Characterization of novel ybjG and dacC variants in Escherichia coli

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A total of 69 strains of Escherichia coli from patients in the Taizhou Municipal Hospital, China, were isolated, and 11 strains were identified that were resistant to bacitracin, chloramphenicol, tetracycline and erythromycin. These strains were PCR positive for at least two out of three genes, ybjG, dacC and mdfA, by gene mapping with conventional PCR detection. Conjugation experiments demonstrated that these genes existed in plasmids that conferred resistance. Novel ybjG and dacC variants were isolated from E. coli strains EC2163 and EC2347, which were obtained from the sputum of intensive care unit patients. Genetic mapping showed that the genes were located on 8200 kb plasmid regions flanked by EcoRI restriction sites. Three distinct genetic structures were identified among the 11 PCR-positive strains of E. coli, and two contained the novel ybjG and dacC variants. The putative amino acid differences in the ybjG and dacC gene variants were characterized. These results provide evidence for novel variants of ybjG and dacC, and suggest that multiple drug resistance in hospital strains of E. coli depends on the synergistic function of ybjG, dacC and mdfA within three distinct genetic structures in conjugative plasmids.

INTRODUCTION

The Escherichia coli YbjG and MdfA proteins are two of the four transmembrane proteins, and MdfA protein is of the major facilitator superfamily (MFS) (Ping et al., 2007), a large, highly conserved family of membrane transporters that have roles in drug resistance. YbjG is similar to the bacitracin-resistance protein BcrC of Bacillus licheniformis. Disruption of this gene causes increased bacitracin sensitivity in E. coli, while overexpression causes increased resistance to bacitracin (Harel et al., 1999; El Ghachi et al., 2005). YbjG has also been identified as a lactoperoxidase–thiocyanate system inducible gene (Sermon et al., 2005). Simultaneous inactivation of ybjG, bacA, and pgpB causes lethality by inducing changes in cell morphology and lysis.

Conversely, overexpression of any of these three genes, or a fourth one, yeiU, leads to increased undecaprenyl pyrophosphatase (C55PP) activity in crude membrane extracts and survival in the presence of bacitracin (El Ghachi et al., 2005). The expression of ybjG is increased in an evgS1 mutant and may be directly regulated by PhoP (Eguchi et al., 2004).

Another E. coli gene within the MFS family, dacC, expresses D-alanyl-D-alanine carboxypeptidase, otherwise known as penicillin-binding protein 6 (PBP6) (Lau et al., 2011). PBP6 is required for proper cell morphology and provides some resistance to penicillin (van der Linden et al., 1992; Santos et al., 2002). PBP6 contains the β-lactam/penicillin-binding domain motif sequences SxxK (SLTK), S/YxyN (SGN), and [K/H][T/S]G (KTG) (Baquero et al., 1996). The C terminus of PBP6 is capable of forming an α-helix and interacts with membranes chiefly through hydrophobic forces (Harris & Phoenix, 1997; Siligardi et al., 1997; Harris et al., 1998, 2002). It is one of four D-carboxypeptidase low-molecular-mass PBPs in E. coli (along with PBP4, PBP5 and DacD) that modify peptidoglycans by removing the terminal D-alanine from their pentapeptide side chains (Baquero et al., 1996).

†These authors contributed equally to this work.

Abbreviations: ICU, intensive care unit; MFS, major facilitator superfamily; PBP, penicillin-binding protein; UppP, undecaprenyl pyrophosphate phosphatase.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are JQ655766 (ybjG) and J0409457 (dacC).
The MdfA protein, also known as Cmr, is a multi-drug efflux protein belonging to the MFS (Paulsen et al., 1996). Transport experiments in an E. coli unc gene mutant have indicated that mdfA confers resistance via an efflux mechanism dependent on the proton motive force (Paulsen et al., 1996). Overexpression of mdfA also results in spectinomycin sensitivity and IPTG exclusion due to unknown mechanisms (Bohn & Bouloc, 1998).

