

Bactobolin Resistance Is Conferred by Mutations in the L2 Ribosomal Protein

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ABSTRACT *Burkholderia thailandensis* produces a family of polyketide-peptide molecules called bactobolins, some of which are potent antibiotics. We found that growth of *B. thailandensis* at 30°C versus that at 37°C resulted in increased production of bactobolins. We purified the three most abundant bactobolins and determined their activities against a battery of bacteria and mouse fibroblasts. Two of the three compounds showed strong activities against both bacteria and fibroblasts. The third analog was much less potent in both assays. These results suggested that the target of bactobolins might be conserved across bacteria and mammalian cells. To learn about the mechanism of bactobolin activity, we isolated four spontaneous bactobolin-resistant *Bacillus subtilis* mutants. We used genomic sequencing technology to show that each of the four resistant variants had mutations in *rplB*, which codes for the 50S ribosome-associated L2 protein. Ectopic expression of a mutant *rplB* gene in wild-type *B. subtilis* conferred bactobolin resistance. Finally, the L2 mutations did not confer resistance to other antibiotics known to interfere with ribosome function. Our data indicate that bactobolins target the L2 protein or a nearby site and that this is not the target of other antibiotics. We presume that the mammalian target of bactobolins involves the eukaryotic homolog of L2 (L8e).

IMPORTANCE Currently available antibiotics target surprisingly few cellular functions, and there is a need to identify novel antibiotic targets. We have been interested in the *Burkholderia thailandensis* bactobolins, and we sought to learn about the target of bactobolin activity by mapping spontaneous resistance mutations in the bactobolin-sensitive *Bacillus subtilis*. Our results indicate that the bactobolin target is the 50S ribosome-associated L2 protein or a region of the ribosome affected by L2. Bactobolin-resistant mutants are not resistant to other known ribosome inhibitors. Our evidence indicates that bactobolins interact with a novel antibiotic target.

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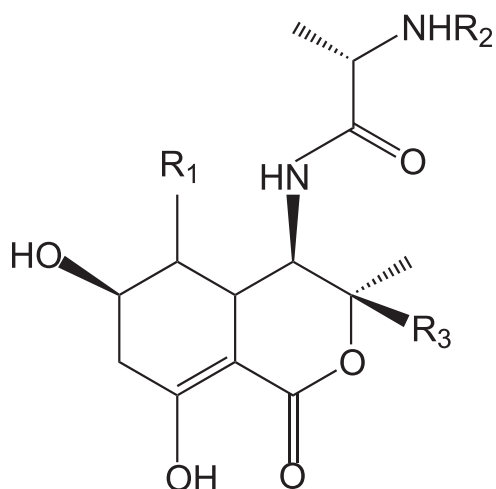
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The saprophyte *Burkholderia thailandensis* has a large genome with at least a dozen gene clusters with predicted polyketide synthase (PKS) and/or nonribosomal peptide synthetase (NRPS) genes (for a detailed list, see reference 1). One of the PKS/NRPS hybrid clusters is involved in the production of eight identified bactobolin compounds (Fig. 1). Some of the bactobolins are potent antibiotics (2, 3). These water-soluble compounds consist of a C₆-polyketide fused to a chlorinated hydroxy-valine residue. Bactobolins A to C were first characterized in the late 1970s as potent, broad-spectrum antibiotics produced by a poorly described pseudomonad. Preparations were toxic to mammalian cells, and perhaps because of this, the interest in bactobolins waned (3–5). We recently discovered five new bactobolins (D to H) and a cluster of genes involved in bactobolin biosynthesis (the genes are BTH_II1222 to BTH_II1242 from genomic coordinates 1445675 to 1482269; see <http://www.burkholderia.com>), which is reasonably well conserved in the related species *Burkholderia pseudomallei* (2, 3, 6). We also showed that bactobolin production is dependent on one of the three acyl-homoserine lactone (acyl-

HSL) quorum-sensing circuits in *B. thailandensis*, the BtaI2–BtaR2–N-3-hydroxydecanoyl HSL (3OHC₁₀-HSL) circuit (6).

The broad-spectrum activities of bactobolins suggest they may have a conserved target (2, 3), and results from one study indicated that bactobolins inhibit protein synthesis (7), as is true for a number of other antibiotics. Although the 50S and 30S subunits of the bacterial 70S ribosome contain 20 and 34 proteins, respectively, the subunits consist predominantly of RNA, and the RNAs serve as the target of most ribosome-inhibiting antibiotics. Antibiotic activity most often occurs at one of three key sites: the codon-anticodon recognition site (A site) on the 30S subunit, the peptidyl-transferase center (PTC) on the 50S subunit, or the peptide exit tunnel on the 50S subunit. Aminoglycoside antibiotics interfere with codon recognition and specificity at the A site. Antibiotics such as chloramphenicol, clindamycin, and linezolid interfere with peptide bond formation at the PTC. Macrolides such as erythromycin block elongation of the growing peptide chain at the peptide exit tunnel (for recent reviews, see references 8 and 9). Several antibiotics can bind the same or an overlapping site, and



