Functional Cloning and Expression of *emeA*, and Characterization of EmeA, a Multidrug Efflux Pump from *Enterococcus faecalis*

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A fragment of chromosomal DNA from *Enterococcus faecalis* ATCC 29212 was cloned using *Escherichia coli* KAM32 host cells lacking major multidrug efflux pumps. *E. coli* KAM32 cells were sensitive to many antimicrobial agents, and the transformed cells harboring a recombinant plasmid became resistant to several structurally unrelated antimicrobial agents such as tetraphenylphosphonium chloride, 4',6-diamidino-2-phenylindole (DAPI), Hoechst 33342, acriflavine, benzalkonium chloride, norfloxacin and ethidium bromide. This suggests that the cloned DNA fragment carries a gene(s) encoding a multidrug efflux pump. Determination of the nucleotide sequence of the cloned DNA revealed a gene designated as *emeA*. The transformed *E. coli* cells showed efflux activity of several antimicrobial agents such as DAPI, Hoechst 33342 and acriflavine. Efflux of DAPI via EmeA was strongly inhibited by reserpine.

Key words EmeA multidrug efflux pump; drug efflux; drug resistance; *Enterococcus faecalis*

Over the past several decades, the emergence of drug resistance in microorganisms, and its association with serious infectious diseases, has increased at an alarming rate.¹⁾ Many bacteria that once appeared to be under control or were believed to be potentially controllable are now causing infections that are increasingly difficult to treat.²⁾ There is a large reservoir of resistance genes in bacterial genomes, and in extra-chromosomal pieces of DNA, that are responsible for different mechanisms of drug resistance such as efflux pumps, enzymes that inactivate antibiotics and which alter antibiotic targets.³⁾ Among them, multidrug efflux pumps are emerging as a major problem in multidrug resistance in bacterial cells.^{4,5)}

The extensive knowledge of the molecular mechanisms underlying microbial antibiotic resistance is required in order to successfully fight the increasing numbers of drug resistant and multidrug resistant (MDR) bacteria.³⁾ Thus, it is important to investigate the multidrug efflux pumps of bacteria at both the genetic and biochemical levels. Gene cloning, expression, and biochemical analysis are useful approaches to the understanding of the mechanism of multidrug efflux pumps.⁶⁾

Enterococci, gram-positive and facultative anaerobic bacteria, are normally found in the human intestine.⁷⁾ Over the past two decades, enterococci have been identified with increasing frequency as causative agents of nosocomial infections, paralleling the correlation of antimicrobial resistance to most commonly used agents. As a result, enterococci have emerged as one of the leading clinical challenges for physicians when identified as the cause of serious or life-threatening infections.⁸⁾ Infections caused by bacteria of the genus *Enterococcus* include urinary tract infections, nosocomial bacteremia, intra-abdominal infections and endocarditis.^{7,9)}

Most enterococci have intrinsic resistance to various antimicrobial agents, including β -lactams, fluoroquinolones, aminoglycosides and glycopeptides.^{10,11)} The rapid emergence of antimicrobial resistance among the enterococci undoubtedly also contributes to their emergence as prominent nosocomial pathogens, making them among the most diffi-

cult to treat. It was hypothesized that resistance to multiple antimicrobial drugs of this strain probably explains to a large extent its prominence in nosocomial infections.¹²⁾

Unlike gram-negative enteric bacteria, an outer membrane does not protect the enterococcal cell. Lynch *et al.* hypothesized that, in the absence of an extracytoplasmic permeability barrier, the most likely mechanism for intrinsic drug resistance in enterococci may be active efflux.¹³⁾ Recently, Jonas *et al.*¹⁴⁾ reported PCR cloning of *emeA* gene encoding a multidrug efflux pump and some properties of the pump, from *Enterococcus faecalis* which causes most enterococcal infections in humans.

In this study, we report functional gene cloning and characterization of the EmeA as multidrug efflux pump, from *E. faecalis*. We found better substrates for the pump and measured their extrusion from cells.

