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Two Highly Similar Multidrug Transporters of *Bacillus subtilis* Whose Expression Is Differentially Regulated

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The *Bacillus subtilis* genome encodes two multidrug efflux transporters sharing 51% sequence identity: Bmr, described previously, and Blt, described here. Overexpression of either transporter in *B. subtilis* leads to a similar increase in resistance to ethidium bromide, rhodamine and acridine dyes, tetraphenylphosphonium, doxorubicin, and fluoroquinolone antibiotics. However, Blt differs widely from Bmr in its expression pattern. Under standard cultivation conditions, *B. subtilis* expresses Bmr but Blt expression is undetectable. We have previously shown that Bmr expression is regulated by BmrR, a member of the family of MerR-like transcriptional activators. Here we show that *blt* transcription is regulated by another member of the same family, BltR. The DNA-binding domains of BmrR and BltR are related, but their putative inducer-binding domains are dissimilar, suggesting that Bmr and Blt are expressed in response to different inducers. Indeed, rhodamine, a substrate of Bmr and Blt and a known inducer of Bmr expression, does not induce Blt expression. Blt expression has been observed only in *B. subtilis* carrying mutation *acfA*, which, as we show here, alters the sequence of the *blt* gene promoter. Unlike *bmr*, which is transcribed as a monocistronic mRNA, *blt* is cotranscribed with a downstream gene encoding a putative acetyltransferase. Overall, the differences in transcriptional control and operon organization between *bmr* and *blt* suggest that the transporters encoded by these genes have independent functions involving the transport of distinct physiological compounds.

Bacteria have developed a number of mechanisms protecting them from environmental toxins and antibiotics, one of the most widespread being the active efflux of the toxic compounds from cells. While some of the known drug efflux transporters, like the tetracycline efflux transporters, show high selectivity for particular toxins, there are many so-called multidrug transporters which are much less selective (8, 13, 14, 17, 18, 21, 26; reviewed in references 12 and 19). Among these is the *Bacillus subtilis* membrane protein Bmr (17), which is structurally similar to tetracycline transporters (25% sequence identity) but does not cause tetracycline efflux. Instead, Bmr causes the efflux of a variety of toxic substances, including such structurally diverse compounds as ethidium bromide, rhodamine and acridine dyes, tetraphenylphosphonium, puromycin, chloramphenicol, doxorubicin, and fluoroquinolone antibiotics (16, 17).

Overexpression of Bmr via intrachromosomal amplification of the *bmr* locus or its expression from a plasmid vector results in increased resistance of *B. subtilis* to all of these drugs, while inactivation of the *bmr* gene results in increased drug sensitivity. Expression of Bmr is regulated at the level of transcription by BmrR, a protein capable of binding the *bmr* promoter and encoded immediately downstream of the *bmr* gene (2). Interestingly, two of the compounds whose efflux is caused by Bmr, rhodamine 6G and tetraphenylphosphonium, bind BmrR, increase its affinity for the *bmr* promoter, and enhance Bmr expression (2). Neither of these compounds is physiologically relevant, however, and a natural inducer of Bmr expression has not been identified.

Neither the mechanism of recognition of dissimilar drugs by multidrug transporters nor the normal physiological functions of these proteins have been elucidated (see reviews in references 7, 12, and 19). In particular, it is not known whether multidrug transporters play solely a defensive role, protecting cells from diverse environmental toxins, or whether each is involved in the transport of a specific natural compound.

Here we describe a second multidrug transporter of *B. subtilis*. This protein, Blt (for Bmr-like transporter), is highly homologous to Bmr and is capable of causing the efflux of the same array of drugs. However, the expression pattern and the genetic surrounding of the *blt* gene differ widely from those of the *bmr* gene, suggesting that Bmr and Blt play distinct roles in bacterial physiology and are normally involved in the efflux of different natural substrates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals. *B. subtilis* BD170 (*trpC2 thr-5*), BD224 (*trpC2 thr-5 recE4*), and 168ACF (*acfA trpC2*) were obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus). *Escherichia coli* HB101 was obtained from Promega. All cells were cultivated in Luria Bertani (LB) medium at 37°C. Plasmids pBEV and pBMR2 have been described in references 16 and 18. *E. coli* expression vector pTrc99a was from Pharmacia. All chemicals were from Sigma Chemical Co., and enzymes for DNA manipulations were from Promega and New England Biolabs.

Drug resistance assay and drug accumulation measurements. The MIC of each drug was determined by growing *B. subtilis* strains in 96-well plates containing serial 1:1.5 dilutions of drugs in LB medium (inoculum, 2×10^5 logarithmic-phase cells per 140 μl in each well). Plates were incubated without lids in a humidified container for 12 h at 37°C, and the MIC of each drug was determined by examining medium turbidity.

BD224, BD224/pBEV, and BD224/pE1 cells in the logarithmic phase of growth were diluted to an optical density at 600 nm of 0.5 in LB medium and incubated with ethidium bromide (5 μg/ml) either in the absence or in the presence of reserpine (10 μg/ml). After 30 min at 37°C, cells from 1 ml of the incubation mixture were pelleted by centrifugation and resuspended in 2 ml of LB medium containing 10 μg of reserpine per ml and the amount of accumulated ethidium bromide was assessed by fluorimetry at an excitation wavelength of 530 nm and an emission wavelength of 600 nm.