Bactobolins

A: R ₁ =OH, R ₂ =H,	R ₃ =CHCl ₂
B: R ₁ =OH, R ₂ =L-Ala,	R ₃ =CHCl ₂
C: R ₁ =H, R ₂ =H,	R ₃ =CHCl ₂
D: R ₁ =H, R ₂ =L-Ala,	R ₃ =CHCl ₂
E: R ₁ =OH, R ₂ =L-Ala-L-Ala,	R ₃ =CHCl ₂
F: R ₁ =H, R ₂ =L-Ala-L-Ala,	R ₃ =CHCl ₂
G: R ₁ =H, R ₂ =H,	R ₃ =CHCl
H: R ₁ =H, R ₂ =L-Ala,	R ₃ =CHCl

FIG 1 Bactobolin structures. Bactobolins A and C vary from B and D at the R₂ position.

consequently changes in a single residue can affect susceptibility to many different antibiotics (10, 11).

To better understand the mechanism of action of the bactobolin antibiotics, we developed conditions to improve bactobolin production by *B. thailandensis*. We purified the most abundant bactobolins and showed they have a broad spectrum of activity that includes bacteria and mouse cells. We then mapped *Bacillus subtilis* bactobolin resistance mutations to a specific region of a 50S ribosomal subunit protein called L2. Our evidence supports the view that the L2 protein is a bactobolin target or directly modifies the target on the ribosome.

RESULTS

Bactobolin production is temperature regulated. We found that, due to bactobolins, fluid from *B. thailandensis* cultures grown at 30°C inhibited growth of *Escherichia coli* in a filter disk assay, whereas fluid from 37°C cultures did not (Fig. 2, top). We posited that the higher activity at 30°C could be because bactobolins are heat sensitive or because production is higher at 30°C. To discriminate between these two possibilities, we grew *B. thailandensis* at 30°C, removed cells by filtration, incubated the culture fluid at temperatures ranging from 30°C to 80°C for 2 h, and then assessed antimicrobial activity by using the *E. coli* filter disk assay. We found that antimicrobial activity in filtered culture fluid was stable up to 60°C (Fig. 2, bottom). Thus, we concluded that production of bactobolins is higher at 30°C than it is at 37°C.

We then used a *B. thailandensis* strain with a chromosomally encoded *lacZ* gene fused to the bactobolin synthesis gene *btaK* (6)

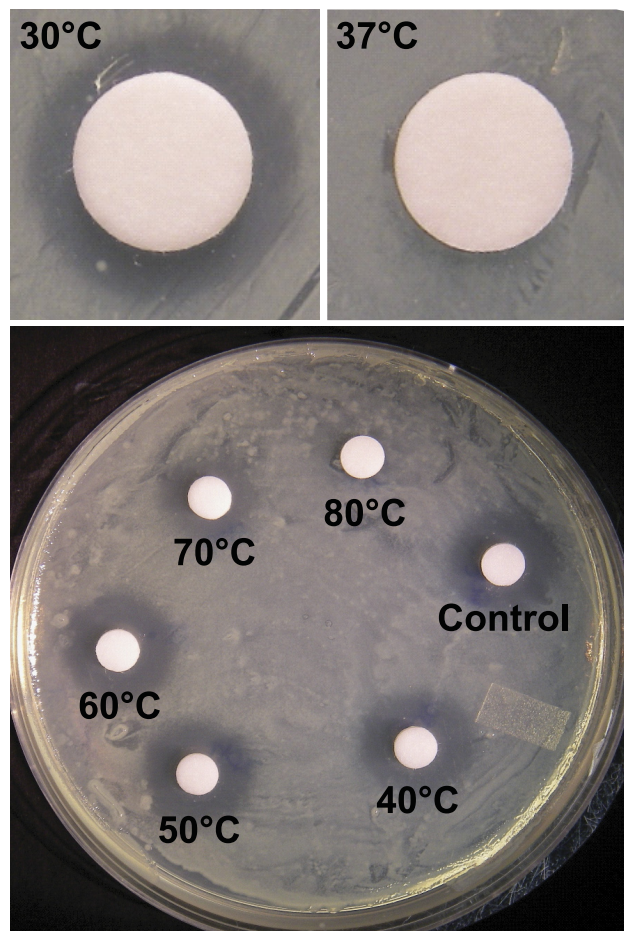


FIG 2 Susceptibility of *E. coli* to antibiotics in *B. thailandensis* culture fluid (top). Paper filter disks were saturated with filtered fluid from *B. thailandensis* cultures grown at 30°C or 37°C, and the filters were placed on a growing lawn of *E. coli* DH10B (bottom). Fluid from *B. thailandensis* cultures grown at 30°C was treated for 2 h at the range of temperatures indicated prior to being applied to the filter disks.

to assess whether enhanced production of bactobolins at 30°C might result from enhanced transcription of a bactobolin synthesis gene(s). The β -galactosidase reporter activity peaked in early stationary-phase cells grown at either 30°C or 37°C, and it was 2- to 3-fold higher at 30°C than at 37°C (Fig. 3). Consistent with a previous study, *btaK* expression was greatly reduced in a *btaR2* quorum-sensing receptor mutant (6). We conclude that bactobolin production is greater at 30°C than at 37°C as a result of enhanced transcription of at least one of the bactobolin biosynthesis genes at 30°C.

