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bmr3, a Third Multidrug Transporter Gene of *Bacillus subtilis*

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A third multidrug transporter gene named *bmr3* was cloned from *Bacillus subtilis*. Although Bmr3 shows relatively low homology to Bmr and Blt, the substrate specificities of these three transporters overlap. Northern hybridization analysis showed that expression of the *bmr3* gene was dependent on the growth phase.

Bacteria possess pumps of low specificity that can extrude structurally unrelated compounds in an energy-dependent manner (12, 16, 20). Mutations in the genes encoding these pumps increase the susceptibilities of the mutant bacteria to diverse cytotoxic compounds (11, 15, 21), and overexpression of these pumps from genes on multicopy plasmids confers multidrug resistance to bacteria (1, 13, 18, 19).

Recently, experimental evidence has accumulated showing that some bacteria have multiple drug efflux pumps with overlapping substrate specificities. *Escherichia coli* has AcrAB and EnvCD efflux pump systems (with more than 65% identical amino acids) and similar substrate specificities (11, 14–16). In the gram-positive bacterium *Bacillus subtilis*, two highly homologous (51% identity) multidrug transporters, Bmr and Blt, have been reported (1, 18). Overexpression of these transporters was shown to result in increased resistance to the same spectrum of substances. It is not yet clear whether these individual pumps have any specific physiological function other than to protect bacteria from various toxins in the environment.

In this article, we report a third multidrug transporter gene of *B. subtilis*. Overexpression of this gene, *bmr3*, on a multicopy plasmid resulted in resistance to puromycin, tosufloxacin, and norfloxacin which are also substrates of the two other multidrug transporters of *B. subtilis*. Bmr3 protein shows moderate homology to EmrB, a multidrug transporter of *E. coli* (13), and to a lesser extent to Bmr and Blt (1, 18). The expression pattern of the *bmr3* gene was dependent on the growth phase and different from those of *bmr* and *blt* genes. Therefore, these three multidrug transporters might have distinct roles under different physiological conditions.

Cloning and sequencing of the *bmr3* gene. The *cmp* gene of *E. coli*, which is located just downstream of the *divE* gene, encodes a 23.5-kDa hypothetical membrane protein with unknown function (24). While attempting to clone a homolog of this gene, we obtained a clone, pH233, from a genomic library of *B. subtilis* 168 cloned into pUC18 that gave a distinct hybridization signal under low-stringency conditions. Sequence analysis showed that the 3.3-kb insert of pH233 contained two open reading frames (ORFs) oriented in opposite directions, as shown in Fig. 1A. Although the 0.7-kb *Hin*dIII fragment of ORF 1 (ORF-1) displayed 50% identity with the *cmp* gene at the level of nucleotide sequence, there was no homology at the level of amino acid sequence. A homology search revealed that the predicted 512 amino acid sequence of ORF-1 showed moderate homology to EmrB (25% identity and 57% similarity), one of the multidrug transporters of *E. coli* (13), and to a putative protein product of the *mmr* gene of *B. subtilis* (22% identity and 56% similarity) previously reported to be a homolog of the methylenomycin-resistant gene of *Streptomyces coelicolor* (17). The homology of ORF-1 to Bmr (15% identity and 49% similarity) and Blt (7% identity and 17% similarity) was relatively low (1, 18). As shown in Fig. 1B, homology to EmrB or Bmr is confined to the N-terminal halves of these proteins. ORF-1 has putative −35, −10, and Shine-Dalgarno sequences in the 5′-upstream region and inverted repeat sequences just downstream of the stop codon, which could function as a transcription termination signal. ORF-1 has two consensus sequences that are conserved in several multidrug transporters: I, the translocase consensus sequence $GXXXD^{R}_{K}XGR^{R}_{K}$ (25); and II, a drug consensus sequence GXhyhyGPXXGG (X is any amino acid; hy is a hydrophobic amino acid) (Fig. 1B) (22). The hydropathy profile of ORF-1 is very similar to that of EmrB, which suggests that ORF-1 is an integral membrane protein with 14 transmembrane segments. These results suggest that ORF-1 encodes a multidrug transporter. Thus, we named the gene *bmr3* to indicate that it is the third multidrug transporter gene of *B. subtilis.*

