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Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor

(peptide antibiotics/thiazole/oxazole/bleomycin)

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ABSTRACT **Drugs that inhibit the activity of DNA gyrase fall almost exclusively into two structural classes, the quinolones and the coumarins. A third class of DNA gyrase inhibitor is defined by the ribosomally synthesized peptide antibiotic microcin B17 (MccB17). MccB17 contains 43 amino acid residues, but 14 of these are posttranslationally modified. Here we describe the characterization of the structure of these modifications. We propose that four cysteine and four serine side chains undergo condensation with the carbonyl group of the preceding residue, followed by α/β dehydrogenation to yield four thiazole and four oxazole rings, respectively. The three proteins implicated in catalyzing these modifications (McbBCD) would constitute the only thiazole/oxazole biosynthetic enzymes identified. These results open up possibilities for the design of DNA gyrase inhibitors and add to the repertoire of posttranslational modifications with potential for protein engineering. *Escherichia coli sbmA* mutants, which lack the inner membrane protein (SbmA) involved in MccB17 uptake, were found to be resistant to bleomycin. Bleomycin is structurally unrelated to MccB17 except for the fact that it contains two thiazole rings. This suggests that thiazole rings are part of the MccB17 structure recognized by SbmA. This observation and the finding that SbmA homologs are widely conserved and can play developmental roles [Glazebrook, J., Ichige, A. & Walker, G. C. (1993) *Genes Dev.* 7, 1485–1497] suggest that thiazole- and oxazole-containing compounds may serve as signaling molecules for a wide variety of bacteria in diverse environments, including pathogen interactions with plant and animal hosts.**

Bacterial DNA gyrase has been extensively exploited as a target in antimicrobial chemotherapy (1). There are two major structural classes of DNA gyrase inhibitors, the quinolones and the coumarins. A third class of gyrase inhibitor is microcin B17 (MccB17), a 43-residue peptide antibiotic that is ribosomally synthesized (2–4). Its peptide nature suggests that it is structurally unrelated to either quinolones or coumarins. Yet, MccB17 inhibits DNA replication and genetic and biochemical evidence strongly indicates that DNA gyrase is the specific target of MccB17 action (5). Similar to quinolones, MccB17 causes double-stranded breaks in the chromosomal DNA in a DNA gyrase-dependent mechanism (5). The possibility that MccB17 could represent an unusual structural type of DNA gyrase inhibitor led us to study its structure.

MccB17 is a glycine-rich peptide antibiotic produced by strains of *Escherichia coli* carrying any one of several naturally occurring plasmids (6, 7). Seven plasmid-encoded genes, constituting an operon and designated *mcbABCDEFG*, are involved in MccB17 production. The first four genes, *mcbABCD*, are required for MccB17 synthesis (8, 9), while *mcbEFG* are required for MccB17 export and immunity (10). The structural gene for MccB17, *mcbA*, encodes a 69-amino acid precursor (see Fig. 1*A*) that undergoes at least two steps of posttranslational modification (3). First, the products of *mcbBCD* mediate side-chain modifications to generate proMccB17 (11). Subsequently, the N-terminal 26 residues are removed, yielding mature MccB17 (11).

Previous chemical analyses of MccB17 had suggested that several residues were posttranslationally modified (3). To determine the structure of the modifications in MccB17 we pursued a series of molecular, chemical, and spectral analyses of the molecule. The results obtained indicate that MccB17 undergoes unprecedented posttranslational modifications involving the backbone of the peptide and leading to the formation of four thiazole rings and four oxazole rings.

MATERIALS AND METHODS

Strains, Plasmids, and Media. The *E. coli* K-12 strain ZK4 (F⁻ *araD139ΔlacU169 rpsL relA thiA recA56*) was used as the host strain for all MccB17 production experiments and as the sensitive strain for MccB17 *in vivo* bioassays (12). ZK4 *sbmA* is resistant to the action of MccB17 because the SbmA protein is involved in the uptake of MccB17 (13). The high-copy plasmid used for wild-type MccB17 production, pPY113, is a pBR322 derivative, with *mcbABCDEFG* cloned into the pBR322 tetracycline-resistance gene. The *mcbABCDEFG* genes are contained in a 6.3-kb *Bam*HI/*Bgl* II fragment. A derivative of pPY113 containing a mutation in *mcbA* that results in the substitution of Ser-39 of MccB17 with Asn was obtained by nitrous acid mutagenesis *in vitro*. The media used for bacterial growth were M63 minimal salts plus 0.2% glucose (14). Ampicillin was used at a final concentration of 150 μg/ml. Bleomycin resistance was determined over bacterial lawns by using filter disks saturated with a 3-mg/ml solution of bleomycin.