Twelve transmembrane domains have been predicted from the hydropathy plot of the protein and confirmed by gene fusion analysis (Edgar & Bibi, 1997; Adler & Bibi, 2002). MdfA can function as a multidrug-resistance transporter and also mediates alkaline resistance. Inverted vesicle fluorescence studies have demonstrated that MdfA catalyses Na\(^{+}\)- or K\(^{+}\)-dependent proton transport (Lewinsson et al., 2004). MdfA has also been implicated in arabinose efflux (Koita & Rao, 2012).

In this study, the ybjG, dacC and mdfA genes and seven other related genes were sequenced from hospital strains of E. coli to identify new variants that might contribute to multidrug resistance. Novel ybjG and dacC gene variants were found in E. coli strains EC2163 and EC2347, which came from the sputum of patients in the intensive care unit (ICU) of Taizhou Municipal Hospital, China. This paper describes the identification of novel ybjG and dacC variants in E. coli and the genetic mapping of three distinct conjugative plasmid structures in which the genes are encoded.

**METHODS**

**Bacterial strains.** A total of 69 non-redundant multidrug isolates of E. coli were recovered from hospitalized patients with clinical infections from February 2010 to November 2011 in the Taizhou Municipal Hospital, which is affiliated with Taizhou University of China. The patients were distributed among six clinical units: the ICU (19/69) and the infection (13/69), neurosurgery (11/69), respiratory (11/69), urology (10/69) and neurology (5/69) units. Strains were isolated from sputum (35/69), blood (15/69), urine (14/69) and venous cannula (5/69), and all isolates were assigned as E. coli using a Vitek GN+ card (bioMérieux); simultaneously, species identification was confirmed by sequence analysis of the 16S–23S rRNA gene intergenic spacer region. The strains EC2163 and EC2347 came from patients in the ICU, and the strains EC2271 and EC2314 were isolated from the infection and urology units, respectively.

**Conjugation and susceptibility testing.** Conjugation experiments were carried out in Luria–Bertani broth with E. coli J53 Azide\(^{R}\) (J53Az\(^{R}\); a strain with resistance to sodium azide) as the recipient; strains of EC2163 and EC2347 that were positive by PCR amplification were used as the donor strains (Wang et al., 2003). Cultures of donor and recipient cells in the exponential phase (0.5 ml of each) were added to 4 ml fresh Luria–Bertani broth and incubated at 37 °C overnight without shaking.

Transconjugants (J53Az\(^{R}\)–EC2163 and J53Az\(^{R}\)–EC2347) were selected on Trypticase soy agar plates supplemented with 300 mg sodium azide l\(^{-1}\) and 0.03 mg ciprofloxacin l\(^{-1}\), and were incubated for 18–24 h at 35 °C. Susceptibility was assessed by determining MIC values. MICs of antibiotics for the donor, recipient and transconjugant strains were compared to determine if the plasmids harbouring ybjG, dacC and mdfA genes were transferred (Table 1). The antimicrobial agents were used in a susceptibility plate using the MicroScan broth dilution method for antibiotics, which comprised bacitracin, amikacin, aztreonam, cefazolin, cefotetan, ceftazidime, ceftriaxone, chloramphenicol, ofloxacin, erythromycin, gentamicin, imipenem, tetracycline and tobramycin. The MICs were determined by broth dilution and interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012).

**PCR amplification and plasmid mapping.** To characterize the plasmids harbouring ybjG, dacC and surrounding genes, as well as the sequences of those genes, plasmid DNA fragments were extracted (Axygen kit) and separated by electrophoresis on 0.6 % agarose gels (60 V, 90 min; Promega). The gels were then digested and plasmid DNA of different sizes was recovered. We used each of the recovered plasmid DNAs as templates for amplification of ybjG, dacC and surrounding genes.