ORF-2 is 1,611 bp long and encodes a 536-amino-acid protein. However, it is uncertain whether the ATG codon at the predicted site is used as an initiation codon because of the lack of sequence information about the 5′-upstream region of ORF-2. A homology search indicated that this putative protein is highly homologous to the lactate permease of *E. coli* (47% identity and 77% similarity) (4).

Mapping of the *bmr3* gene on the chromosome of *B. subtilis*. Using the complete *Not*I and *Sfi*I physical map of the *B. subtilis* 168 chromosome constructed by Itaya and Tanaka (10), we located the *bmr3* gene on the chromosome by pulsed-field gel electrophoresis (PFGE) with CHEF DR-II (Bio-Rad Laboratories). Preparation of genomic DNA of *B. subtilis* 168 and digestion by *Not*I and *Sfi*I were carried out as described by Itaya and Tanaka (10). Electrophoresis of *Sfi*I fragments was done on a 1% agarose gel (ultra pure agarose gel; Bio-Rad Laboratories) at 14°C in 0.5× TBE (Tris-borate-EDTA) with a 30- to 150-s pulse time at 6 V cm^{-1} for 20 h. In the case of *Not*I fragments, the conditions were identical, except that the pulse and running times were 5 to 20 s and 24 h, respectively. Hybridization of PFGE-separated *Sfi*I and *Not*I fragments from *B. subtilis* 168 genomic DNA with a 3.3-kb *Hin*cII fragment from pH233 revealed 460- and 57-kb bands, respectively. From this result, we concluded that the *bmr3* gene maps to the 29N fragment in the 414-kb BS fragment of the *Sfi*I and *Not*I restriction map of *B. subtilis* (10). The *amyR* gene is located in the same fragment.