MccB17 Production and Purification. The procedure for production and purification of MccB17 was adapted from that described previously (3). Wild-type and mutant microcin-producing strains were grown for MccB17 production at 37°C in Erlenmeyer flasks on rotary platform shakers (New Brunswick Scientific). Cells were harvested after the culture had been at least 6 hr in stationary phase. Cell pellets were resuspended in 1/5th of the original culture vol of 100 mM acetic acid/1 mM EDTA, boiled for 10 min, and centrifuged. The supernatant was loaded on a preparative C_{18} column, washed with water, and eluted with 15% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA), followed by 50% ACN/0.1%

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Abbreviations: ACN, acetonitrile; TFA, trifluoroacetic acid.

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TFA. MccB17 eluted in the 15–50% ACN fraction. This fraction was dried, resuspended in dimethyl sulfoxide (DMSO), and filtered through 0.45-μm nylon filters (MSI, Cameo 3N). This was further purified by reverse-phase HPLC, using a C_{18} column, and eluting with a 5–30% ACN gradient in 0.1% TFA. The major HPLC peak of wild-type or mutant MccB17 was isolated, dried, resuspended in DMSO, and stored at −70°C.

Proteolysis of MccB17 and Purification of Fragments. Proteinase K was used for proteolysis of MccB17. [^{35}S]Cysteine-labeled MccB17 was prepared by feeding [^{35}S]cysteine to a ZK4 pPY113 culture in M63 glucose plus ampicillin. The cells were harvested and the ^{35}S-labeled MccB17 was purified. A fraction of the labeled sample (≈0.05 μg) was added to 3 μg of unlabeled MccB17 and the combined sample was digested with proteinase K (100 μg/ml) in 50 μl of 100 mM sodium borate (pH 8.0) for 22 hr at 37°C. The fragments were separated by HPLC using a C_{18} column and a 2–20% ACN gradient with 0.1% TFA. The eluate was monitored at 254 nm and fractions were collected throughout the gradient and assayed for radioactivity.

Based on these results, an exhaustive preparative digest of ≈100 mg of MccB17 was conducted over 14 days while monitoring progress by HPLC. Several additions of fresh proteinase K were required. The reaction mixture was filtered through a Centricon 3 separation device and the filtrate was collected and lyophilized. The resulting material was then separated by HPLC. The major 254-nm absorbing peaks eluted in a cluster at 2–3% ACN. UV and fluorescence spectra and ^{1}H NMR spectra in ^2H_2O were recorded for the major fractions.

Characterization of the Cysteines in MccB17. To characterize the state of the cysteine residues, several analyses were conducted. First, MccB17 was subjected to denaturing conditions by boiling in 2% SDS, followed by treatment with 100 mM dithiothreitol to effect reduction of any existing disulfide bonds, and subsequently treated with 0.5 M iodoacetic acid (pH 8.5) to effect carboxymethylation of any sulfhydryl groups. HPLC analysis of material removed during each step of the protocol was identical with virgin MccB17. In addition, these treatments did not diminish the antibiotic activity of the MccB17 sample. MccB17 was also subjected to performic acid oxidation, followed by acid hydrolysis, phenyl isothiocyanate derivatization, and analysis by HPLC. This treatment yielded no cysteic acid, which would be expected from performic acid oxidation of free sulfhydryls or disulfide bonds.

Amino Acid Analysis, N-Terminal Sequencing, Mass Spectrometry, and NMR Spectroscopy. Amino acid composition analysis and performic acid oxidation were carried out by Dan Strydom and Wynford Brome at the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School. N-terminal sequencing was completed by William Lane at the Harvard University Microchemistry Facility. Fast atom bombardment mass spectrometry and matrix-assisted laser desorption mass spectrometry were carried out by Steve Martin and Ioannis Papayannopoulos at the National Institutes of Health Mass Spectrometry Facility at the Massachusetts Institute of Technology. The NMR spectroscopy and data analysis were carried out at the NMR Facility in the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School.