Bactobolins A and C are potent and have broad-spectrum activity. We determined the MICs of purified bactobolins A, B, and C for a diverse set of pathogenic and environmental bacteria (Table 1). We found that both bactobolin A and bactobolin C were particularly active against many Gram-positive and *Proteobacteria* species, including vancomycin-intermediate resistant *Staphylococcus aureus* isolates (VISA) (bactobolin A MIC, 3 μ g/ml). Bactobolin A was generally more potent than bactobolin C. We also tested bactobolin B and found it had relatively little or no measurable activity against the bacteria we tested (Table 1). Several species, including two close relatives of *B. thailandensis*, *B. pseu-*

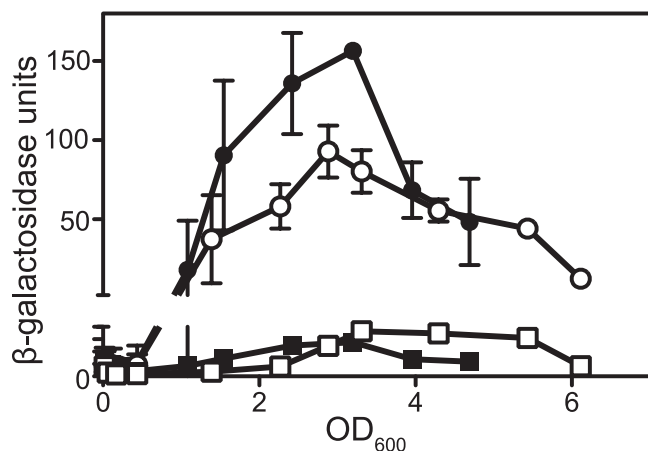


FIG 3 Temperature-dependent expression of *btaK* and acyl-HSLs. Closed symbols and bars represent cultures grown at 30°C, and open symbols and bars represent cultures grown at 37°C. Expression of a chromosomal *btaK-lacZ* reporter in the wild type (circles) or the *btaR2* mutant (squares) is shown. The data are the means for three biological replicates, and the error is the range.

domallei and *Burkholderia mallei*, were not susceptible to bactobolins. There may be a conserved resistance mechanism in *B. thailandensis*, *B. pseudomallei*, and *B. mallei*. Of note, *B. mallei* has lost 120 kb of DNA containing, among many other open read-

ing frames, the bactobolin biosynthetic gene locus. Thus, the resistance of these three species may not be encoded within the bactobolin gene locus or adjacent DNA.

Previous studies have shown that bactobolin preparations are toxic to mammalian cells (7). Because these preparations may have included more than one of the eight bactobolins produced by *B. thailandensis*, we assessed the cytotoxicities of purified bactobolins A, B, and C. We also assessed the toxicity of bactobolin D, a compound we only recently discovered (3). The concentration of bactobolin A required to cause a 50% decrease in viability, the ID_{50} , of cultured mouse NIH 3T3 fibroblast cells was 0.6 $\mu\text{g/ml}$, comparable to findings in a previous study (7). The ID_{50} of bactobolin C was similar (0.7 $\mu\text{g/ml}$). Bactobolins B and D were less cytotoxic (ID_{50} s, 1.5 and 1.7 $\mu\text{g/ml}$, respectively). Thus, for bactobolins A to C, cytotoxicity correlates with antibacterial activity (Table 1). The more active bactobolins A and C do not have an alanine at the R_2 position (Fig. 1). We suspect that alanine at the R_2 position may reduce the bioactivity of bactobolins. The correlation of activities with mouse cells and bacterial cells indicates that the target of bactobolin activity is conserved in biological systems.