Molecular biology techniques. Transformation of *B. subtilis* with plasmids and linear DNA fragments was performed as described in reference 5. Plasmids were isolated from logarithmic-phase *B. subtilis* by using the Wizard DNA purification

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system (Promega) with a slight modification: the cell resuspension solution was supplemented with lysozyme (1 mg/ml), and cells were incubated for 15 min at 37°C prior to alkaline lysis. Plasmids were sequenced with 18-mer synthetic oligonucleotide primers and a Sequenase 2.0 kit (United States Biochemical Corp.). Both regular and inosine sequencing reactions were performed. Sequencing of the chromosomal region of 168ACF cells (see Fig. 2B) was performed with the *fmol* DNA Sequencing System (Promega) after first amplifying this region by PCR with 168ACF DNA as the template. In parallel, the same region of the BD170 genome was sequenced by using the same techniques. Sequence comparisons with databases were performed first with the BLAST algorithm (3), available from the National Center for Biotechnology Information through the Internet, and then with the programs IMAGENE (Query Logic Inc., Morton Grove, Ill.) and MACAW (National Center for Biotechnology Information).

Cloning of the *blt* gene. Chromosomal DNA from *B. subtilis* BD170 was partially digested with *Sau*3A and ligated into expression vector pBEV, which had previously been cut with *Bam*HI. *B. subtilis* BD224 was transformed with this ligation mixture and plated on LB medium plates containing 5 μg of ethidium bromide per ml. A plasmid from one of the resultant clones, pE1, contained the *blt* gene, as well as other sequences (see Results). Plasmid pBLT, which contains only the *blt* gene under control of the pBEV promoter, was constructed by cloning the *Rsa*I-generated fragment of the pE1 insert (see Fig. 2A) into the *Bam*HI site of pBEV by using *Bam*HI linkers.

Disruption of the *blt* gene in the *B. subtilis* chromosome. Erythromycin resistance gene *emr* was amplified from transposon Tn*917* by PCR. The primers used were designed to introduce the *Sac*II and *Sph*I sites upstream and downstream of the gene, respectively. The *blt* gene was subcloned from pBLT into the *Bam*HI site of pBluescriptKS$^-$, from which the *Sac*II site had been eliminated by a cutting-filling-ligation reaction. By using this construct, the *emr* gene was then cloned between the *Sac*II and *Sph*I sites of *blt* (see Fig. 2A). The disrupted *blt* gene was then cut out from the resulting plasmid by *Bam*HI digestion and used for transformation of BD170 cells with selection for erythromycin resistance. Integration of the *emr* gene into the chromosomal *blt* gene was confirmed by PCR analysis of chromosomal DNAs isolated from selected clones by using primers upstream and downstream of the integration site.

Cloning of the *bltR* gene, its expression in *E. coli*, and gel mobility shift assay. To express BltR in *E. coli*, the *bltR* gene in pE1 was cloned into isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pTrc99a. Here, the *bltR* gene was amplified by PCR with pE1 as the template. The direct primer created an *Afl*III site, which included the first ATG codon of *bltR*, while the reverse primer created a *Bam*HI site downstream of the *bltR* coding sequence. After digestion with *Afl*III and *Bam*HI, the PCR product was cloned between the *Nco*I (compatible with *Afl*III) and *Bam*HI sites of pTrc99a. The absence of PCR-generated mutations in the cloned *bltR* gene was confirmed by sequencing. Expression of BltR in *E. coli* HB101 cells transformed with the resultant plasmid was induced by 1 mM IPTG and, after 2 h, reached ca. 1 to 3% of the total bacterial proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

For gel mobility shift assays, HB101 cells expressing BltR or control HB101 cells were incubated with IPTG for 2 to 3 h, collected by centrifugation, resuspended in lysis buffer (100 mM Tris-HCl, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 5% glycerol, 2 mM 2-mercaptoethanol, pH 7.5), and lysed in a French pressure cell (Aminco) at 10,000 lb/in^2. The lysate was cleared by centrifugation (10,000 × *g*, 15 min) and mixed with an equal volume of saturated ammonium sulfate. Precipitated proteins were redissolved in GSA buffer (10 mM Tris-HCl, 50 mM KCl, 5% glycerol, 50 μg of bovine serum albumin per ml, 1 mM cysteine, pH 7.5) to a final protein concentration of ca. 1 mg/ml. Six microliters of this protein solution, either neat or diluted further in GSA, was mixed with 1 μl of herring sperm DNA (200 μg/ml in GSA) and 1 μl of the DNA probe (ca. 1,000 cpm). The probe, a 46-mer oligonucleotide containing the *blt* promoter (see Fig. 6A), was prepared by a PCR in which one of the primers had been prelabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (23). In some experiments, rhodamine 6G was added to a final concentration of 20 μM. All mixtures were incubated on ice for 30 min prior to polyacrylamide gel electrophoresis, which was performed as previously described (2).

Northern (RNA) blot analysis. RNA was isolated from *B. subtilis* by following the previously described protocol for RNA isolation from gram-negative bacteria (6), with the exception that protoplasts were obtained by incubating cells with lysozyme at 10 mg/ml (10 min, 37°C) and the RNA was additionally purified by precipitation with 2 M LiCl. DNA probes labeled with ^{32}P were prepared from PCR products corresponding to the central regions of the *blt*, *bltD*, and *bmr* coding sequences by using the Prime-a-Gene labeling kit (Promega). Agarose gel electrophoresis of RNA, blotting, and hybridization were performed essentially as previously described (23).