MATERIALS AND METHODS

Bacteria and Growth *E. faecalis* ATCC 29212, *Escherichia coli* KAM32 (Δ acrB, Δ ydhE)⁶⁾ were used in this study. *E. faecalis* cells were grown in Brain Heart Infusion (BHI) medium (Difco Co.) and *E. coli* cells were grown in L medium¹⁵⁾ at 37 °C. Measurement of the optical density at 650 nm was used to monitor cell growth.

Cloning and Sequencing Chromosomal DNA was prepared from *E. faecalis* cells by the method of Berns and Thomas.¹⁶⁾ The DNA was partially digested with *Sau*3AI and the fragments from 4 to 10 kbp were separated by sucrose density gradient centrifugation. Plasmid pBR322 DNA was digested with *Bam*HI, dephosphorylated with bacterial alkaline phosphatase (BAP) and then ligated to the chromosomal DNA fragments by using a ligation kit, Ver. 2 (TaKaRa Co.). Competent cells of *E. coli* KAM32 were transformed with the recombinant plasmids and were spread onto agar plates containing L broth, 60 μ g/ml ampicillin, 10 μ g/ml tetraphenylphosphonium chloride (TPPCI) and 1.5% agar. The plates were incubated at 37 °C for 24 h. Candidate colonies were replica plated, and plasmids were isolated from each of

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the candidates. Plasmid DNAs were re-transformed into the KAM32 cells and spread onto the same type of plate. Plasmids were isolated from each of the retransformants and used for restriction mapping and sequencing. The nucleotide sequence of the gene was determined by the dideoxy chain termination method¹⁷⁾ with an automated DNA sequencer (ALF Express, Pharmacia Biotech.). The nucleotide sequence data has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB091338.

Drug Susceptibility Test The minimal inhibitory concentrations (MICs) of various drugs were determined in Muller-Hinton broth (Difco) by the standard 2-fold dilution method.¹⁸⁾ Cells were incubated in the test medium at 37 °C for 24 h and the growth was examined by visual inspection.

Fluorometric Assay of EmeA-Mediated Efflux of Drugs Fluorometric assays of EmeA-mediated efflux of 4',6-diamidino-2-phenylindole (DAPI), Hoechst 33342 and acriflavine were carried out essentially as described previously for ethidium.¹⁹⁾ DAPI binds to double-stranded DNA, resulting in a substantial increase in fluorescence.²⁰⁾ Hoechst 33342, when bound to membrane lipids and/or DNA, are highly fluorescent²¹⁾; acriflavine binds to DNA resulting in fluorescence quenching.⁶⁾ *E. coli* KAM32 cells harboring recombinant plasmids were grown in L broth at 37 °C to the exponential phase of growth. The cells were harvested and washed with modified Tanaka buffer (pH 7.0),²²⁾ containing 2 mM MgSO₄. Cells were loaded with 4.2 μM acriflavine by incubating for 1–2 h at 37 °C in the presence of 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). For loading of DAPI (5 μM) or Hoechst 33342 (1 μM), dinitrophenol (DNP, 5 mM) instead of CCCP was used and incubated for 10 h at 37 °C. Cells were washed three times, and resuspended with 0.1 M MOPS-tetramethylammonium hydroxide (TMAH) (pH 7.0) containing 2 mM MgSO₄, 20 mM KCl and each substrate of the same concentration as indicated above. Energy-dependent efflux was assayed following the addition of glucose to a final concentration of 20 mM. Fluorometric measurements were performed at 37 °C using a Hitachi 2000 fluorescence spectrophotometer. The excitation and emission wavelengths used for each of the fluorescent compounds are as follows: DAPI, 332 nm and 463 nm; Hoechst 33342, 355 nm and 457 nm; and acriflavine, 468 nm and 499 nm, respectively.

