Functional Gene Cloning and Characterization of MdeA, a Multidrug Efflux Pump from Staphylococcus aureus

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A DNA fragment conferring drug resistance was cloned from the chromosomal DNA of Staphylococcus aureus N315 using a drug hypersensitive Escherichia coli KAM32 as the host. Although E. coli KAM32 cells were sensitive to many antimicrobial agents, transformed cells harboring a recombinant plasmid carrying the DNA region became resistant to several structurally unrelated antimicrobial agents, such as tetracyphenolphosphonium chloride, Hoechst 33342 and norfloxacin. These results suggest that the cloned DNA fragment carries a gene(s) encoding a multidrug efflux pump. We partially determined the nucleotide sequence of the cloned DNA and found the mdeA gene within it. The E. coli cells transformed with the mdeA gene showed efflux activity of Hoechst 33342. On the other hand, S. aureus cells transformed with mdeA showed elevated resistance to doxorubicin, daunorubicin, tetracyphenolphosphonium chloride, Hoechst 33342, ethidium bromide and rhodamine 6G. Elevated energy-dependent efflux of ethidium was observed with transformed S. aureus. We found that the mdeA gene was expressed under normal growth conditions in S. aureus N315.

Key words  MdeA; multidrug efflux pump; drug resistance; Staphylococcus aureus

Infectious diseases pose a great threat for human beings. After World War II, many antibiotics have been discovered, developed, and used for the treatment of patients infected with pathogenic bacteria. It had been expected that infectious diseases might be well controlled by using the antibiotics. However, drug resistant bacteria appeared soon after the use of antibiotics. Nowadays, bacteria that show resistance against multiple drugs appeared and are called multidrug resistant bacteria. It is very difficult to treat patients infected with multidrug resistant bacteria. Only a few antimicrobial agents are effective on the multidrug resistant bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci (VRE) and so on. Thus, emergence and widespread occurrence of multidrug resistant bacteria are big problems for the health of human beings.

There are several mechanisms of drug resistance in bacteria; 1) inactivation of the drug by degradative- or modification-enzymes, 2) alteration of the drug target, 3) appearance of alternative enzymes that are not inhibited by the drug, 4) reduced membrane permeability of the drug, and 5) active extrusion of the drug from cells. Among these mechanisms, drug extrusion has been recognized as the major mechanism for multidrug resistance. Multidrug efflux pumps extrude structurally unrelated multiple drugs. Thus, multidrug efflux pumps are most important for bacterial escape from the toxicity of compounds of various structures.

In order to successfully control multidrug resistant bacteria, extensive knowledge of the properties is required of the multidrug efflux pumps and of the molecular mechanisms underlying microbial antibiotic resistance. MRSA occupies the highest percentage of bacterial isolates that are drug resistant in hospitals.11

Although only five multidrug efflux pumps encoded by the chromosomal DNA of S. aureus have been reported,2–5 there are many (more than 30) genes for putative multidrug efflux pumps judging from the genome sequence data.11 The five reported multidrug efflux pumps are, NorA,2–5 SepA,6 MdeA,7 NorB8 and MepA.9,10 The NorA pump has been well characterized.2–6 However, the properties of the other pumps are not as well elucidated. We have been trying to clone as many genes for the multidrug efflux pumps as possible from the chromosome of S. aureus in order to gain insight into the complete picture of the multidrug efflux pumps in this microorganism. We employed two strategies for the gene cloning of the multidrug efflux pumps; 1) functional cloning using the drug hyper-susceptible Escherichia coli strain as a host, and 2) PCR cloning of all possible genes for multidrug efflux pumps using the S. aureus drug susceptible strain as a host. It is advantageous to use a strain of S. aureus for which the genome sequence is available as a source of chromosomal DNA.11 Here we report the functional cloning of a gene for a multidrug efflux pump, which was identified as MdeA, and the characterization of the pump in cells of both E. coli and S. aureus.

MATERIALS AND METHODS

Bacteria and Growth S. aureus N315 (an MRSA strain), of which the genome project has been completed and the genome information available,11 was used as a source of chromosomal DNA. Escherichia coli strain KAM3212 (a drug hyper-susceptible strain) lacking two multidrug efflux pumps AcrAB and YdhE, and S. aureus RN4220 (a methicillin sensitive strain), were used as hosts. S. aureus N315 cells and RN4220 cells were grown in a nutrient medium (NISSUI Co.), and E. coli KAM32 cells were grown in L medium (1% polypepton, 0.5% sodium chloride, 0.5% yeast extract) at 37°C. Growth of cells was monitored turbidimetrically at 650 nm.