Reverse transcription-PCR assay. RNA for reverse transcription-PCR assays was additionally purified by CsCl gradient centrifugation and contained no DNA, as determined by ethidium bromide staining of an agarose gel. To synthesize cDNA, 1 μg of RNA was annealed with 100 pmol of 18-mer oligonucleotide p1 (see Fig. 2A) in 20 μl of reaction buffer and the reaction was performed as described in reference 23 by addition of 10 U of reverse transcriptase (Seikagaku America, Rockville, Md.). In control reactions, 1 μg of DNase-free RNase A was added 15 min prior to the addition of reverse transcriptase. One microliter of each reaction mixture was then used as the template for a PCR in which oligonucleotides p1 and p2 or p1 and p3 (see Fig. 2A) were used as primers. PCR products were analyzed by agarose gel electrophoresis.

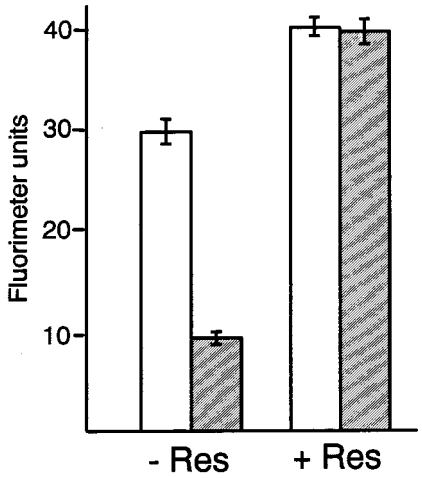

FIG. 1. Accumulation of ethidium bromide in *B. subtilis* BD224 (□) and BD224/pE1 (▨) cells in the absence or presence of reserpine (Res). Cells were incubated with ethidium bromide (5 μg/ml) with or without reserpine (10 μg/ml) for 30 min at 37°C, and then dye accumulation was assessed fluorimetrically.

Nucleotide sequence accession number. The sequence reported here (see Fig. 2A) has been submitted to GenBank and assigned accession no. L32599.

RESULTS

Identification of the *blt* gene. The gene for multidrug transporter Blt was isolated from a library of *B. subtilis* genomic fragments cloned into expression vector pBEV (18). *B. subtilis* BD224 (Rec$^-$) was transformed with this library and selected for growth on plates containing an inhibitory concentration of ethidium bromide, a toxic dye whose efflux is caused by Bmr. One of the clones selected, E1, demonstrated resistance not only to ethidium bromide but also to other known substrates of Bmr, namely, norfloxacin, rhodamine, and tetraphenylphosphonium. A plasmid isolated from this clone, pE1, when transformed into fresh BD224 cells, conferred the same multidrug resistance phenotype, indicating that pE1, and not the E1 chromosomal DNA, contained a resistance determinant.

Resistance was found to correlate with reduced drug accumulation. As Fig. 1 demonstrates, BD224/pE1 cells, when incubated with ethidium bromide, accumulated three times less of this toxic dye than did control BD224 cells. It is noteworthy that transformation of BD224 with vector pBEV alone did not change the accumulation of ethidium bromide (data not shown). When the incubation was conducted in the presence of reserpine, an inhibitor of Bmr-mediated drug efflux (1, 17), BD224/pE1 cells accumulated as much ethidium as did control cells (Fig. 1).

To test the possibility that pE1 leads to overexpression of the *bmr* gene, we performed Northern analysis, which indicated that the amount of *bmr*-specific RNA was not increased in BD224/pE1 cells (data not shown). We then sequenced the entire 3,723-bp pE1 insert. Sequence analysis revealed the presence of three complete, nonoverlapping open reading frames, each with an initiation codon preceded by a Shine-Dalgarno sequence (Fig. 2A). The putative protein product of one of the open reading frames showed strong homology (51% sequence identity) to Bmr (Fig. 3) and therefore was termed *blt*.

To test if the putative Blt transporter, alone, is responsible for the aforementioned multidrug resistance phenotype, we

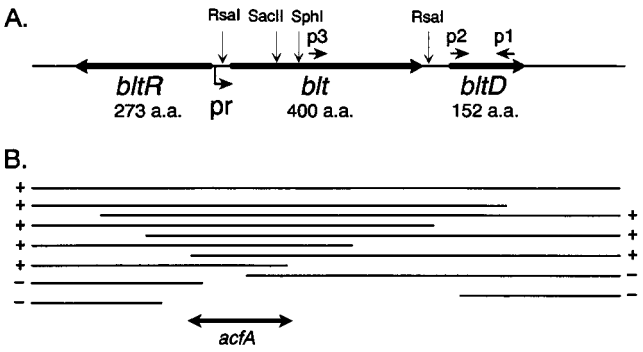

FIG. 2. (A) Organization of the open reading frames in the pE1 insert (3,723 bp). The lengths of the putative proteins encoded by the open reading frames, the putative *blt* promoter (pr), some restriction sites used for cloning, and primers p1, p2, and p3 are indicated. aa, amino acids. (B) Transfer of the *acfA* mutation by DNA fragments obtained by PCR amplification of the indicated regions of 168ACF chromosomal DNA. Competent *B. subtilis* BD170 bacteria were transformed with 1 μg of each of the PCR products and plated on 10 μg of ethidium bromide per ml. Plus signs indicate those products which yielded hundreds and thousands of colonies. Minus signs indicate products which yielded fewer than 10 colonies. The arrowed line at the bottom shows the conservative conclusion made as to the location of the *acfA* mutation.