RESULTS

Functional Cloning and Sequencing of the *emeA* Drug-Resistance Gene In order to clone an *E. faecalis* gene responsible for multidrug resistance, partially digested chromosomal DNA fragments from *E. faecalis* were ligated into the cloning vector pBR322, and transformed into *E. coli* KAM32 (Δ *acrB*, Δ *ydhE*). We obtained four recombinant plasmids using TPPCI as an antimicrobial agent for selection. Restriction analysis revealed that these four plasmids contained the same DNA region. One of them, pTEF7, was used for further analysis (Fig. 1). The insert of pTEF7 was about 2.9 kbp long. Sequencing of the DNA insert in plasmid pTEF7 revealed that it contained two complete open reading frames (ORF1 and ORF2) and one of them, ORF2, was found to be almost same as *emeA*¹⁴⁾ (locus EF1078),²³⁾ which

encodes a multidrug resistance protein.

We constructed several hybrid plasmids carrying various portions of the insert DNA region from pTEF7. The resulting hybrid plasmids were introduced into *E. coli* KAM32 cells, and the transformants were tested for sensitivity or resistance to TPPCI (Fig. 1). A plasmid pTEF71 contained the whole region of *emeA* and a part of ORF1. *E. coli* KAM32 cells harboring plasmid pTEF71 showed resistance to TPPCI. A plasmid pTEF72, which carried only *emeA* located downstream from the tet promoter of pBR322, conferred drug resistance. Plasmid pTEF73, carrying most of the *emeA* gene, and plasmid pTEF78, carrying ORF1, did not confer drug resistance. Thus, it is clear that *emeA* alone is enough for the drug resistance if it is appropriately expressed.

We determined the nucleotide sequence of the whole *emeA* region because we cloned the gene from *E. faecalis* ATCC 29212, not from *E. faecalis* V583. We found that there were some differences (37 different nucleotides at various positions) in the sequence between the *emeA* gene of *E. faecalis* ATCC 29212 and the EF1078 locus of *E. faecalis* V583 (97% identity in the nucleotide sequence). The *emeA* gene was preceded by a possible promoter-like sequence and a ribosome-binding sequence (Shine–Dalgarno sequence). The gene was followed by a putative transcription terminator-like (inverted repeat) sequence. The *emeA* gene, 1179 bp in length, specifies a putative 393-amino acid protein with a calculated molecular mass of 42.1 kDa. The identity in the amino acid sequence between EmeA from strain ATCC29212 and V583 was 97.7%. Only nine amino acid residues (at the position 141–149) were different. Hydropathy analysis²⁴⁾ of EmeA predicted a highly hydrophobic protein with 12 membrane-spanning regions and hydrophilic sequences at both the –NH₂ and –COOH termini of the protein (data not shown).

The deduced amino acid sequence of the EmeA showed 80, 47, 46 and 35% identities and 89, 64, 66, and 61% similarities with putative Blt of *Lactococcus lactis*,²⁵⁾ Bmr of *Bacillus subtilis*,²⁶⁾ Blt of *B. subtilis*,²⁷⁾ and NorA of *Staphylococcus aureus*,²⁸⁾ respectively, as suggested by Jonas *et al.*¹⁴⁾

Drug Susceptibility Studies in *E. coli* To define the contribution of EmeA towards mediating drug resistance, the

Fig. 1. Restriction Map of Plasmid pTEF7 and Its Derivatives

Horizontal bars indicate DNA regions derived from the chromosomal DNA of *E. faecalis*. Plus signs (+) on the right hand side indicate that *E. coli* KAM32 cells harboring each plasmid grew on an L plate containing 10 μg/ml TPPCI and 60 μg/ml ampicillin. Minus signs (-) indicate that cells did not grow on the same plates. The arrow indicates the position and direction of the *emeA* gene. Restriction sites are shown at the top.

protein was expressed in *E. coli* KAM32, which is hypersensitive to many drugs due to deficiencies of the major multidrug efflux pumps AcrAB and YdhE.⁶ The drug susceptibility in cells of KAM32/pTEF71 (carrying *emeA*) and KAM32/pBR322 (control) are shown in Table 1. Plasmid pTEF71 in KAM32 cells conferred resistance to several structurally unrelated drugs. The MICs of TPPCI, DAPI, Hoechst 33342, acriflavine, benzalkonium chloride, norfloxacin, ciprofloxacin, and ethidium bromide in *E. coli* KAM32/pTEF71 were 16-, 8-, 8-, 4-, 8-, 8-, 4-, and 2-fold higher, respectively, than the control. Thus, we conclude that the EmeA is a protein conferring multidrug resistance, perhaps a multidrug efflux pump. No significant change in the MIC was observed with the other drugs tested in this study (Table 1).