The PCR system and parameters that were used to characterize gene expression in EC2163, EC2347 and other strains have been described previously (Sulavik et al., 2001). The primers used to amplify ybjG, dacC and surrounding genes (gstB, deoR, mdfA, ybjH, ybjI, ybjK, ybjK and ybjJ) are listed in Table 2. Plasmid fragments from the E. coli strains were cloned by digesting total DNA with EcoRI, ligating it into EcoRI-digested plasmid pBKCMV and transforming the recombinant plasmids into E. coli DH10B. Recombinant plasmids were selected on Trypticase soy agar plates containing amoxicillin (50 g l\(^{-1}\)) and kanamycin (30 g l\(^{-1}\)). The initial positions of the recombinant plasmids were determined and the estimated size of the recombinant plasmid DNA was referenced (Kitagawa et al., 2005). The cloned DNA fragments of the recombinant plasmids were sequenced and analysed as described previously (Santos et al., 2002). The genetic structures associated with these genes for the 11 positive E. coli strains are shown in Fig. 1.

**RESULTS**

**Isolation of antibiotic-resistant strains of E. coli**

To identify novel variants of E. coli antibiotic-resistance genes, we isolated E. coli from patients with clinical infections at the Taizhou Municipal Hospital, China. The majority of the 69 isolates of E. coli came from the ICU (27.5 %), infection unit (18.8 %) or neurosurgery unit (15.9 %), and most of the strains were isolated from sputum (50.7 %), blood (21.7 %) or urine (20.3 %). The strains EC2163, EC2347, EC2271 and EC2314 were four of the representative strains in the 11 strains of E. coli that were positive by PCR amplification.

All of the 69 isolates of E. coli were sensitive to the antibiotics amikacin, aztreonam, cefazolin, ceftazidime, ceftriaxone, ofloxacin, gentamicin, imipenem and tobramycin, but 11 of the 69 isolates of E. coli were resistant to bacitracin, chloramphenicol, erythromycin and tetracycline. The MIC values for each of the 11 resistant isolates were similar to those of EC2163 and EC2347, as listed in Table 1. Conjugation experiments for strains EC2163 and EC2347 were performed seven and five times, respectively, until plasmids were successfully transferred into J53Az\(^{R}\), an azide-resistant strain of E. coli J53. The susceptibility-testing results showed that the MIC values for the antibiotics bacitracin, chloramphenicol, erythromycin and tetracycline transconjugants (J53Az\(^{R}\)–EC2163 and J53Az\(^{R}\)–
Identification of novel variants of the \textit{ybjG} and \textit{dacC} genes in EC2163 and EC2347

DNA from the 11 isolates that displayed resistance to bacitracin, chloramphenicol, erythromycin and tetracycline was subjected to PCR amplification using the primers listed in Table 2. By sequencing and BLASTN (Altschul et al., 1990) analysis of PCR-positive results, we discovered a novel \textit{ybjG} gene variant in EC2163 and a novel \textit{dacC} gene variant in EC2347. A comparison of the putative amino acids for these novel gene variants and highly homologous sequences is shown in Fig. 1. JQ655766 had more than one putative amino acid difference from other YbjG proteins (Fig. 1A); and JQ409457 had only one putative amino acid difference (Asp59Ala) from other DacC proteins, such as GenBank accession nos. NP_415360 and NP_752850 (Fig. 1B).