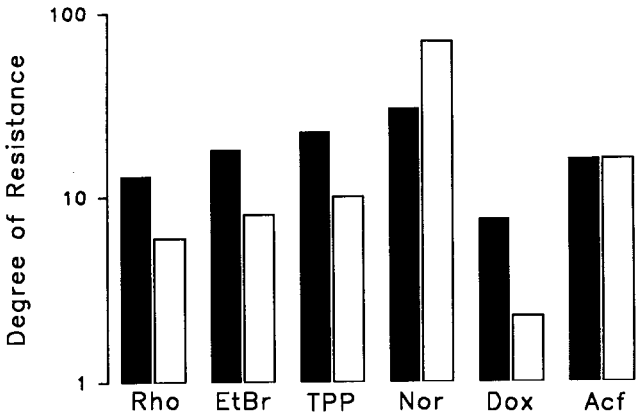

FIG. 4. Drug resistance of *B. subtilis* BD224 transformed with either pBMR2 (overexpression of Bmr [■]) or pBlt (overexpression of Blt [□]). Degree of resistance is defined as the ratio of the MIC for BD224/pBMR2 or BD224/pBLT to the MIC for BD224. Rho, rhodamine 6G; EtBr, ethidium bromide; TPP, tetraphenylphosphonium; Nor, norfloxacin; Dox, doxorubicin; Acf, acriflavine.

cloned the *Rsa*I-generated DNA fragment containing *blt* (Fig. 2A), into pBEV. The resulting Blt-expressing plasmid (pBLT), when cloned into *B. subtilis* BD224, conferred resistance to the same diverse drugs as the original pE1 plasmid, confirming that *blt* alone is responsible for the observed multidrug resistance. We then compared the drug resistance profiles of BD224 transformed with pBEV containing either the *blt* gene (pBLT) or the *bmr* gene (pBMR2; reference 16). As Fig. 4 demonstrates, Bmr and Blt protect cells from the same drugs with only small quantitative differences in resistance levels. We have been unable to find any toxic compound transported by one transporter but not the other.

Expression of Blt is not normally detectable. We have shown previously that *bmr* is transcribed under standard cultivation conditions and that disruption of the chromosomal *bmr* gene by a chloramphenicol resistance gene leads to an increase in bacterial sensitivity to the Bmr-transported drugs (2, 17). To estimate the contribution of Blt to the drug resistance profile of *B. subtilis*, we created a strain of *B. subtilis*, BD170/*blt*::*emr*, in which the chromosomal *blt* gene is disrupted by an erythromycin resistance gene. This strain did not differ from control BD170 in either growth rate (data not shown) or sensitivity to ethidium bromide: the MIC of ethidium bromide for both of these strains was 3.0 μg/ml. In the same experiments, the MIC of ethidium bromide for strain BD170/*bmr*::*cat*, in which the *bmr* gene is disrupted (2), or BD170/*bmr*::*cat blt*::*emr*, in which both transporter genes are inactivated, was only 1.3 μg/ml. This result shows that a moderate level of ethidium bromide resistance is present in wild-type *B. subtilis* and that Bmr, not Blt, is responsible for this. That Blt does not contribute to ethidium bromide resistance indicates that it may be silent or expressed in only very small amounts in BD170. Indeed, Northern hybridization with RNA from BD170 grown under standard cultivation conditions repeatedly failed to detect *blt*-specific transcripts (see Fig. 7A).

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Bmr    1 MEK----KNITLTILLTNLFIAFLGIGLVIPVTPTIMNELHLSGTAVGYMVACFAITQLI
           * *          ***:*:*:*******:*** *: *  :****:::**:** ***:***
Blt    1 MKKSINEQKTIFIILLSNIFVAFLGIGLIIPVMPSFMKIMHLSGSTMGYLVAAFAISQLI

Bmr   57 VSPIAGRWVDRFGRKIMIVIGLLFFSVSEFLFGIGKTVEMLFISRMLGGISAPFIMPGVT
          ** *********** **::*** **:** :**:*  * : : **:***:** ****:**
Blt   61 TSPFAGRWVDRFGRKKMIILGLLIFSLSELIFGLGTHVSIFYFSRILGGVSAAFIMPAVT

Bmr  117 AFIADITTIKTRPKALGYMSAAISTGFIIGPGIGGFLAEVHSRLPFFFAAAFALLAAILS
          *:;*****;* * **:**:************** ***:*    *:*****;* **:**: *
Blt  121 AYVADITTLKERSKAMGYVSAAISTGFIIGPGAGGFIAGFGIRMPFFFASAIALIAAVTS

Bmr  177 ILTLREPERNPENQEIKGQKTGF------KRIFAPMYFIAFLIILISSFGLASFESLFAL
          :  *:*     *  :             **   *:*****:*: : :***:::*:::*:*
Blt  181 VFILKESLSIEERHQLSSHTKESNFIKDLKRSIHPVYFIAFIIVFVMAFGLSAYETVFSL

Bmr  231 FVDHKFGFTASDIAIMITGGAIVGAITQVVLFDRFTRWFGEIHLIRYSLILSTSLVFLLT
          * *******  *** :**  :**: : **:** :     ** ::*   **  : * *: *
Blt  241 FSDHKFGFTPKDIAAIITISSIVAVVIQVLLFGKLVNKLGEKRMIQLCLITGAILAFVST