EmeA-Mediated Efflux of Various Fluorescent Antimicrobial Agents It has been reported that EmeA mediates efflux of ethidium from cells.¹⁴ We further tested whether EmeA mediates efflux of structurally dissimilar compounds DAPI, Hoechst 33342 and acriflavine (Fig. 2). In the cases involving DAPI and Hoechst 33342, fluorescence of dilute solution increases when each are bound to DNA,^{20,21} indicating that the fluorescent dye is accumulated in the cells. On the other hand, the fluorescence of acriflavine decreases when it binds to DNA.⁶ In Fig. 2, energy-dependent effluxes of DAPI and Hoechst 33342 are shown as rapid decrease in

their fluorescence intensities, whereas efflux of acriflavine is observed as a rapid increase in the fluorescence. These results showed that structurally different chemicals (at least three substrates listed here) were extruded *via* EmeA. Thus, EmeA is indeed a multiple drug efflux pump.

EmeA-mediated efflux of all three of the substrates tested was abolished by the addition of CCCP (data not shown), a proton conductor, suggesting that a proton-motive force drives the EmeA-mediated extrusion of several structurally dissimilar antimicrobial agents.

Subsequently, we tested the effect of monovalent cations, such as Na⁺, Li⁺ or K⁺, in the efflux process because several multidrug efflux pumps are coupled with Na⁺.^{6,19,29} The addition of either KCl, NaCl or LiCl showed no significant difference in the efflux activity compared to the control (data not shown). Thus, we conclude that the drug efflux *via* the EmeA pump is an H⁺-coupled one.

Inhibition of EmeA-Mediated Drug Efflux by Reserpine Reserpine is known as an inhibitor of a wide range of multidrug efflux pumps. These systems include P-glycoprotein³⁰ (a member of the ABC super-family), Bmr from *B. subtilis*,³¹ LmrP from *L. lactis*,³² and NorA from *S. aureus*.³³ (members of major facilitator superfamily (MFS)), and EmrE from *E. coli*.³⁴ (a member of small multidrug resistance super-family). Jonas *et al.*¹⁴ reported that reserpine (at 20 µg/ml; roughly 33 µM) only slightly inhibited efflux of ethidium *via* EmeA. We investigated the effect of reserpine on the EmeA-mediated efflux of DAPI. We observed a very strong inhibition of EmeA activity due to reserpine (Fig. 3). The concentration of reserpine giving 50% inhibition (IC₅₀) of the initial rate of EmeA-mediated DAPI efflux was calculated to be 15 µM. Similar strong inhibition by reserpine was observed with ethidium as an efflux substrate (data not shown). The reason for the difference with the observation by Jonas is not clear at the present time.

DISCUSSION

Recently, the nucleotide sequencing of the entire chromosome and plasmids of *E. faecalis* V583 has been completed and are available on the web.²³ Based on the sequence information, Jonas *et al.*¹⁴ reported on the PCR cloning of a gene *emeA* encoding a multidrug efflux pump from *E. faecalis* strains OG1RF and V583, and reported some of the properties of the EmeA pump. They reported that EmeA was re-

Table 1. MICs of Various Drugs in *E. coli* KAM32 Harboring Either Control Plasmid or Plasmid Carrying *emeA* Gene

Drug	MICs (µg/ml)	
	KAM32/pBR322	KAM32/pTEF71
TPPCI	8	128
DAPI	<0.06	0.5
Hoechst 33342	0.5	4
Acriflavine	1	4
Benzalkonium	0.015	0.12
Ethidium bromide	2	4
Norfloxacin	0.03	0.25
Ciprofloxacin	0.004	0.015
Chloramphenicol	1	1
Erythromycin	4	4
Tetracycline	0.5 ^{a)}	0.5
Rhodamine 6G	8	8

a) Value with KAM32 not harboring pBR322.