Table 2. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide sequence (5′→3′)</th>
<th>Location</th>
<th>Reference</th>
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<tr>
<td>\textit{ybjG}</td>
<td>GCCCTGGAAAATTGGAATCTTCTC</td>
<td>\textit{ybjG} gene, forward primer</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>CCGETCAGCGACCCAGCCTTGGG</td>
<td>\textit{ybjG} gene, reverse primer</td>
<td></td>
</tr>
<tr>
<td>\textit{mdfA}</td>
<td>GCCCAAAATAAATTTAGCTTGGG</td>
<td>\textit{mdfA} gene, forward primer</td>
<td>Kitagawa et al. (2005)</td>
</tr>
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<td></td>
<td>CCCTTTCGTAGAAATTTCCCAT</td>
<td>\textit{mdfA} gene, reverse primer</td>
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<tr>
<td>\textit{gstB}</td>
<td>GCCATTACGCTGTGGGTTGCGAA</td>
<td>\textit{gstB} gene, forward primer</td>
<td>Kitagawa et al. (2005)</td>
</tr>
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<td></td>
<td>CGCTAAAAGGGAATCTACCAAC</td>
<td>\textit{gstB} gene, reverse primer</td>
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<tr>
<td>\textit{dacC}</td>
<td>GCCAGCGCAATATCTCTCTCTTCT</td>
<td>\textit{dacC} gene, forward primer</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>CCAGAGAAGAGAGCGGAAACCCA</td>
<td>\textit{dacC} gene, reverse primer</td>
<td></td>
</tr>
<tr>
<td>\textit{deoR}</td>
<td>GCCGAAACGAGCTGCAAGAGCG</td>
<td>\textit{deoR} gene, forward primer</td>
<td>Kitagawa et al. (2005)</td>
</tr>
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<td></td>
<td>CCATACATCAACTTAATGCGCTG</td>
<td>\textit{deoR} gene, reverse primer</td>
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<td>\textit{ybjH}</td>
<td>GCCATTATGAAAAATTGTCTACT</td>
<td>\textit{ybjH} gene, forward primer</td>
<td>Kitagawa et al. (2005)</td>
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<td>CAATGTTGATGTCATGCCATCTG</td>
<td>\textit{ybjH} gene, reverse primer</td>
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<td>GCCAGCTAAATATTATAGGCGGT</td>
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<td>CTTTGGTCAAAATTGCGCTTCT</td>
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<td>\textit{ybjJ}</td>
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<tr>
<td>\textit{ybjK}</td>
<td>GCCGGTGCTGCTGCTAAGTGCCTCGGA</td>
<td>\textit{ybjK} gene, forward primer</td>
<td>Kitagawa et al. (2005)</td>
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<tr>
<td></td>
<td>CCACCCTGCAACCCCTCTCACCAT</td>
<td>\textit{ybjK} gene, reverse primer</td>
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<td>\textit{ybjL}</td>
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<td></td>
<td>CCTCCTAATCCTCGCCATAACAT</td>
<td>\textit{ybjL} gene, reverse primer</td>
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Fig. 1. Partial alignment of the amino acid sequences with high homology to the novel \textit{ybjG} and \textit{dacC} genes deposited in GenBank. Accession numbers are indicated on the left. (a) Alignment of the amino acids for the novel \textit{ybjG} variant (JQ655766), which we identified from \textit{E. coli} strain EC2163, with other \textit{ybjG} that had significant homology (>98\% nucleotide identity). (b) Alignment of amino acids for the novel \textit{dacC} variant (JQ409457), which was identified from \textit{E. coli} strain EC2347, with other \textit{dacC} that had significant homology (>99\% nucleotide identity).
Preparation of recombinant plasmids to identify the genetic structure of drug-resistance regions by plasmid mapping

To examine the context of the novel ybjG and dacC genes, we characterized the region of the plasmids harbouring ybjG, dacC and surrounding genes by EcoRI restriction digest and cloning. The recombinant plasmid inserts were approximately 8200 bp. Three types of genetic structures (A, B and C) were identified in the recombinant plasmids of the 11 positive strains. Eight were of genetic structure A, and strain EC2163 was one of them. Genetic structure A comprised two partial-length and six full-length genes, including the novel ybjG gene, as well as the dacC and mdfA genes. Two positive strains, EC2271 and EC2314, were of genetic structure B. Genetic structure B comprised three full-length genes, ybjG, dacC and mdfA. Only one positive strain, EC2347, was of genetic structure C. This structure had two full-length genes, including mdfA and the novel dacC variant (Fig. 2).

DISCUSSION

In this study, we isolated drug-resistant E. coli strains from hospitalized patients to identify novel gene variants and genetic structures associated with multidrug resistance.