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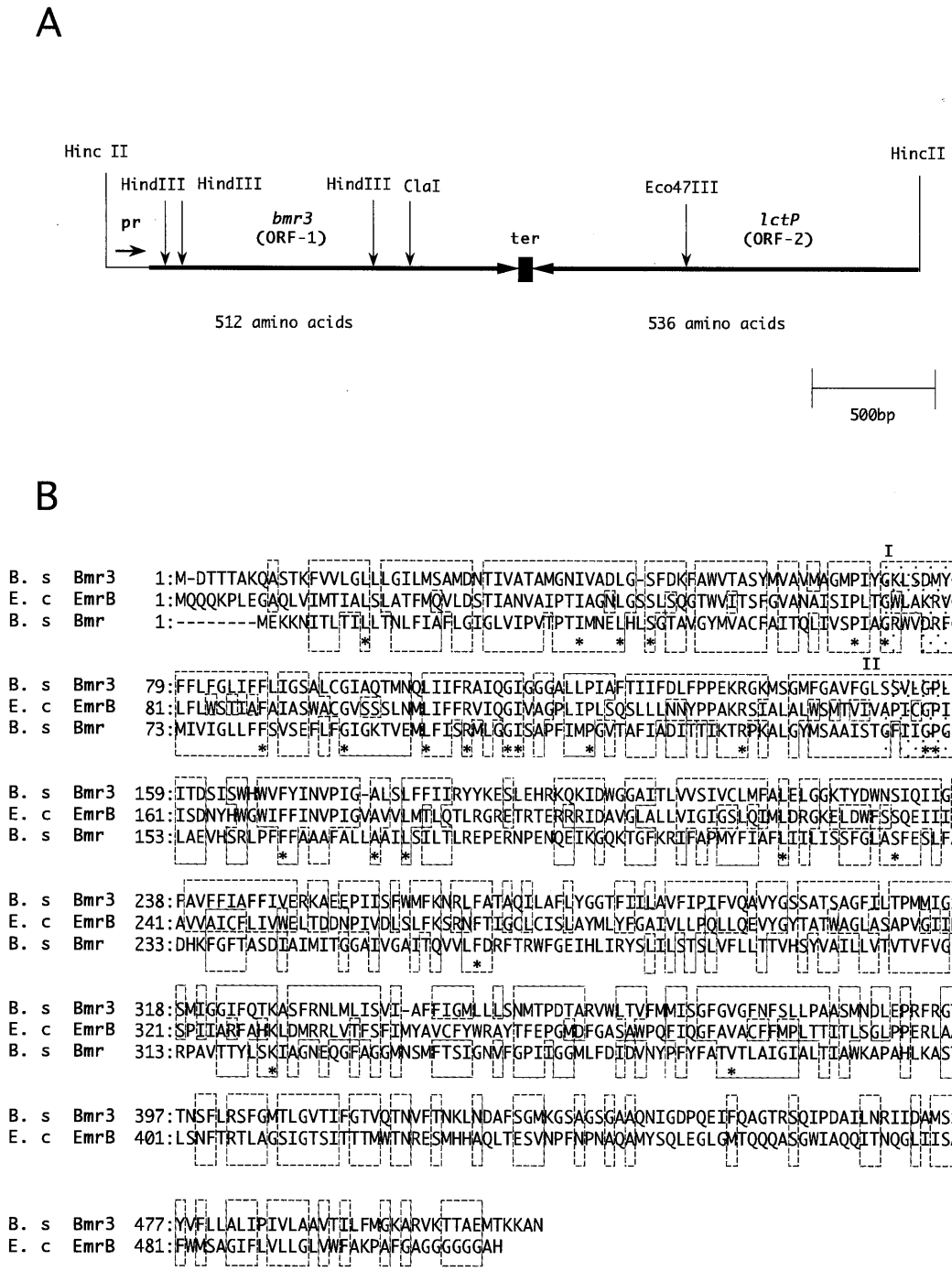

FIG. 1. (A) Restriction map and ORFs of the pH233 insert. The ORFs are shown by a thick horizontal line. Putative promoter (pr) and termination (ter) sites of transcription are indicated. ORF-1 corresponds to *bmr3*, and ORF-2 corresponds to *ltcP*. (B) Amino acid sequence alignment of Bmr3, EmrB, and Bmr from *B. subtilis* (B. s) and *E. coli* (E. c). The positions containing identical amino acids in the three sequences are shown by an asterisk. Identical residues and conservative replacements with respect to Bmr3 are boxed. Two consensus sequences are boxed and shown on a stippled background: I, translocase consensus sequence $GXXXD^{R}_{K}XGR^{R}_{K}$; II, drug consensus sequence GXhyhyGPXXGG (X is any amino acid; hy is a hydrophobic amino acid). Homology search was carried out by the program GENETYX (Software Development Co., Ltd.).

Drug resistance of the strain transformed with a multicopy plasmid carrying the *bmr3* gene. On the basis of sequence analysis, we predicted that ORF-1 encodes a multidrug transporter. In order to verify this hypothesis, we examined the drug resistance of *B. subtilis* ISW1214 (9) transformed with a multicopy plasmid pHY300PLK (8) carrying the 2.4-kb *Hin*cII-*Eco*47III fragment from pH233 (pHY*bmr3*). For comparison, we cloned the *bmr* gene obtained by PCR amplification into the same restriction site of pHY300PLK (pHY*bmr*). Strain ISW1214/pHY*bmr3* showed the same level of resistance to puromycin as that of strain ISW1214/pHY*bmr*, whereas strain ISW1214/pHY*bmr3* was more resistant than strain ISW1214/