Bmr  291 TVHSYVAILLVTVTVFVGFDLMRPAVTTYLSKIAGNEQGFAGGMNSMFTSIGNVFGPIIG
          :  ::::****  :*::***:***:*: ** :***:*** :**** :**:**:*** :*
Blt  301 VMSGFLTVLLVTCFIFLAFDLLRPALTAHLSNMAGNQQGFVAGMNSTYTSLGNIFGPALG

Bmr  351 GMLFDIDVNYPFYFATVTLAIGIALTIAWKAPAHLKAST   389
          *:***::: *** **   : :*::**: **        *:
Blt  361 GILFDLNIHYPFLFAGFVMIVGLGLTMVWKEKKNDAAALN  400
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FIG. 3. Alignment of Bmr and Blt amino acid sequences. Asterisks indicate identical residues; colons indicate conservative substitutions.

BltR, the likely transcriptional regulator of Blt expression. Sequence analysis has suggested that expression of Blt and Bmr is controlled by different, albeit homologous, regulatory proteins. A putative protein product of a second open reading frame of the pE1 insert, termed *bltR* (Fig. 2A), showed sequence homology with BmrR, the transcriptional regulator of *bmr* expression (2). Unlike the *bmrR* gene, however, which is located immediately downstream of *bmr* and oriented in the same direction as *bmr*, the *bltR* gene is located upstream of *blt* and oriented inversely to it.

As evident from sequence comparison (Fig. 5), BltR, as well as BmrR, belongs to the family of bacterial transcriptional activators, which includes MerR from different bacterial species (25), $TipA_L$ from *Streptomyces lividans* (9), SoxR from *E. coli* (4), and NolA from *Bradyrhizobium japonicum* (22). The members of this family of regulators have homologous N-terminal domains (Fig. 5) involved in promoter recognition but dissimilar C-terminal domains involved in the binding of specific inducer molecules modulating transcriptional activation (9, 25). The C-terminal region of BltR shows no significant sequence homology with the C-terminal region of BmrR or with any other sequence in the current databases.

Analysis of sequences immediately upstream of the *blt* coding sequence revealed the presence of a single promoter-like element (Fig. 6A). This putative promoter has the same peculiar characteristics as the *bmr* promoter: the spacing region between the −35 and −10 boxes contains an imperfect inverted repeat and is longer than in most other promoters, specifically, 19 bp instead of the normal 16 or 17 bp. The

FIG. 5. Amino acid sequence alignment of the N-terminal putative DNA-binding regions of BltR, BmrR, and TipA$_L$ from *S. lividans* (SwissProt accession no. P32184), MerR from *B. subtilis* (SwissProt accession no. P22853), NolA from *B. japonicum* (SwissProt accession no. P22537), and SoxR from *E. coli* (SwissProt accession no. P22538). A dark background indicates identity to the corresponding residue in BltR; a shaded background indicates a conservative substitution.

sequence of the inverted repeat is, however, entirely different from that in the *bmr* promoter.

We have previously shown that BmrR binds specifically to the *bmr* promoter (2). To test if BltR similarly binds the putative *blt* promoter, we first expressed BltR in *E. coli* by cloning it into the IPTG-inducible expression vector pTrc99a (see Materials and Methods). BltR was precipitated from the *E. coli* lysates at 50% saturation of ammonium sulfate and, after redissolution in a low-ionic-strength buffer, tested for the ability to retard the electrophoretic mobility of a PCR-generated 46-bp DNA fragment containing the putative *blt* promoter (Fig. 6A). Figure 6B demonstrates that protein preparations from cells expressing BltR caused a gel mobility shift of the putative *blt* promoter, while similar preparations obtained from control *E. coli* cells did not. It should be noted that these binding reactions were performed in the presence of a large excess of herring sperm DNA, indicating that the binding of BltR to the putative *blt* promoter was, indeed, sequence specific.

We have previously shown that one of the Bmr substrates, rhodamine 6G, increases Bmr expression via direct binding of