Isolation of spontaneous bactobolin-resistant *B. subtilis* mutants. Our approach toward identification of the cellular target of bactobolins was to isolate spontaneous bactobolin-resistant *Bacillus subtilis* mutants and then define the mutations leading to resistance. We chose *B. subtilis* 3610 for this analysis because it is

TABLE 1 Antimicrobial activities of bactobolins

Species and strain	MIC ($\mu\text{g per ml}$) ^a			Reference or source
	A	B	C	
<i>Bacillus cereus</i> ATCC 14579	13	ND	38 (13)	ATCC ^b
<i>Bacillus subtilis</i> 3610 ^c	0.39	12.5	1.56	ATCC
<i>Burkholderia cenocepacia</i> K56-2	50	ND	>100	56
<i>Burkholderia kururiensis</i> M130	13	ND	50	Brazil ^d
<i>Burkholderia mallei</i> ATCC 23344	100	ND	ND	57
<i>Burkholderia pseudomallei</i> 1026b	>100	ND	ND	58
<i>Burkholderia pseudomallei</i> 1258b	>100	ND	ND	Thailand ^d
<i>Burkholderia pseudomallei</i> E0274	>100	ND	ND	Thailand ^d
<i>Burkholderia vietnamiensis</i> G4	0.78	ND	6	59
<i>Candida krusei</i> ATCC 14243	>100	ND	>100	60
<i>Chromobacterium violaceum</i> CV017	2 (0.8)	ND	13	61
<i>Escherichia coli</i> 100110	38 (13)	ND	ND	UW ^d
<i>Escherichia coli</i> 090428	50	ND	ND	UW ^{d,e}
<i>Flavobacterium johnsoniae</i> CI04	6	ND	25	62
<i>Haemophilus influenzae</i> ATCC 10211	>100	ND	>100	ATCC
<i>Klebsiella pneumoniae</i>	19 (6)	>100	>100	63
<i>Mycobacterium marinum</i> BAA535	6	>100	50	64
<i>Proteus mirabilis</i> HI4320	>100	>100	>100	65
<i>Pseudomonas aeruginosa</i> PA14	50	>100	>100	66
<i>Pseudomonas aeruginosa</i> ATCC 25873	100	>100	>100	ATCC
<i>Pseudomonas fluorescens</i> 2-79	0.19	9.3(3)	2	67
<i>Ralstonia pickettii</i> ATCC 27511	2	ND	19 (6)	68
<i>Salmonella enterica</i> serovar Typhimurium ATCC 14028s	25	ND	100	ATCC
<i>Staphylococcus aureus</i> (VISA) T27857	3	ND	ND	UW ^d
<i>Staphylococcus aureus</i> (VISA) M32276	2	ND	ND	UW ^d
<i>Staphylococcus aureus</i> (VISA) T57502	3	ND	ND	UW ^d
<i>Streptococcus pyogenes</i> MGAS5005	0.6 (0.2)	ND	2	69

^a MICs for bactobolins A, B, and C were determined with two independent experiments. The range is indicated in parentheses.

^b American Type Culture Collection.

^c Data previously reported (3).

^d Environmental isolate from Brazil, NCBI GenBank database, accession number AJ238360, 1999; human infection (1258b) or environmental (E0274) isolate from the Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand (unpublished); human infection isolates from the UW Medical Center Clinical Microbiology Laboratory.

^e Imipenem resistant.

TABLE 2 Susceptibilities of *B. subtilis* bactobolin-resistant mutants to bactobolin A, bactobolin B, and other antibiotics

Mutation ^b	MIC ($\mu\text{g per ml}$) ^a						
	Bact A	Bact B	Cm	Clin	Ery	Kan	Lin
None	3 (1)	50	7 (5)	4 (2)	0.7 (0.6)	2 (1)	2 (3)
E236A	17 (6)	>200	5 (2)	3 (3)	0.3 (0.1)	2 (1)	1 (1)
235G ^c	100	>200	3 (1)	2 (1)	0.3 (0.3)	2 (1)	1 (0.2)
E236Q	21 (6)	>200	5 (2)	4 (3)	0.3 (0.1)	2 (1)	1 (1)

^a MICs were determined with three independent experiments, and the range is indicated in parentheses. Antibiotics are bactobolin A (Bact A), bactobolin B (Bact B), chloramphenicol (Cm), clindamycin (Clin), erythromycin (Ery), kanamycin (Kan), and linezolid (Lin).

^b Strains shown are wild-type *B. subtilis* 3610 and the bactobolin-resistant mutants of 3610 with the indicated amino acid changes in L2 (encoded by *rplB*), corresponding with the following base changes (in order of appearance in the table): A708C, 706GGT, and G706C.

^c Two 235G variants were initially identified, and results with the two were identical.

particularly sensitive to bactobolin A and bactobolin C (Table 1), and because in comparison to sensitive members of the *Proteobacteria*, for example, it seemed less likely that there would be a spontaneous bactobolin permeability mutation rather than a target mutation. We spread *B. subtilis* cells on LB (Luria-Bertani) agar containing fluid from 30°C-grown *B. thailandensis* cultures (about 7.5 μl culture fluid per ml LB agar and about 10^8 cells per plate, as described in Materials and Methods). Colonies arose after 2 days at 37°C at a frequency of about 3×10^{-9} cells. We isolated four mutants by streaking on LB agar containing *B. thailandensis* culture fluid for further study. We compared the bactobolin A MICs for each mutant to that of the parent. The MIC for the parent was 3 $\mu\text{g per ml}$, and the four mutant MICs were 17, 21, 100, and 100 $\mu\text{g per ml}$ (Table 2).