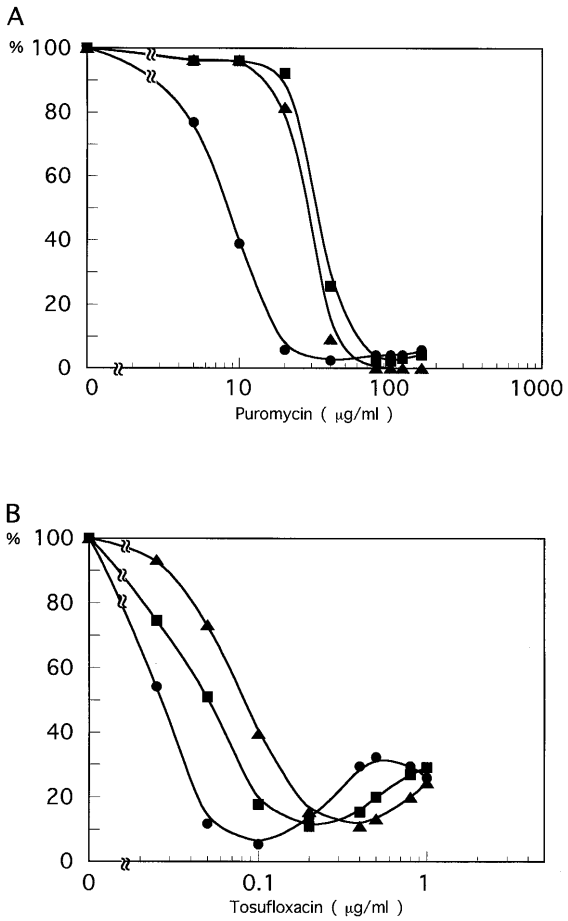

FIG. 2. Drug resistance of *B. subtilis* ISW1214. The ISW1214 strain was transformed with pHY300PLK (circles), pHY*bmr3* (triangles), or pHY*bmr* (squares). Resistance to puromycin (A) or tosufloxacin (B) is shown. Cells were grown in L medium to early log phase (optical density at 530 nm [OD_{530}], 0.2). Two milliliters of culture was added to an equal volume of L medium containing various concentrations of drug, and growth was monitored by measuring OD_{530} after 2 h. Growth was expressed as the percent increase in the OD_{530} over that observed in the absence of the drug.

pHY*bmr* to tosufloxacin (Fig. 2). The Bmr-overproducing strain showed increased resistance to all quinolones, such as norfloxacin, ofloxacin, and levofloxacin, whereas the strain overexpressing the *bmr3* gene showed no resistance to ofloxacin or levofloxacin (Table 1). For all the strains tested, there was a critical concentration of tosufloxacin that gave maximum growth inhibition—concentrations higher than this were not as effective (Fig. 2B). This phenomenon, termed the paradoxical effect (7), has been seen with other quinolones but not with other drugs. Bmr-overproducing strains have been reported to show a high level of resistance to acriflavine, ethidium bromide, and tetraphenylphosphonium bromide (18). Bmr3-overproducing cells also showed a 30 to 50% increase in relative resistance to these drugs (Table 1). In spite of the high sequence similarity between Bmr3 to EmrB, overproduction of Bmr3 did not result in an increase in resistance to nalidixic acid or carbonyl cyanide *m*-chlorophenylhydrazone. Although the substrate specificity of Bmr3 appears to be somewhat narrower than those of Bmr and Blt, it nevertheless overlaps to a certain extent with those of Bmr and Blt.

Disruption of the *bmr3* gene on the *B. subtilis* chromosome. We constructed *B. subtilis* ISW1214/*bmr3*::*cat* in which the *bmr3* gene was disrupted by a chloramphenicol acetyltransferase gene, *cat*. The *Hin*cII-generated 3.3-kb fragment containing the *bmr3* gene cloned in pBR322 was cut by *Cla*I within the *bmr3* gene (Fig. 1A). The 1.8-kb *Eco*RI fragment from pSGMU37 (5) containing the *cat* gene was then cloned into this site. The disrupted *bmr3* gene was isolated from the resulting plasmid and used to transform *B. subtilis* ISW1214 with selection for chloramphenicol resistance. PCR and Southern hybridization analysis showed that the native *bmr3* gene on the chromosome had been replaced by the disrupted *bmr3* gene by homologous recombination.