Fig. 2. Fluorometric Assays of Drug Efflux

Efflux of DAPI (A), Hoechst 33342 (B), and acriflavine (C) from cells of KAM32/pBR322 or cells of KAM32/pTEF71 (carrying *emeA*) were measured. Energy-starved cells were loaded either with DAPI, Hoechst 33342 or acriflavine. Glucose (20 mM) was added at time point indicated by downward arrow to energize the cells. Efflux of DAPI or Hoechst 33342 is represented by a rapid decrease in the fluorescence, whereas efflux of acriflavine was observed as a rapid increase in the fluorescence.

Fig. 3. Inhibition of EmeA-Mediated DAPI Efflux by Reserpine

Energy-starved cells of KAM32/pTEF71 were loaded with DAPI. Cells were resuspended in 0.1 M MOPS-TMAH (pH 7.0) containing 2 mM MgSO_4 , 20 mM KCl and 5 μM DAPI. After incubation for 5 min, 20 mM glucose was added (indicated by an arrow) to energize the cells. Efflux of DAPI from the cells was measured either in the absence or presence of various concentrations (0 to 35 μM) of reserpine.

sponsible for resistance to ethidium bromide, norfloxacin, acriflavine, clindamycin and erythromycin, but not against benzalkonium chloride. On the other hand, EmeA derived from strain ATCC 29212 and expressed in *E. coli* cells showed resistance to TPPCl, DAPI, Hoechst 33342, norfloxacin, ethidium bromide and benzalkonium chloride, but not against erythromycin. These differences may be due to the distinctiveness of the organisms involved (*E. faecalis* vs. *E. coli*). It seems possible that *E. faecalis* cells possess another pump (or other resistance mechanisms) for benzalkonium. Erythromycin is not very effective against *E. coli* because of the presence of an outer membrane, a known permeability barrier for large molecules.

Ethidium bromide is often used as a substrate during the fluorometric assay of multidrug efflux pump activities. In the case of EmeA, it seemed that fluorescent DAPI, Hoechst 33342 and acriflavine were much better substrates (Table 1). Thus, we investigated the efflux properties of these fluorescent antimicrobial agents. We clearly observed an energy-dependent efflux of these compounds. We previously demonstrated that acriflavine was a good substrate for the fluorometric assay of the multidrug efflux pump, VmrA.⁶⁾ We also found that DAPI was a good substrate for the fluorometric assay of multidrug efflux pumps (J. Chen *et al.*, will be published elsewhere). Now we can use these fluorescent probes for the measurement of multidrug efflux pump activities, depending upon their substrate specificities.

Comparison of the deduced amino acid sequences of the two EmeAs revealed that there were differences at 9 positions. These differences may be due to variation in the original strains. The conserved sequences of the 12 putative transmembrane segments (TMS) of the MFS, motifs A, B, C, D2 and G,³⁾ were all conserved well in the EmeA sequence at the corresponding positions (data not shown). Pao *et al.*³⁵⁾ classified current members of the MFS into 17 (possibly 18) distinct families. Bmr and Blt of *B. subtilis* and NorA of *S. aureus*, homologues of EmeA from *E. faecalis*,¹⁴⁾ were included in family 3, the Drug: H^+ antiporter (DHA12) family, of the MFS according to this classification. Therefore, it seems that the EmeA protein is a member of the DHA12 group of the MFS judging from the sequence homology and the conserved domains.

Klyachko *et al.*³⁶⁾ reported that various substitutions of

residues Phe-143 and Phe-306 of Bmr not only reduced its sensitivity to reserpine but also significantly changed its substrate specificity, and these two amino acid residues are likely to be involved in substrate recognition. These two Phe residues are conserved at corresponding positions (positions 143 and 315) in the EmeA protein.

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