Identification of mutations in bactobolin-resistant *B. subtilis* mutants. As a first approach, we performed a shotgun sequence analysis by using an Illumina sequencing platform. We identified single mutations in two of the four isolates but none in the two isolates with the highest levels of resistance. The mutations we identified were both *rplB* (BSU1190) missense mutations (Table 2) (E236A and E236Q). The *rplB* product is the 50S ribosome-associated protein L2. Because two of the four bactobolin-resistant mutants had *rplB* mutations, we reasoned that there might be *rplB* mutations in the other two mutants that were not detected by using the Illumina sequencing platform. Thus, we PCR amplified and sequenced *rplB* from each of our four mutants. In the first two, mutations coding for E236A and E236Q were confirmed, and no other mutations were identified. In the remaining two, we identified an identical 3-bp insertion (Table 2) (coding for 235G). These insertion mutations were overlooked by our whole-genome sequencing method, likely due to the stringency of the alignment method used (12). The insertion mutation resulted in a higher level of resistance to bactobolin A than did either of the base substitution mutations (Table 2). We were unable to sufficiently assess resistance to the other six bactobolins due to their weak activities (2, 3). All four mutants showed a small (<10%) reduction in growth rate in LB broth relative to that of their parent (data not shown), suggesting there is a cost associated with harboring these bactobolin resistance mutations.

To determine whether expression of a mutant L2 protein alone can confer bactobolin resistance, we placed the L2 235G gene behind a *lac* promoter and moved it into a neutral site (*amyE*) in

TABLE 3 Activities of bactobolin A against engineered *B. subtilis* strains

Strain ^b	MIC ($\mu\text{g per ml}$) ^a	
	–IPTG	+IPTG ^c
<i>B. subtilis</i> JH624	3	2 (1)
<i>B. subtilis</i> JH624 <i>spec</i>	3	4 (2)
<i>B. subtilis</i> JH624 L2 <i>spec</i>	6	6
<i>B. subtilis</i> JH624 L2 ^{235G} <i>spec</i>	4 (1)	17 (6)

^a MICs are the means for three independent experiments, with the ranges indicated in parentheses.

^b We used the naturally competent *B. subtilis* JH642 and JH642 derivatives with a chromosomal copy of a spectinomycin-resistant gene (*spec*), with *spec* plus an IPTG-inducible wild-type *rplB* gene (L2 *spec*), or with *spec* plus a mutant *rplB* gene encoding L2^{235G} (L2^{235G} *spec*).

^c IPTG was at a 1 mM concentration.

B. subtilis JH642. We then compared the MICs of this strain grown with or without isopropyl- β -D-thiogalactopyranoside (IPTG) induction of the *lac*-promoter-controlled (235G) *rplB* mutant with the MIC of a control (*B. subtilis* JH642 with a wild-type *lac*-promoter-controlled *rplB* gene). For the control, the MIC was similar to that of the parent grown with or without IPTG. The strain with the *lac*-promoter-controlled 235G gene showed a substantially increased MIC when grown in the presence of IPTG (Table 3). The resistance was intermediate between those of susceptible strains and the 235G isolate. This was presumably because our engineered strain contains two copies of *rplB*, a wild type and a mutant gene.

Bactobolin-resistant mutants remain susceptible to other antibiotics that target ribosomes. Many antibiotics target the ribosome, but we are not aware of any role of L2 in conferring resistance to antibiotics that interfere with protein synthesis. Nevertheless, it may be that the *rplB* mutations we selected confer resistance to other antibiotics that target the ribosome. Thus, we tested the effects of several ribosome-inhibiting antibiotics on *B. subtilis* 3610 and on strains carrying the three different *rplB* mutations conferring bactobolin resistance we identified (Table 2). The antibiotics tested were kanamycin, which targets the A site of the 30S subunit, chloramphenicol, clindamycin and linezolid, which target the peptidyl transferase center of the 50S subunit, and erythromycin, which targets the peptide exit tunnel of the 50S subunit (8). The sensitivities of *B. subtilis* to these antibiotics were not altered appreciably in the L2 mutants. If anything, the mutants were slightly more sensitive to some of the antibiotics. These results support the view that the site of bactobolin interaction is specific for this group of ribosome inhibitors.