B. subtilis ISW1214/*bmr3*::*cat* showed the same level of resistance to tosufloxacin and puromycin as that of the control strain ISW1214. This result indicates that the *bmr3* gene on the chromosome is not essential for growth and that Bmr with a similar substrate specificity may contribute to the intrinsic resistance to drugs in the absence of a functional *bmr3* gene.

B. subtilis ISW1214 is a derivative of strain 168 which was selected for its higher sensitivity to tetracycline (9). However, the genetic background of this phenotype has not been elucidated. In order to confirm that the observed results were not particular to the genetic background of strain ISW1214, we used strain BD170 as a host cell for the various recombinant plasmids and tested their resistance to puromycin, tosufloxacin, and norfloxacin. The results obtained with this strain were similar to those described above (data not shown).

Identification of Bmr3 protein in maxicells of *E. coli*. A maxicell method was used to detect the gene product of *bmr3* in *E. coli*, since the genes of *B. subtilis* can usually be expressed in *E. coli*. Recombinant plasmids, pBR322-*bmr3* and pBR322-*bmr3*::*cat*, as well as pBR322 vector alone, were transformed into *E. coli* JM109. ^{35}S-labeled maxicells were prepared as described by M. Tadayyon et al. (23). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at three different concentrations of acrylamide, 7.5, 12.5, and 18%. Labeled protein bands of plasmid-borne gene products were analyzed by a Fuji Bas2000 Imaging Analyzer. One prominent labeled band was detected in the strain transformed with pBR322-*bmr3* and not in the strain transformed with the vector alone at all gel concentrations. This labeled band was not present in the sample from the strain transformed with pBR322-*bmr3*::*cat*. The molecular mass of Bmr3 calculated from the predicted amino acid composition was 57 kDa. The apparent molecular mass of the predicted Bmr3 protein in-

TABLE 1. Drug resistance of *B. subtilis* ISW1214 transformed with pHY*bmr3* or pHY*bmr*

Drug[b]	Relative resistance[a]	
	pHY*bmr3*	pHY*bmr*
Puromycin	3.3	4.0
Tosufloxacin	3.2	2.0
Norfloxacin	1.7	6.5
Levofloxacin	1.1	2.1
Ofloxacin	1.0	2.1
Ethidium bromide	1.4	5.4
Acriflavine	1.5	
TPP[c]	1.3	

[a] Relative resistance was determined by dividing the IC_{50} (the drug concentration required to inhibit growth by 50%) for cells harboring pHY*bmr3* or pHY*bmr* by the IC_{50} for control cells harboring pHY300PLK.

[b] Puromycin and norfloxacin were purchased from Sigma Chemical Co. Tosufloxacin was obtained from Toyama Chemical Co., Ltd. Levofloxacin and ofloxacin were from Daiichi Chemical Co., Ltd.

[c] TPP, tetraphenylphosphonium bromide.