RESULTS AND DISCUSSION

To characterize the modifications present in MccB17 we began by performing standard amino acid analysis of the molecule. The results obtained (Fig. 1*B*) showed that of the 43 predicted residues, four serines and six glycines were unaccounted for, suggesting that these residues were either

A

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          -20        -10
MELKASEFGVVLSVDALKLSRQSPLG

1         10        20        30        40
VGIGGGGGGGGGGSCGGQGGGCGGCSNGCSGGNGGSGGSGSHI
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B

Amino acid	Predicted	Analysis
Asx	2	2.00
Glx	1	1.07
Ser	**6**	**1.76**
Gly	**26**	**19.77**
His	1	1.12
Val	1	0.95
Ile	2	1.79
Cys	**4**	**0**

FIG. 1. Amino acid sequence and composition of MccB17. (*A*) Predicted amino acid sequence of the primary translation product of *mcbA* was derived from the nucleotide sequence of the gene (3). N-terminal sequencing of MccB17 identified V1 (as designated in the figure) as the N-terminal residue in mature MccB17. The four cysteine and four serine residues that are modified in MccB17 are underlined and in boldface type. (*B*) Amino acid composition using acid hydrolysis and phenyl isothiocyanate derivatization of MccB17 revealed the absence of four serines and six glycines. To characterize the state of the cysteine residues, several analyses were conducted as described.

modified or excised. Since any cysteine residues would be lost in standard amino acid analysis, additional analyses were required to characterize the state of the four cysteine residues predicted from the gene sequence. Reduction and carboxymethylation of MccB17 had no effect on its antibiotic activity or its retention time in HPLC. MccB17 was also subjected to performic acid oxidation, followed by acid hydrolysis and amino acid analysis. This treatment yielded no cysteic acid, which would be expected from performic acid oxidation of free sulfhydryls or disulfide bonds. These results provided strong evidence that neither free sulfhydryls nor disulfide bonds were present in MccB17.

N-terminal amino acid sequencing showed that mature MccB17 lacked the N-terminal 26 amino acids of the precursor (3). In addition, N-terminal sequencing was completely blocked after glycine residue 12, suggesting that glycine residue 13 was modified in some way that prevented its reaction with the Edman reagent or its subsequent release.

Further indication of posttranslational side-chain modifications came from spectroscopic analyses. MccB17 exhibited a UV absorbance maximum, between 275 and 280 nm, uncharacteristic of any of the residues predicted from the nucleotide sequence and indicating the presence of a chromophore(s). The spectrum of a purified proteolytic fragment of MccB17 that retained UV absorbing properties showed an absorbance maximum at 276.8 nm and a corresponding fluorescence maximum at 337.6 nm, suggesting that the modifications included conjugated heterocycles.

Fast atom bombardment mass spectrometry analysis indicated a monoisotopic mass of 3093.6 for MccB17. The mass of the unmodified 43-amino acid peptide would be 3253.3 mass units, indicating a loss of 159.7 mass units as a result of posttranslational modification of the peptide.

^{1}H NMR spectroscopy of MccB17 revealed eight aromatic or olefinic protons uncharacteristic of any of the predicted amino acid residues (Fig. 2*A*). The spectrum was recorded after treating the sample with a 25% $^2H_4$-acetic acid/^2H_2O mixture. This replaces the exchangeable amide protons with

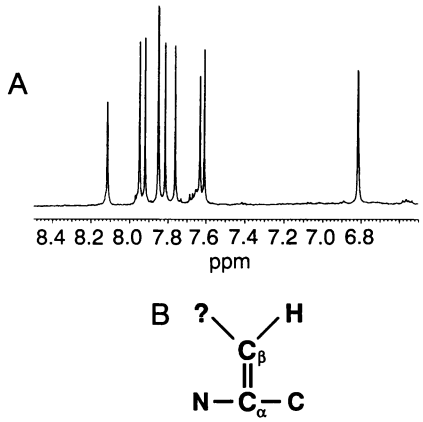

FIG. 2. Results from NMR analyses of MccB17. (*A*) One-dimensional ^{1}H NMR spectrum of MccB17 in the amide and aromatic proton region. (*B*) Additional ^{1}H, ^{13}C, and ^{15}N NMR analyses defined eight modified residues as dehydroalanyl moieties, which are located within the backbone of MccB17. Each of the four serines and four cysteines that is missing is modified to yield the structure shown. The C^{β} protons correlate to the eight aromatic/olefinic protons whose resonances are shown in *A*.