Gene Cloning and Sequencing Chromosomal DNA was prepared from S. aureus N315 by the method of Berns and Thomas13 The DNA was partially digested with a re-
striction enzyme Sau3AI, and the fragments ranging in size from 4 to 10 kbp were separated by sucrose density gradient centrifugation. Plasmid pBluescript II KS (+) (TOYOBO Co.) was digested with BamHI, dephosphorylated with bacterial alkaline phosphatase and ligated with the chromosomal DNA fragments by using a Ligation Kit Ver. 2 (TaKaRa Co.). Competent cells of E. coli KAM32 were transformed with recombinant plasmids and were spread onto agar plates containing L broth, 9 µg/ml of tetraphenylphosphonium chloride (TPPCl), 100 µg/ml ampicillin 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 the candidates. Plasmids were reintroduced into E. coli KAM32 cells and spread onto the same type of plate. The plasmids were then isolated from each of the transformants that appeared on the plate. One of the candidate plasmids possibly carrying a gene responsible for TPPCl resistance was designated as pBTP3. For sub-cloning, the insert in the pBTP3 plasmid was digested with NsiI and blunt ended with T4 polymerase (TaKaRa Co.) and ligated to the HincII site of pSTV28, a vector. The resulting recombinant plasmid, designated as pSTP2, was introduced into E. coli KAM32 cells, and the transformants were tested for sensitivity or resistance to TPPCl. The pSTP2 plasmid was digested with EcoRI and PsI, and ligated to the corresponding sites of pRT5, a shuttle vector between E. coli and S. aureus. The resulting recombinant plasmid was introduced into S. aureus RN4220 cells. The resulting plasmid was designated as pRTP2.

The nucleotide sequence was determined by the di-deoxy chain termination method using a DNA sequencer (ALF Express, Pharmacia Biotech.).

Drug Susceptibility Testing The minimal inhibitory concentrations (MICs) of various drugs were determined in Mueller-Hinton (MH) broth (Difco) containing different drugs at various concentrations as reported previously. Cells were incubated in the medium at 37 °C for 24 h, and the growth was examined by visual inspection.

Hoechst 33342 Efflux Assay in E. coli Cells Cells of E. coli KAM32 harboring control plasmid or recombinant plasmid were grown in 10 ml of L broth. After harvesting, the cells were washed with modified Tanaka buffer, in which sodium salts were replaced with potassium salts, and then resuspended in the same buffer containing 1 µM of Hoechst 33342 and 5 mM 2,4-dinitrophenol (DNP) and incubated at 37 °C for 10 h. Cells were washed with 0.1 M Mops-tetramethylammonium hydroxide (pH 7.0) containing 2 mM MgSO₄, and resuspended in the same buffer. Changes in fluorescence intensities of Hoechst 33342 were measured with a fluorescent spectrometer at excitation and emission wavelengths of 355 nm and 457 nm, respectively. The cell suspension was incubated at 37 °C for 5 min, and then glucose was added as an energy source to the cells.

Ethidium Efflux Assay in S. aureus Cells Cells were harvested at the late exponential phase of growth and washed twice with 20 mM HEPEs–NaOH buffer (pH 7.0). Cells were then suspended in HEPEs–NaOH buffer containing carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 20 µM and ethidium bromide at 5 µM, followed by incubation at 37 °C for 30 min. The cells were centrifuged, washed, and resuspended in the HEPEs–NaOH buffer, and changes in fluorescence intensities were measured with a fluorescent spectrometer at 530 nm (excitation) and 600 nm (emission) wavelengths.