DISCUSSION

Our results show that among the bactobolins produced by *B. thailandensis*, bactobolin A and bactobolin C are potent broad-spectrum antibiotics (Table 1). By isolating and studying spontaneous *B. subtilis* bactobolin-resistant mutants, we provide evidence that resistance to bactobolins occurs through the ribosome 50S subunit L2 protein, the product of *rplB*. The L2 protein is quite conserved (Fig. 4), and there is in fact a homolog in the 50S subunit of the eukaryotic ribosome called L8e. The resistance mutations we found map to a particularly well-conserved region of the protein. The conservation of L2 (L8e) may account for the broad-spectrum activity exhibited by bactobolin A and bactobolin C. We imagine that the conserved eukaryotic L8e protein is in-

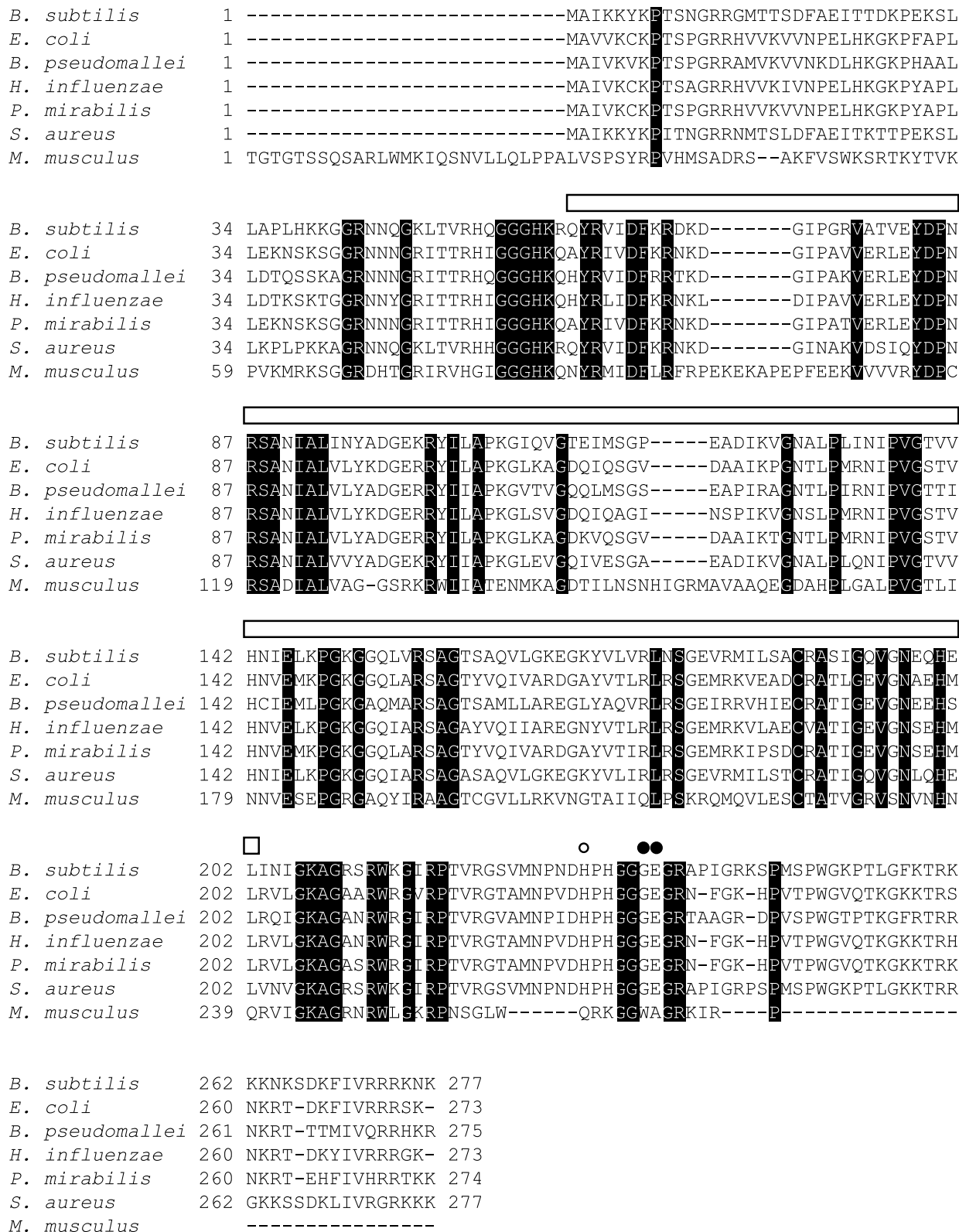


FIG 4 Multiple alignments of L2 proteins. Conserved amino acids are in black. The bar above the residues corresponding to *B. subtilis* L2 61 to 202 represents the central RNA-binding domain (21). The open circle above *B. subtilis* L2 H230 (previously reported as H229) indicates a residue known to be essential for PTC (peptidyl-transferase center) function (23, 27). The filled circles above *B. subtilis* G235 and E236 indicate the residues corresponding with the mutations identified in this study (235G, E236A, and E236Q) (Table 2). The L2 sequences of *B. pseudomallei* and *B. thailandensis* were identical. The aligned sequences are from *B. subtilis* 3610, *E. coli* K-12, *B. pseudomallei* K96243, *H. influenzae* 6P18H1, *P. mirabilis* HI4320, *S. aureus* COL (methicillin-resistant *S. aureus* [MRSA]), and *Mus musculus* mitochondria.

volved in the observed cytotoxic activity in eukaryotes. The fact that potencies of bactobolins A to C in fibroblasts correlate with potency in bacteria is consistent with the idea that L8e plays a role in the eukaryotic activity of bactobolins.