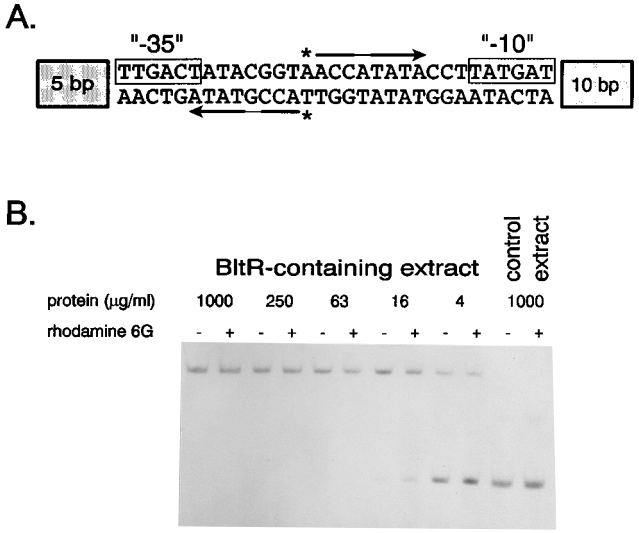

FIG. 6. Gel mobility shift analysis of the binding of BltR to the putative *blt* promoter. (A) Structure of the PCR-generated DNA promoter fragment used as a probe. The sequence of the putative *blt* promoter with the −35 and −10 consensus boxes is shown. The arrows indicate the imperfect inverted repeat located inside the 19-bp promoter spacing region. The asterisks indicate the base pair deleted in the *acfA* mutant of *B. subtilis*. (B) Gel mobility shift assay. Before electrophoresis, the labeled DNA fragment shown in panel A was incubated with different concentrations of a BltR-containing protein fraction isolated from BltR-expressing *E. coli*. In the right two lanes, a similarly prepared protein fraction isolated from control *E. coli* was used. BltR retards the mobility of the *blt* promoter-containing DNA fragment. Rhodamine 6G (20 μM) added to the incubation mixture (lanes labeled with plus signs) did not change the affinity of BltR for the promoter. This is in contrast to BmrR, whose affinity for the *bmr* promoter has been shown to increase fourfold in the presence of rhodamine (2).

BmrR (2). Furthermore, rhodamine increases the affinity of BmrR for the *bmr* promoter, as detected in gel shift experiments (2). Although rhodamine is also a substrate of the Blt transporter, it does not exert similar effects in the Blt-BltR system. First, Northern blot experiments demonstrated that rhodamine does not induce *blt* transcription (data not shown). Second, as shown in Fig. 6B, the affinity of BltR for the putative *blt* promoter is not affected by rhodamine. As of yet, we have not identified an inducer of Blt expression.

Mutation *acfA*, which alters the sequence of the *blt* promoter, causes Blt expression. In search of a *B. subtilis* strain expressing Blt, we analyzed strain 168ACF, which carries spontaneous acriflavine resistance mutation *acfA* (11). That acriflavine is a substrate of both Blt and Bmr (Fig. 4) suggested that overexpression of either of these two multidrug transporters could be responsible for the resistance phenotype of these cells. This hypothesis was substantiated by the observation that 168ACF cells are resistant not only to acriflavine but also to several other multidrug transporter substrates, including rhodamine 6G, ethidium bromide, tetraphenylphosphonium, and norfloxacin, and that this resistance was reversible by the Bmr and Blt inhibitor reserpine (data not shown).

To determine if Bmr or Blt is involved in the *acfA*-induced multidrug resistance phenotype, we transformed *B. subtilis* BD170/*bmr*::*cat* and BD170/*blt*::*emr*, in which either the *bmr* or *blt* gene was disrupted, with DNA from 168ACF cells. Transformants were selected on plates containing ethidium bromide, to select for *acfA* transfer, and either chloramphenicol or erythromycin, to ensure that the host transporter gene, *bmr* or *blt*, remained disrupted. Transformation of BD170/*bmr*::*cat* cells yielded hundreds of resistant colonies, while transformation of BD170/*blt*::*emr* cells yielded no colonies, indicating that an intact *blt*, but not *bmr*, gene is essential for the *acfA* phenotype. This was directly confirmed by Northern analysis of the 168ACF and BD170 RNAs, in which the *bmr* transcripts were present in similar amounts (data not shown) while an abundance of *blt* transcripts was found in 168ACF (Fig. 7A), indicating that the *acfA* mutation is associated with *blt* expression.

Interestingly, when BD170/*blt*::*emr* cells transformed with the 168ACF DNA were selected on ethidium bromide alone, they yielded large numbers of colonies. None of these colonies, however, retained erythromycin resistance, suggesting that during homologous recombination, an intact wild-type *blt* gene was transferred along with the *acfA* mutation. This close genetic linkage of the *acfA* mutation with the *blt* gene strongly indicated that this mutation is localized in the vicinity of *blt*.

The location of the *acfA* mutation in the *blt* locus was determined in the following way. First, by PCR, we amplified the entire *blt* locus (schematically shown in Fig. 2A), using as the template chromosomal DNA isolated either from control BD170 cells or from 168ACF cells. The amplified *blt* locus from the 168ACF cells, but not that from BD170 cells, effectively conferred the ethidium bromide resistance phenotype when transformed into wild-type BD170 cells (Fig. 2B). Next, the same experiment was performed with a series of PCR

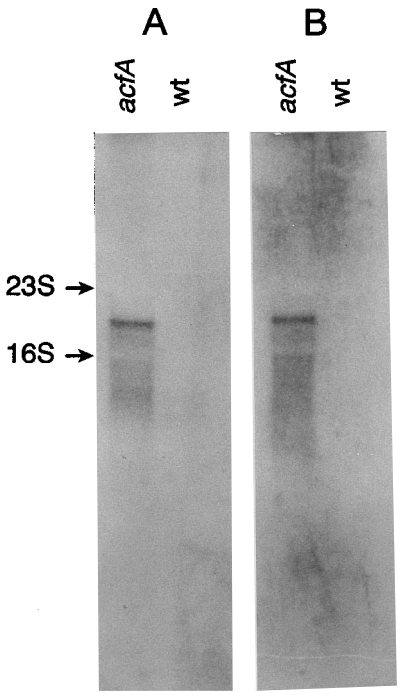

FIG. 7. Northern blot analysis of RNAs isolated from *B. subtilis* 168ACF (*acfA*) and BD170 (wild type [wt]). The probes used were specific for *blt* (A) and *bltD* (B). The same amount of RNA (5 μg) was loaded in each lane, and this was confirmed by ethidium bromide staining of the gel. The 23S and 16S rRNA bands are indicated. Both *blt* and *bltD* are transcribed in 168ACF as a 2.0-kb transcript.

products progressively truncated on either side. As Fig. 2B indicates, the *acfA* mutation is evidently located either in the 5′ region of the *blt* coding sequence or immediately upstream of it. Direct sequencing of this entire region in the PCR products obtained from control and 168ACF DNAs identified a single mutation in 168ACF DNA: a single base pair deletion in the spacer region located between the −35 and −10 promoter consensus motifs of the *blt* promoter (Fig. 6A). As described in the Discussion, the shortening of this spacer region explains the high-level expression of *blt* seen in 168ACF cells.