RT–PCR Analysis Cells of S. aureus N315 were harvested at the exponential phase of growth. Total cellular RNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen Inc., U.S.A.), treated with RNase-free DNase (Promega, U.S.A.) (1 U of enzyme/µg RNA for 2 h at 37 °C) and re-purified using the same kit. A 1 ng sample of DNase-treated RNA was used as a template for RT-PCR with the OneStep RT-PCR Kit (Qiagen Inc., U.S.A.) according to the manufacturer’s protocol. Primer pairs specific for and internal to mdeA, norA or rpsL (encoding a constitutively expressed gene for ribosomal protein; a control) genes were used for RT-PCR. Primers used for mdeA were GCCATGTAGCACAAGA (forward) and GGAGCGACA- ACATGGAAAAG (reverse); for norA were CTGCTATGTTG- AATGCTTGGT (forward) and TCGCTGACATGAGC- CAAA (reverse); and for rpsL were CACCCAAAAA- CGTGGTGTATG (forward) and TGGTTGTTGATAAAC- GCACAG (reverse). The reaction mixtures were incubated for 30 min at 50 °C, followed by 15 min at 95 °C, and 28 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. A 5 µl sample of each reaction product was analyzed by Agarose X (3% w/v) gel electrophoresis for the presence of expected RT-PCR products (for mdeA 168 bp, for norA 90 bp, and for rpsL 102 bp).

RESULTS

We cloned a gene responsible for resistance to TPPCl from the chromosomal DNA of S. aureus N315. Introduction of a plasmid pBTP3 carrying the gene into E. coli KAM32 cells that originally showed hyper-susceptibility to many antimicrobial agents conferred elevated resistance to (higher MIC of) TPPCl. We found that there were 3 open reading frames (ORFs) derived from the chromosome of S. aureus in the recombinant plasmid pRTP3. We sub-cloned the ORFs and found that ORF SA2203 was responsible for the elevated resistance. The deduced amino acid sequence of the protein encoded by the SA2203 showed 99% identity with MdeA of S. aureus Buttle strain. Therefore we conclude that the ORF SA2203 in S. aureus N315 corresponds to mdeA of the Buttle strain. Some difference in the deduced amino acid sequences might be due to differences in the two strains. The MdeA protein has been reported to be a multidrug efflux pump belonging to the MF family with 14 predicted transmembrane segments. Thus, it seems that MdeA of N315 also possesses 14 predicted transmembrane segments.

We constructed a plasmid pSTP2 which carries the mdeA gene (SA2203) and its putative promoter, and analyzed the properties of MdeA in E. coli cells. We determined the MIC values of 20 antimicrobial agents: ampicillin, erythromycin, streptomycin, tetracycline, norfloxacin, ciprofloxacin, ofloxacin, nalidixic acid, acriflavine, etidium bromide, rhodamine 6G, 4′,6-diamidino-2-phenylindole, benzalkonium, chlorhexidine, sodium dodecyl sulfate, doxorubicin, daunorubicin, triclorans, TPPCl and Hoechst 33342. Cells of E. coli KAM32/pSTP2 showed elevated MICs of Hoechst 33342 (16-fold), TPPCl (8-fold) and norfloxacin (2-fold) compared with cells of E. coli KAM32 (Table 1). No change in the MIC value was observed with 17 antimicrobial
agents. The mdeA gene is located in the multicloning site of vector pSTV28 in the opposite direction to the lac promoter. We also constructed a plasmid in which the mdeA gene is located downstream from the lac promoter in the same direction. We observed no difference in the MIC values between the two types of cells harboring either one of the plasmids (data not shown). Thus, we conclude that the putative promoter present upstream from the mdeA gene can function as a promoter in E. coli cells.

We investigated efflux activity of MdeA. We prepared energy-starved cells and loaded with Hoechst 33342. Addition of glucose as an energy donor elicited a large efflux of energy-starved cells and loaded with Hoechst 33342. At the time point indicated by an arrow, glucose (final concentration, 10 mM) was added to energize the cells. The fluorescence of Hoechst 33342 was monitored with a fluorescence spectrometer at 37 °C over time. The downward deflection indicates the efflux of Hoechst 33342 from the cells.

We cloned the mdeA gene using E. coli cells as the host, because E. coli is a very convenient host for gene cloning, and analyzed some properties in these E. coli cells. However, since mdeA is a gene from S. aureus, it is important to analyze the properties of MdeA in cells of S. aureus. We constructed a plasmid pRTP2 which carries the mdeA gene using pRIT5 as a shuttle vector between E. coli and S. aureus,[14] and introduced the resulting plasmid pRTP2 into cells of S. aureus RN4220. We determined the MIC values of the 20 antimicrobial agents described above with S. aureus RN4220/pRTP2 (carrying the mdeA gene) and RN4220/pRIT5 (control). Cells of RN4220/pRTP2 showed elevated MICs of doxorubicin (8-fold), daunorubicin (4-fold), ethidium bromide (4-fold), Hoechst 33342 (4-fold), TPPC1 (4-fold), ciprofloxacin (2-fold), acriflavine (2-fold) and rhodamine 6G (2-fold) compared with cells of RN4220/pRIT5 (Table 2). These increases in the MICs were reproducible. The mdeA gene is located downstream from the spa promoter of S. aureus in the opposite direction in the pRTP2 plasmid. We also constructed a plasmid in which the mdeA gene is located in the downstream region of the spa promoter with the same direction. However, we observed no difference in MIC values between cells harboring either one of the plasmids (data not shown). Therefore we conclude that the mdeA gene is expressed from its own promoter, and the spa promoter gave no significant effect on the expression of the mdeA gene in the plasmid.