Three types of genetic structures, A, B and C, were identified in the recombinant plasmids of EC2163, EC2271, EC2314 and EC2347 and seven other resistant E. coli hospital strains. Genetic structure A has been reported previously (Santos et al., 2002); however, structures B and C are novel. Our study suggests that multiple drug resistance for the 11 PCR-positive strains depends on the genetic synergy between ybjG, dacC and mdfA. This was demonstrated by conjugation, susceptibility testing and plasmid mapping. The dacC and mdfA genes were both encoded within all three structures; however, ybjG was only encoded within structures A and B.

We showed that the ybjG gene is encoded within structures A and B from strains EC2163, EC2271 and EC2314. The coding product of the ybjG gene is undecaprenyl pyrophosphate phosphatase (UppP) (El Ghachi et al., 2005; Keseler et al., 2005; Kitagawa et al., 2005). UppP activity is essential for the synthesis of undecaprenyl phosphate, a C55 lipid carrier for cell wall synthesis. Therefore, a ybjG, pgpB and bacA triple mutant is lethal (Edgar & Bibi, 1997). Overexpression of ybjG, pgpB or bacA genes results in increased bacitracin resistance. Compared with Gram-positive species, E. coli and other Gram-negative species are not very sensitive to bacitracin, most likely because of the presence of the additional outer membrane barrier and limited uptake (Harel et al., 1999; Chalker et al., 2000; El Ghachi et al., 2005). However, increased expression of the latter three genes confers even greater resistance.

We also showed that the dacC and mdfA genes are encoded within all three genetic structures. Expression of dacC is induced upon entry into the stationary phase by BolA (Santos et al., 2002) under the regulation of the low-density lipoprotein receptor related protein (an apolipoprotein E binding protein) (Tani et al., 2002), but is also upregulated under basic conditions with oxygen limitation (Hayes et al., 2006) and upon treatment with cefsulodin and amdinocillin (Laubacher & Ades, 2008). Deletions in dacC are demonstrated to be viable and to have no obvious growth defects, though dacC mutants and dacCl/dacA double mutants show defects in morphology and cell division when bolA is overexpressed (Santos et al., 2002). The MdfA protein, also known as Cmr, is a multi-drug efflux protein.

Fig. 2. Genetic structure of the recombinant plasmids of 11 positive strains of E. coli. All of the recombinant plasmid inserts were approximately 8200 bp in length. The EcoRI restriction site that was used to clone the inserts is labelled with the corresponding gaattc sequence. Three types of genetic structures (A, B and C) were identified in the recombinant plasmids of the 11 positive strains. (a) Eight positive strains contained the sequence of genetic structure A, including strain EC2163. Structure A comprised two partial-length and six full-length genes, including mdfA and the novel ybjG and dacC gene variants. (b) Two positive strains, EC2271 and EC2314, contained sequence of genetic structure B, with three full-length genes, ybjG, dacC and mdfA, in the recombinant plasmids. (c) One positive strain, EC2347, contained sequence of genetic structure C, with two full-length genes, dacC and mdfA.
Novel ybjG and dacC genes were identified in recombinant plasmids derived from E. coli strains EC2163 and EC2347, respectively. The ybjG gene variant was identified in genetic structure A and the dacC variant in genetic structure C, and these were deposited in GenBank as JQ655766 and JQ409457, respectively. JQ655766 encodes a putative protein of 198 amino acid residues. Compared with corresponding high-homology ybjG genes from GenBank (>98% nucleotide identity), this putative protein had more than two amino acids that differed from other ybjG proteins, with the exception of GenBank no. NP_415362, which differed by only one amino acid. Variable amino acid positions included Val43Ala and Tyr141Asn. The novel dacC gene, JQ409457, showed one putative amino acid difference at site Asp59Ala compared with corresponding high homology (99% nucleotide identity) to NP_415360 from GenBank. How these variants of ybjG and dacC may affect their function is unknown. In addition, how ybjG, dacC, mdfA and similar genes function to synergistically promote drug resistance awaits further investigation.

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