Based on sequence conservation and its importance in the ribosome, L2 is thought to be one of the most evolutionarily ancient ribosome-associated proteins (13). L2 is required for the association between the 50S and 30S ribosomal subunits and for full activity of the PTC (14). In fact, L2 is one of only a few proteins that are necessary for elongation of peptides (15–18). L2 is comprised of a central RNA-binding domain (RBD) (residues 61 to 202) (Fig. 4), which localizes to the outside of the ribosome, and flexible N and C termini that extend into the ribosome and are proximal to the PTC (19–22). The central RBD interacts with domain IV of the 23S rRNA and mediates contacts between the 50S and 30S subunits (18, 23–26). The C terminus of L2, in particular residue H230 (reported previously as H229), is critical for peptidyl transferase activity (27). This region is thought to stabilize the PTC-containing domain V of the 23S rRNA, allowing movement of tRNAs through the ribosome (23, 27). The bactobolin-resistant mutations mapped just in this region (Table 2; Fig. 4). The association of this L2 region with the PTC suggests that the bactobolins may inhibit peptidyl transfer specifically. The activities of other antibiotics that target the PTC are not altered by the L2 mutations (Table 2). In fact, relatively few ribosomal proteins have known roles in antibiotic resistance, and these include L4, L6, L11, L16, L22, S5, S12, and S17 (28–37). Although in a few cases the antibiotics may interact directly with a ribosomal protein (38, 39), it is common that the mutated protein alters an rRNA target (40–43). For example, mutations in the S12 ribosomal protein confer resistance to streptomycin by causing structural changes in its rRNA target (40). Most antibiotics target rRNAs (44). Proteins comprise much less total ribosome surface area than rRNAs, and they are not directly involved in the catalytic functions. In the case of bactobolin, direct interaction could be with L2 itself or an rRNA affected by L2. Our results support the view that the interaction site is novel because resistance mutations map to L2 and because the mutations do not confer resistance to other classes of antibiotics that target the ribosome.

Although bactobolin A and bactobolin C are potent antibiotics, they are also equally toxic to mouse fibroblast cells. This mammalian toxicity excludes development of these molecules as antibacterial therapeutics. Nevertheless, because of our identification of a specific ribosomal protein as a target of bactobolins coupled with our developing understanding of bactobolin genetics and chemistry, these molecules or derivatives of these molecules have potential uses in biology and in applications. Bactobolins may be of use in efforts to better understand ribosome function. Although it may not be possible to develop bactobolin derivatives that specifically target bacterial ribosomes, the bactobolins may also find utility if they can be targeted to specific cells, for example, tumor cells, via developing technologies.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and reagents. We used *B. thailandensis* strain E264 (45) and *E. coli* DH10B for genetic manipulations. Our *B. thailandensis* *btaK-lacZ* chromosomal insertion mutant (*btaK126::ISlacZ/hah-TC*) was from a sequence-defined transposon mutant library (L. Gallagher and C. Manoil, unpublished). This mutant has a transposon insertion in the coding sequence of *btaK* after bp +2237 relative to the

predicted *btaK* translational start site. We obtained bactobolin-resistant mutants of *B. subtilis* 3610 (46) as described. We used the easily transformed *B. subtilis* JH642 (47) to create *rplB* diploids. Other bacteria are listed in Table 1 or described in the text.

Bacteria were grown in tryptic soy broth (TSB), cation-adjusted Mueller-Hinton broth (MHB), or morpholinepropanesulfonic acid (MOPS)-buffered LB broth or, for one experiment, starch agar (33 g tryptose blood agar base and 10 g Argo pure corn starch per liter). When appropriate, the following antibiotics were used (per ml): 100 μ g trimethoprim and spectinomycin at 50 μ g (*E. coli*) or 100 μ g (*B. subtilis*). We added isopropyl- β -D-thiogalactopyranoside (IPTG) (1 mM) as appropriate. Bactobolins were isolated from *B. thailandensis* culture fluid as described elsewhere (2, 3).

We measured β -galactosidase activity with a Tropix Galacto-Light Plus chemiluminescence kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Genomic DNA, PCR and DNA fragments, and plasmid DNA were purified using the DNeasy blood and tissue kit, PCR or plasmid purification kits, or gel extraction kit (Qiagen) according to the manufacturer's protocol.