We measured ethidium efflux activity in S. aureus RN4220 cells harboring pRTP2 carrying the mdeA gene. We observed elevated energy-dependent efflux of ethidium from cells of S. aureus RN4220/pRTP2 (Fig. 2). Little efflux was observed with cells of RN4220/pRIT5 (control), which would be due to efflux pumps present in the RN4220 strain. Thus, it is clear that MdeA is a functional drug efflux pump in cells of S. aureus.

Although it became clear that MdeA is functional in S. aureus cells when mdeA is introduced in a multicopy-number plasmid, it is important to test whether mdeA is expressed from the chromosome in the original N315 cells. Therefore, we measured expression of the mdeA gene in cells of S. aureus N315 grown in a nutrient medium by the RT-PCR method. We found that mdeA is indeed expressed in S. aureus N315 (Fig. 3). It seemed that the expression level was roughly similar to that of norA, a gene for a multidrug efflux pump, NorA, a major multidrug efflux pump in S. aureus.
Among the 20 antimicrobial agents tested, Hoechst 33342, TPPC1 and norfloxacin were found to be substrates for MdeA produced in E. coli cells. On the other hand, doxorubicin, daunorubicin, ethidium bromide, Hoechst 33342, TPPC1, ciprofloxacin, acriflavine and rhodamine 6G were found to be substrates for MdeA in S. aureus cells. The MIC values in S. aureus RN4220 cells were higher than those in E. coli KAM32 cells. Although we do not know the exact reason for this discrepancy in substrate specificity, there are several possibilities. The presence of the outer membrane in E. coli, but not in S. aureus, may affect this property of the pump, or differences in membrane component(s) (or cellular component) in the two bacteria may affect the property. A difference in substrate specificity when expressed in S. aureus and in E. coli has not been reported with NorA. Thus, a factor which modulates the property of MdeA may be present in cells of S. aureus or E. coli.

Huang et al. reported that benzalkonium chloride was a substrate for MdeA in the S. aureus Buttle strain. However, we were unable to repeat this result. We noticed that there were some differences in the deduced amino acid sequences (5 residues among 479 residues) in the MdeAs derived from S. aureus Buttle strain and S. aureus N315 strain. The different residues are as follows; T81S in putative trans-membrane segment 3 (TMS3), F159L in putative loop region between TMS5 and TMS6, I234V in TMS8, A235V in TMS8, and F338I in TMS11. These differences may affect the substrate specificity in MdeA. They also reported that fluoroquinolone was not a substrate for MdeA. However, we observed some changes in MIC values of fluoroquinolones between S. aureus RN4220 and RN4220 harboring a plasmid carrying the mdeA gene, and between E. coli KAM32 and KAM32 harboring a plasmid carrying mdeA. Thus, it seems that fluoroquinolones are moderate substrates for MdeA.

We found that the gene mdeA was expressed in cells of S. aureus N315 to a certain level. The expression level of mdeA was roughly similar to that of norA. It has been reported that disruption of the norA gene of S. aureus resulted in a decrease in the MIC values of several antimicrobial agents. These results indicate that NorA is involved in the intrinsic resistance of S. aureus against several antimicrobial agents. Also, it has been reported that over-expression of norA resulted in elevated resistance against several drugs. Thus, it is likely that MdeA is also involved in intrinsic resistances against multiple antimicrobial agents in S. aureus N315, and further elevated expression due to mutation in the promoter region of mdeA will result in elevated resistances against multiple drugs. Since there are many genes for putative multidrug efflux pumps in S. aureus, over-expression of one or some of such gene(s) should result in more elevated MIC values of multiple antimicrobial agents. This suggests that super MRSAs that show much higher resistance to many antimicrobial agents may emerge. Therefore we believe that analyses of whole multidrug efflux pumps in S. aureus are important.

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