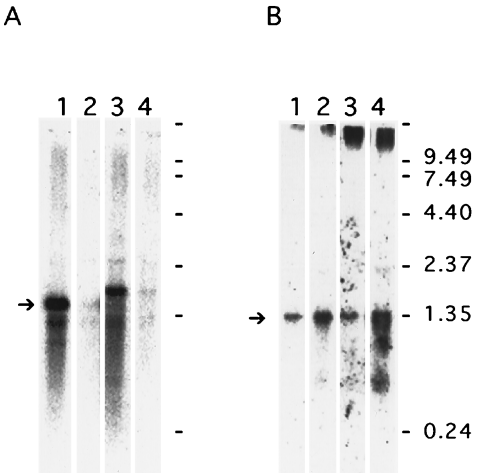

FIG. 3. Northern hybridization analysis of RNA isolated from *B. subtilis* BD170 and BD170/*bmr3*::*cat*. Five micrograms of RNA dissolved in solution A (20 mM sodium acetate, 0.5% sodium dodecyl sulfate, 10 mM EDTA; pH 5.5) was loaded in each lane and subjected to electrophoresis in a 1.5% agarose gel containing 4% formaldehyde. The ^{32}P-labeled *bmr* and *bmr3* probes were prepared from PCR-generated DNA fragments with a multiprime DNA labeling system (Amersham). The probes used were specific for *bmr3* (A) and *bmr* (B). Strains BD170 (lanes 1 and 2) and BD170/*bmr3*::*cat* (lanes 3 and 4) were used. RNA was isolated from early-log-phase cultures (OD_{530}, 0.3) (lanes 1 and 3) and late-log-phase cultures (OD_{530}, 1.6) (lanes 2 and 4). The positions of *bmr3* and *bmr* transcript are indicated by arrows. The positions of molecular size markers (in kilobases) are indicated to the right of panel B.

creased with the concentration of the gel, as reported for various integral membrane protein previously (6). In 7.5 and 12.5% acrylamide gels, the predicted Bmr3 migrated faster than the 45-kDa marker protein, whereas in 18% acrylamide gels, it migrated between the 66- and 45-kDa marker proteins.

Expression pattern of the *bmr3* gene. To determine whether the putative promoter sequence located upstream of the *bmr3* coding region is functional in *B. subtilis*, a fusion gene, p*bmr3-lacZ*, was constructed and inserted into the vector pHY300PLK. In this construct, the reporter gene, *lacZ*, is transcribed from the *bmr3* promoter. When this recombinant plasmid, pHYp*bmr3-lacZ*, was transformed into *B. subtilis* BD170 or ISW1214, significant expression of β-galactosidase activity was detected, indicating that the putative promoter was functional in *B. subtilis.*

Northern hybridization analysis was carried out on RNA isolated from strain BD170 or ISW1214 grown in antibiotic medium 3 by the method described by Ambulos et al. (3). In strain BD170, a 1.6-kb band was detected by the *bmr3*-specific probe (Fig. 3A, lane 1). The size of the *bmr3* gene transcript indicated that the *bmr3* gene was transcribed from the promoter located just upstream of the coding region. In strain BD170/*bmr3*::*cat*, the 1.6-kb transcript disappeared and was replaced by a 2.1-kb band which could correspond to the transcript of the *bmr3*::*cat* fusion gene (Fig. 3A, lane 3). We also found that the level of the *bmr3* transcript was drastically decreased when RNA was isolated from cells in late log phase (Fig. 3A, lane 2). The same expression pattern was obtained with the *bmr3-cat* fusion gene inserted into the chromosome (Fig. 3A, lane 4), indicating that lower expression of the *bmr3* gene in late log phase was dependent on the *bmr3* promoter. For a control, the same RNA samples were hybridized with the *bmr*-specific probe (Fig. 3B). During early log phase, the level of the 1.35-kb *bmr* transcript was found to be similar to that of the *bmr3* gene. However, unlike the *bmr3* gene transcript, the level of the *bmr* gene transcript increased in late log phase (Fig. 3B, lanes 2 and 4). The same results were obtained with strain ISW1214 (data not shown). It has been reported that the transcription of the *bmr* gene is positively regulated by BmrR and that an inducer such as rhodamine 6G can increase the binding of BmrR to the promoter region of the *bmr* gene (2). It remains to be determined whether there is a positive or negative transcriptional regulator specific to the *bmr3* gene. Ahmed et al. reported that the *blt* gene was not expressed under normal growth conditions (1).

It is not yet clear why *B. subtilis* has three multidrug transporters with overlapping substrate specificities. It is reasonable to assume that the presence of multiple drug resistance efflux pumps with overlapping substrate specificities but with different expression patterns enables bacteria to respond more precisely to environmental changes. Further analysis of the regulatory mechanisms governing the expression of these genes should lead to the identification of their physiological function.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to DDBJ and assigned accession no. D50098.

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