enzyme (15). However, it does not show much similarity with other adenylating enzymes. Sequence and structural homology analysis indicate BlsK does not belong to any known protein family. In particular, HHpred-based analysis could only find some relevant domain templates for partial amino acid region of BlsK. Interestingly, through genetic and biochemical experiments, BlsK has been confirmed to be responsible for the leucyl transfer in an aa-tRNA–dependent manner. The finding of BlsK as a leucyl transferase adds an additional functional member to the aminoacyl transferases of the aa-tRNA–dependent protein family.

During the past decade, a number of aa-tRNA–dependent enzymes have been found to be involved in eesthr119.8(n(m)1561(f)12.r(so)18.1(m)

of enzymes, indicating that it may represent a different type of aa-tRNA–dependent aminoacyl transferase. As with the majority of other biosynthetic pathways that contain an aa-tRNA–dependent aminoacyl transferase, the BS biosynthetic cluster does not include aa-tRNA synthetase-encoding genes. Thus, BlsK might hijack the leucyl-tRNA^{Leu} generated by LeuRS in the cell that is primarily used for protein synthesis with an unknown mechanism.

Another unique feature of BlsK is that the enzyme contains an iron–sulfur cluster that is necessary for aminoacyl transferase activity. The dependency of BlsK activity on a [3Fe-4S] cluster is consistent with the relatively low activity of purified BlsK with the CFE compared with the CFE from *E. coli* BL21 (DE3, Δ*pepN*)/pET44b-*bl K*. In the purification process, the [3Fe-4S] cluster in BlsK was gradually degraded. However, the exact role of the iron–sulfur cluster in the aa-tRNA–dependent leucyl transfer process remains unknown. In many proteins, [3Fe-4S] clusters are involved in electron transfer reactions (33). BlsK catalyzes amide formation, which does not involve electron transfer. It is possible that the iron–sulfur cluster of BlsK plays an important role in stabilization of the structure of BlsK. When BlsK was over-produced in iron-deficient condition, it formed inclusion bodies, indicating the structural importance of the iron–sulfur cluster in BlsK (*SI Appendi* , Fig. S12). Another possible function of the iron–sulfur cluster of BlsK is substrate recognition. It has

$FeCl_3$ (23 mM, dissolved in 1.2 mM HCl) were added, mixed thoroughly until the solution was colorless, and centrifuged at 12,000 rpm for 15 min. The absorption at 670 nm of the supernatant was measured with a Biotek Synergy 2 plate reader. The standard curve of S content was measured with S standard solution reagents (Na_2S 0.2 mg/mL, dissolved in 0.1 M NaOH). In order to re-confirm the content of Fe and S, ICP-MS (iCAP Q, Thermo Fisher Scientific) was used. UV-visible scanning of BlsK was performed on the Biotek Synergy 2 plate reader (Biotek) between 300 to 700 nm. X-band EPR spectra of BlsK were recorded with Bruker EMX plus 10/12 (Bruker Corp.), equipped with an Oxford ESR910 Liquid Helium cryostat spectrometer (Oxford Instruments plc) at 10K. The CD spectra of BlsK and its mutant proteins were measured using a CD spectrophotometer (J-815, Jasco International, Co. Ltd.). The assay buffer (50 mM Tris·HCl buffer pH 8.0, 100 mM NaCl, 10 mM DTT, and 10% glycerol) was used for the EPR, CD, and UV-visible scanning experiments.

Enzymatic Assay of BlsK and HPLC/LC-MS Analysis. For in vitro assay of BlsK, the reactions were conducted in 100 μL Tris·HCl buffer pH 8.0 (50 mM pH, 8.5, 100 mM NaCl) containing 10 μM BlsK, 2 μM LeuRS, 1 mM ATP, 10 mM Leu, 250 μM DBS, 1 mg/mL $tRNA_S$, 20 mM $MgCl_2$, 10 mM KCl, and 10 mM DTT at 37 °C for various incubation times. For the preparation of CFE used in the activity assay, the *E. coli* strain BL21 (DE3, Δ*pepN*)/pET44b-*blsK* was used. After induction by IPTG for 16 h, the appropriate amount of cells was collected and resuspended in cell lysate buffer (50 mM Tris HCl pH 8.0, 0.3 M NaCl, 10% glycerol), sonicated, and centrifuged at 4 °C at 12,000 rpm for 30 min. The supernatant was used as the CFE. CFE of the *E. coli* strain BL21 (DE3, Δ*pepN*) was similarly prepared (without IPTG induction). The assays were stopped by adding an equal volume of chloroform to denature the protein, and the aqueous fractions were collected after centrifugation. The reaction products were analyzed by HPLC and LC-MS using the TC-C18 (2) column (4.6 mm × 2resus3(m(NaCl,)-ctio.7(5-1.282(NaCC)-338Agil(ifhe)-4)-39.3(super99.6(f9(ow(1)-8.9re)-4(a(5-1.(at)-48(entr)-10.563.i7(were1(h8.8(ana-1.29[(st4[(After)-37

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