***blt* is cotranscribed with a downstream gene, *bltD*, which encodes a putative acetyltransferase.** The size of the *blt*-specific mRNA (2.0 kb; Fig. 7A) exceeds the size of the *blt* gene itself (1.2 kb). Considering that there is no putative transcriptional terminator downstream of *blt*, it was tempting to speculate that the *blt* transcript contains not only the Blt coding sequence but also an open reading frame located downstream of *blt* and named, for this reason, *bltD*. Indeed, Northern analysis with a *bltD*-specific probe detected a transcript whose size exactly coincided with that of the *blt* transcript and whose expression was detectable only in 168ACF cells and not in control BD170 cells (Fig. 7B).

The hypothesis that *blt* and *bltD* are cotranscribed as a single transcript was confirmed by reverse transcription-PCR analysis. RNA isolated from 168ACF cells was annealed with reverse primer p1 (Fig. 2A), corresponding to the downstream region of *bltD*, and cDNA was synthesized by reverse transcriptase. Two PCRs were then performed, each using this cDNA as the template and p1 as one primer. For the second primer, either p2, corresponding to the upstream part of *bltD*, or p3, corresponding to the middle part of *blt* (Fig. 2A), was used. Indicating that both genes are transcribed as a contiguous mRNA, each PCR yielded a single product of the expected size (data not shown). Importantly, no PCR products were detected when reverse transcriptase was omitted from the cDNA synthesis reaction, or when the RNA was pretreated with DNase-free RNase A before the addition of reverse transcriptase. These controls served as evidence that the template for the PCR was cDNA and not chromosomal DNA, which can sometimes contaminate RNA preparations. Overall, these results indicate that *blt* and *bltD* are cotranscribed as a dicistronic mRNA species, therefore constituting a new operon.

The putative protein product of *bltD* exhibits an interesting sequence homology. Database searches revealed significant similarity between the C-terminal region of BltD and the C-terminal regions of several bacterial acetyltransferases (Fig. 8), including enzymes involved in the inactivation of antibiotics, i.e., aminoglycosides and bactothricins; an enzyme acetylating spermidine; *B. subtilis* protein Pai1, which affects protease production and is also likely to be an acetyltransferase (10); and *E. coli* proteins RimJ and RimL, which acetylate the N termini of specific ribosomal proteins, thereby protecting cells from certain antibiotics detrimental to translation. Considering the extent of this sequence homology, it is reasonable to conclude that BltD is likely to be an acetyltransferase. More importantly, however, the putative acetyltransferase activity of BltD may shed some light on the normal physiological function of Blt.

DISCUSSION

Here we identify and partially characterize a new *B. subtilis* operon containing a gene for the second multidrug transporter described in these bacteria, Blt. This transporter is structurally highly similar (51% sequence identity) to the previously described *B. subtilis* multidrug transporter, Bmr. *B. subtilis* strains overexpressing either Bmr or Blt display resistance to the same spectrum of drugs, with only small quantitative differences in their resistance levels.

Despite these similarities between Bmr and Blt, their expression patterns are disparate. Unlike the transcription of *bmr*, transcription of *blt* is undetectable in wild-type *B. subtilis*. Moreover, disruption of *blt*, in contrast to disruption of *bmr*, has no effect on bacterial sensitivity to drugs. The difference in

FIG. 8. Sequence alignment of the C-terminal region of the putative BltD protein with the C-terminal regions of the following bacterial acetyltransferases: Pai1 from *B. subtilis* (SwissProt accession no. P21340); spermidine-N1 acetyltransferase from *E. coli* (SpAT; GenBank accession no. D25276); aminoglycoside N6′-acetyltransferase from *Serratia marcescens* (AGAT; SwissProt accession no. P20092); nourseothricin acetyltransferase from *Streptomyces noursei* (NTAT; PIR accession no. JN0662); RimL (SwissProt accession no. P13857) and RimJ (SwissProt accession no. P09454) from *E. coli*, which acetylate the N termini of ribosomal proteins L7/L12 and S5, respectively. A dark background indicates identity to the corresponding residue in BltD; a shaded background indicates a conservative substitution.

the levels of expression of the two multidrug transporters is likely due to the fact that the transcription of their genes is under different controls. It has been established previously that Bmr expression is regulated by BmrR, a protein which is encoded in the vicinity of the *bmr* gene and interacts directly with the *bmr* promoter (2). Here we show that the expression of Blt is regulated by a different protein, BltR, which is encoded in the vicinity of the *blt* gene, binds the *blt* promoter, and, like BmrR, is a member of the MerR family of transcriptional activators.