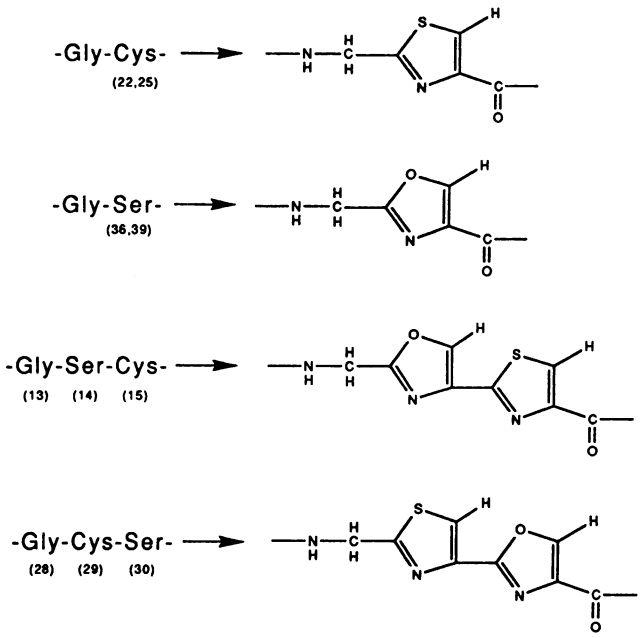

FIG. 3. Proposed modifications in MccB17. Numbers correspond to residue position in the MccB17 sequence (see Fig. 1*A*).

^{2}H, which is not detected in ^{1}H NMR. The resonances of these eight protons are flanked by the two histidine proton resonances at 6.82 and 8.12 ppm. (Only seven peaks are clearly visible because the peak at 7.83 ppm represents two overlapping singlets.) Additional ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy indicated that each of the eight aromatic/olefinic protons was attached to the C^{β} of a dehydroalanyl moiety (Fig. 2*B*). In addition, these dehydroalanyl residues were demonstrated to be β-substituted with an aprotic atom, which was neither carbon nor nitrogen. Analysis by two-dimensional ^{1}H NMR permitted the assignment of 11 of 43 residues (V1, G2, I3, G4, Q18, S26, N27, N33, S41, H42, and I43). These assigned residues include all the nonglycyl residues, except four cysteines (C15, C22, C25, C29) and four serines (S14, S30, S36, S39). The absence of the four cysteine and four serine residues predicted from the gene sequence implicated these residues as the precursors of the eight dehydroalanyl moieties. Several additional ^{13}C and ^{15}N resonances uncharacteristic of the predicted amino acids were also identified. These NMR results are a summary of extensive spectroscopic analyses to be presented elsewhere (J.L., P.Y., and R.K., unpublished results).

From these data we propose that MccB17 contains four thiazole and four oxazole rings, which are posttranslationally generated (Fig. 3). These proposed structures are consistent with the modification of four serine, four cysteine, and six glycine residues, the loss of 160 mass units from the peptide (eight rings formed, each accounting for the loss of 20 mass units), the presence of strong chromophoric moieties, including both UV absorbance and fluorescence characteristic of conjugated heterocycles, and the eight dehydroalanyl structures. In addition, the aromatic/olefinic proton resonances in MccB17 (Fig. 2*A*), as well as the unusual ^{13}C and ^{15}N resonances, are consistent with the corresponding resonances in known thiazole rings (15–17). Similar structural analyses of MccB17, arriving at the same conclusions, were performed independently by Bayer *et al.* (18).