Genetic manipulations. We used standard procedures for DNA manipulations (48). To assess *btaK-lacZ* activity in a *btaR2* mutant background, an unmarked, in-frame *btaR2* deletion was introduced into the *btaK-lacZ* reporter strain by using the deletion construct pJRC115 *btaR2* and methods described previously (49). For ectopic expression of L2 and L2^{235G} in *B. subtilis*, the genes were placed under control of the IPTG-inducible promoter on pDR111 (50), and the resulting plasmids were integrated into the nonessential *B. subtilis* *amyE* locus. To clone pDR111 expressing each L2 allele, the genes were amplified from genomic DNA isolated from *B. subtilis* 3610 or the 235G variant, respectively, by using primers that incorporated an upstream ribosome binding site, AAG GAGG (51), and restriction sites (SphI and NheI) into the product. These amplicons were cut with SphI and NheI and ligated to SphI-NheI-digested pDR111. In both cases, *rplB* was downstream of the pDR111-encoded $P_{\text{hyperspank}}$ IPTG-inducible promoter (50). The resulting constructs were used to transform the naturally competent *B. subtilis* JH624 by established methods (52). Transformants were selected on spectinomycin LB agar, and disruption of *amyE* was verified by lack of hydrolysis on starch agar.

MIC and cytotoxicity assays. The antimicrobial activities of purified bactobolins or bactobolin-containing *B. thailandensis* supernatant were assessed by using a MIC assay according to the 2003 guidelines of the Clinical and Laboratory Standards Institute (CLSI) and as described elsewhere (3). Fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 μ M minimal essential medium (MEM) with nonessential amino acids and incubated at 37°C with 5% CO₂. Briefly, microtiter dish wells were seeded with 10⁴ cells in 100 μ l medium and grown to 50% confluence (24 h). Cells were then treated with fresh bactobolin-containing medium. After 48 h, we determined cell viability with alamarBlue according to the manufacturer's instructions (Invitrogen). We determined the inhibitory dose causing 50% loss of viability (ID₅₀).

Screen for bactobolin-resistant mutants. We used *B. thailandensis* culture fluid as a source of bactobolin to isolate bactobolin-resistant *B. subtilis*. *B. thailandensis* was grown to stationary phase (optical density at 600 nm [OD₆₀₀] of 8 to 10) in LB broth, and the culture fluid was clarified by microcentrifugation and filtered through a 0.22- μ m-pore membrane. Filtered culture fluid was stored at 4°C for up to 1 month prior to use. LB agar plates were supplemented with the minimal concentration of culture fluid required to inhibit growth of a lawn of stationary-phase *B. subtilis* after 2 days. This varied with each batch of culture fluid but was approximately 7.5 μ l culture fluid per ml medium. Culture fluid-supplemented plates were spread with approximately 1 \times 10⁸ stationary-phase *B. subtilis* cells. Colonies that grew after 2 days were verified by streaking on fresh fluid-supplemented plates.

Illumina sequencing and analysis. Whole-genome resequencing of our wild-type strain, 3610, and its bactobolin-resistant *B. subtilis* deriva-

tives was as described previously (53). For each genome, a random-fragment library was constructed using a custom paired-end protocol. Briefly, genomic DNA (gDNA) samples were sheared by using a Bioruptor UCD-200 sonication device (Diagenode Inc. Denville, NJ) and end repaired using an End-It DNA end repair kit (Epicentre). Repaired fragments were subjected to A tailing using *Taq* DNA polymerase (Roche Inc., Chicago, IL). Custom “Y” adaptors (sequences available upon request) were added by using T4 DNA ligase (New England Biolabs, Beverly, MA). Libraries were size selected by using automated electrophoresis on a Pippen Prep system (Sage Science, Beverly, MA) and assessed for size range and concentration using a Qubit fluorometer (Invitrogen Inc., Carlsbad, CA) and a Bioanalyzer system (Agilent Inc., San Diego, CA). Sequencing was done on an Illumina GAIIX genome analyzer (Illumina, San Diego, CA) (paired-end 76-bp reads).

The Burrows-Wheeler Alignment software tool was used to align reads from sequencing (54). The sequence alignment of each variant was compared with sequence of a close relative of the parent strain, *B. subtilis* 168 (GenBank accession number CM000487), by using the SAMtools mpileup method (SourceForge). This yielded a list of single-nucleotide polymorphisms (SNPs). We used a custom script to identify each SNP as nonsynonymous or synonymous and as genic or intergenic. We narrowed the SNP list to changes that were present in a bactobolin-resistant variant but not in the resequenced 3610 parent. Remaining SNPs were verified by using the genome viewer IgV, version 1.5 (55). Mutations identified by this method were verified by sequencing PCR-amplified products.

L2 sequence alignments. Amino acid sequences of selected L2 proteins were obtained from the Integrated Microbial Genome Database (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) and aligned using the software tool Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) with default settings.

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