Members of this family of regulatory proteins have homologous N-terminal DNA-binding domains, and the promoters recognized by them have a peculiar feature: the −35 and −10 consensus motifs are separated by 19 bp instead of the usual 16 or 17 bp. The C-terminal domains of these proteins are unrelated, however, and are thought to be involved in the binding of molecules which induce transcriptional activation and which are distinct for each member of this family of regulators. For example, it has been demonstrated that the homologs of BmrR and BltR, MerR and $TipA_L$, bind the inducer molecules, mercury ions and thiostrepton, respectively, through their C-terminal domains. Similarly, we have recently found that rhodamine, an artificial compound inducing Bmr expression, binds not only to BmrR but also to its C-terminal domain expressed individually in *E. coli* (15a).

The C-terminal domains of BmrR and BltR show no signs of sequence homology, suggesting that the inducers which normally interact with BmrR and BltR are distinct. This hypothesis is supported by experiments in which we tested if rhodamine, a compound enhancing Bmr expression via interaction with BmrR, functions as an inducer in the Blt-BltR system. Although rhodamine is transported by Blt, it neither induced *blt* transcription in vivo nor affected the affinity of BltR for the *blt* promoter, indicating that the inducer-binding domains of BmrR and BltR are indeed functionally distinct. This difference in the inducer specificities of BmrR and BltR can explain why Bmr, and not Blt, is normally expressed in wild-type *B. subtilis*. Apparently, the inducer molecule activating Bmr expression via binding to BmrR is normally present in limited amounts in culture medium or is produced by *B. subtilis* itself, while the inducer of Blt expression is normally absent, implying that expression of Blt occurs only under unknown environmental conditions.

We have identified a strain of *B. subtilis*, 168ACF, carrying an *acfA* mutation, which constitutively expresses Blt. Mapping of this mutation followed by direct DNA sequencing identified its molecular nature: a deletion of a single base pair in the spacer region of the *blt* promoter. The structure of the *blt* promoter, and of other promoters controlled by the members of the MerR family of regulators, explains how this deletion may lead to *blt* transcription. It is believed that the unusually long spacer region of these promoters renders these promoters, in the absence of an inducer, inactive because the −35 and −10 binding sites for RNA polymerase are located on different sides of the DNA helix. Numerous experiments performed with MerR (summarized in reference 25) have shown that this protein binds the promoter spacer region and, upon binding of the inducer, mercury ion, partially untwists DNA. It is hypothesized that, as a result, the −35 and −10 promoter elements acquire proper spatial orientation on the DNA helix for RNA polymerase binding. Interestingly, single base pair deletions in the spacer region cause the MerR-regulated promoter to become constitutively active, no longer requiring MerR or mercury ions (15). Apparently, shortening of the spacer region to a more normal length is sufficient for proper spatial alignment of the promoter consensus elements. Thus, it is reasonable to suggest that with the *acfA*-associated deletion, the observed *blt* expression occurs by a similar mechanism.

The finding that the *acfA* mutation is located within the *blt* locus helped us to determine the position of *blt* on the *B. subtilis* chromosome: like the previously mapped *acfA* mutation (11), *blt* is located at 230° on the map. Interestingly, another known acriflavine resistance mutation, *acfB*, has been mapped to 215° on the *B. subtilis* map (24), which is in very close proximity to the position of the *bmr* gene (216°; reference 17).

Another interesting result derived from the analysis of the *acfA* mutant of *B. subtilis* is the finding that the *bmr* and *blt* genes differ in operon organization. While the *bmr* promoter directs expression of Bmr alone, the *blt* promoter controls expression of two proteins: Blt and a putative BltD protein displaying strong homology to acetyltransferases. Such an operon organization strongly suggests that Blt and BltD perform different aspects of the same biochemical process. For example, the natural substrate of Blt may be either the molecule which BltD acetylates or the product of this acetylation.

From the observation that Bmr and Blt cause the efflux of structurally dissimilar drugs, it is tempting to assume that their role lies in the protection of cells from diverse environmental toxins. It is not clear, then, why a bacterial genome, with its generally economical organization, encodes two seemingly redundant protective efflux pumps with nearly identical substrate specificities. The differences seen in the regulation of transcription and the operon organization of the genes encoding Bmr and Blt prompt us to hypothesize that Bmr and Blt have different physiological functions and that their unknown natural substrates are, likewise, specific and distinct. Coexpression of Blt with BltD, a protein homologous to acetyltransferases, which are highly specific for particular substrates, strongly supports this hypothesis.

Interestingly, *Staphylococcus aureus* expresses NorA, a close homolog of Bmr (44% sequence identity; reference 16) and Blt (37% identity; data not shown), which is also capable of causing the efflux of the same broad spectrum of drugs (18). Importantly, the *norA* gene is flanked by open reading frames not homologous to any of the genes surrounding either *bmr* or *blt* (compare GenBank records D90119 [*norA*], L25604 [*bmr*], and L32599 [*blt*]), which, following the same logic, suggests that the function of NorA differs from that of either Bmr or Blt.

The hypothesis that Bmr, Blt, and NorA perform specific functions parallels the recent findings concerning another multidrug transporter, MexAB of *Pseudomonas aeruginosa*. Besides having the ability to cause the efflux of a variety of antibiotics (21), a specific physiological role has been attributed to MexAB, i.e., its expression is induced by the absence of iron in medium, and it causes the efflux of pyoverdine, the oligopeptide which mediates iron uptake by *P. aeruginosa* cells (20).

Further biochemical and genetic analyses of Bmr and Blt and of the mechanisms regulating their expression can lead to identification of their normal physiological substrates.

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