Four different types of modifications are present in MccB17 (Fig. 3). Two Gly-Cys residue pairs (G21-C22 and G24-C25) both yield a single thiazole ring. Two Gly-Ser residue pairs (G35-S36 and G38-S39) both yield a single oxazole ring. The adjacent oxazole-thiazole rings and adjacent thiazole-oxazole rings both result from the modification of tripeptides, Gly-Ser-Cys (G13-S14-C15) and Gly-Cys-Ser (G28-C29-S30), respectively. The precise assignment of the four modified serines was possible because the two unmodified serine residues, S24 and S41, could be assigned in the NMR analyses. We had previously hypothesized that the posttranslational modifications generated covalent crosslinks in MccB17 and that these crosslinks facilitated stable folding of MccB17 (11). Although thiazole/oxazole rings clearly would not form covalent crosslinks, they may constrain the peptide chain in some other way. One possibility is that MccB17 contains a coordinated metal ion, since thiazole/oxazole rings are capable of complexing metals (19).

A pathway for the formation of the thiazole and oxazole moieties is proposed in Fig. 4. This is based on an additional

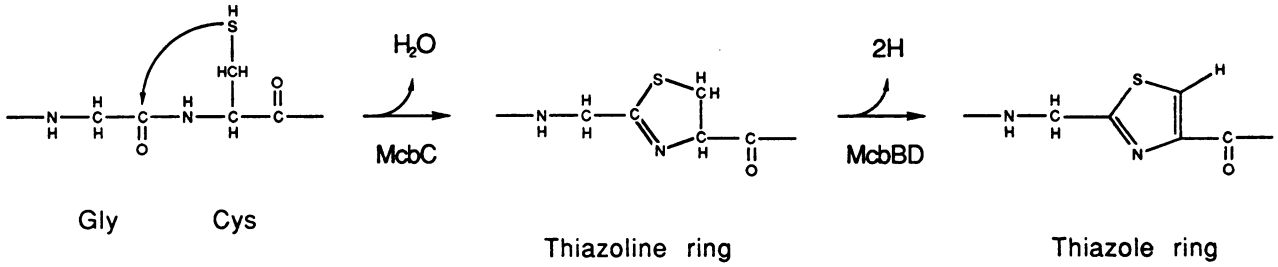

FIG. 4. Proposed two-step pathway for formation of a thiazole ring from a Gly-Cys dipeptide. Formation of oxazoline rings is also postulated to be catalyzed by McbC. This step, however, may require a different enzyme and could therefore be catalyzed by McbB or McbD, in which case the remaining protein (McbB or -D) could catalyze the dehydrogenation. Formation of a single thiazole (or oxazole) ring results in the loss of 20 mass units (4 H, 1 O).

and very compelling observation. McbC, one of the MccB17-modifying enzymes, shows extensive amino acid sequence similarity with a protein, TfxB (20), which is required for synthesis of a thiazoline-containing peptide antibiotic, trifolitoxin (21). Trifolitoxin is also ribosomally synthesized and the thiazoline ring is generated posttranslationally from a Gly-Cys residue pair. The McbC/TfxB homology provides support for the proposed modifications in MccB17 and suggests a pathway for their biosynthesis. We propose that McbC catalyzes the initial cyclic condensation to yield thiazoline and that McbB and/or McbD then catalyzes the subsequent dehydrogenation of the α,β C-C bond. The formation of oxazoline intermediates could also be catalyzed by McbC or by McbB or McbD, in which case the remaining protein (McbB or -D) could catalyze the dehydrogenation. Computer-assisted searches of protein sequence data bases with the amino acid sequence of McbB and McbD yielded no significant sequence similarities with any other proteins.

Further support for the proposed modifications of MccB17 came from mass spectrometry and amino acid analysis of a MccB17 mutant peptide, S39N, in which one of the modified serine residues (S39) is replaced with an asparagine residue. The total mass of the S39N peptide was 47 mass units higher than wild-type MccB17, corresponding precisely to replacing serine with asparagine (+27) and blocking the formation of one oxazole ring (+20) (Fig. 3). In addition, amino acid analysis of S39N showed the presence of one additional glycine, which is predicted if the formation of a single oxazole ring is blocked. Not surprisingly, the S39N substitution results in a >99% reduction in antibiotic activity. This suggests that the modification is essential for activity.

The presence of thiazole and oxazole rings in MccB17 has several implications. First, thiazole or oxazole rings are unprecedented in ribosomally synthesized polypeptides. In addition, although thiazole and oxazole rings have been known in natural products for decades, here we report the identification of enzymes involved in the biosynthesis of these rings. As a result, MccB17 and its modifying enzymes, McbBCD, provide an excellent system in which to characterize the pathway and enzymology of thiazole and oxazole ring biosynthesis and to identify the structural signals that target a peptide or protein for modification. Since posttranslational modifications can greatly enhance the structural diversity of peptides or proteins, they offer promising possibilities for protein engineering (22).

The presence of thiazole and oxazole rings in MccB17 indicates that it is structurally unrelated to the two major classes of DNA gyrase inhibitors, the quinolones and the coumarins (Fig. 5) (23). In addition, mutations that confer resistance to MccB17 (5) fall in *gyrB* near the C-terminal end of the gyrase B subunit and are clearly distinct from the quinolone and coumarin resistance-determining regions. These results suggest that the molecular mechanism of gyrase inhibition by MccB17 may be different, even though the outcome of MccB17 action closely resembles that of the quinolones, functioning as a poison and generating double-stranded breaks in DNA (5, 23). Thus, the elucidation of the structure of the MccB17 modifications may provide the foundation for development of additional poisons of DNA gyrase and the closely related eukaryotic type II topoisomerases (24). The clinical efficacy of antimicrobial agents and anticancer drugs that target type II topoisomerases illustrates the usefulness of drugs with this target (23, 24).

A possible clue regarding the role of the thiazole and oxazole rings in MccB17 function comes from studies of the anticancer drug bleomycin, a non-ribosomally synthesized peptide antibiotic that contains adjacent thiazole rings. Bleomycin is not a gyrase inhibitor but binds directly to DNA and generates double-stranded breaks (19). The adjacent thiazole rings have been shown to be involved directly in facilitating its binding to DNA (19). Given that the target of MccB17 is DNA gyrase, it seems reasonable to postulate that the thiazole/oxazole rings may also facilitate interaction of MccB17 with DNA either prior to interaction with gyrase or as part of its mechanism of gyrase inhibition.

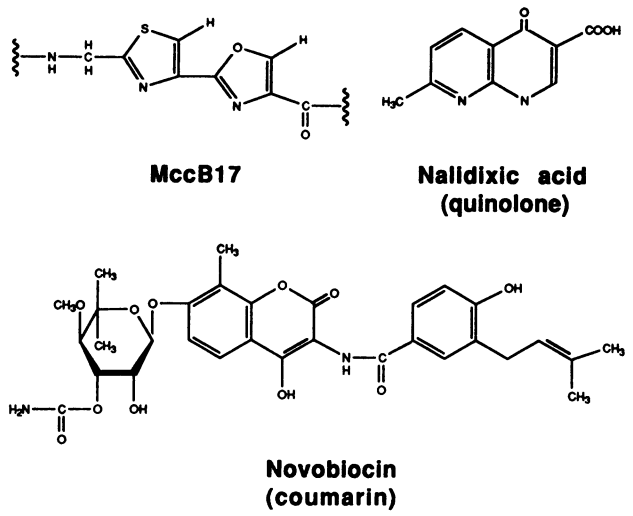

FIG. 5. MccB17 modifications compared to the two major structural classes of DNA gyrase inhibitors.

Finally, we discovered that *E. coli* mutants that lack the MccB17 receptor SbmA are not only resistant to MccB17 (13) but also show increased resistance to bleomycin. This suggests that thiazoles/oxazoles might be the key structural feature recognized by SbmA. This is particularly intriguing because homologs of SbmA are conserved in a wide variety of bacteria, including Gram-negative and Gram-positive species, plant symbionts, plant and animal pathogens, and free-living bacteria (25). In *Rhizobium meliloti* the SbmA homolog (BacA) is essential for proper bacteroid development during the establishment of symbiotic nitrogen-fixing nodules in plant roots (25). There is precedent for the involvement of thiazole-containing plant compounds in plant–microbe interactions, among them some phytoalexins whose synthesis is induced in response to wounding or microbial attack (26, 27). These results suggest that thiazole- or oxazole-containing compounds may serve as signaling molecules for bacteria in a wide variety of environments, including interactions with plant and animal hosts.

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