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Research paper

Whole transcriptome analysis reveals potential novel mechanisms of low-level linezolid resistance in *Enterococcus faecalis*

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ABSTRACT

Linezolid is an oxazolidinone antibiotic commonly used to treat serious infections caused by vancomycin-resistant enterococcus. Recently, low-level linezolid resistant *Enterococcus faecalis* strains have emerged worldwide, but the resistant mechanisms remain undefined. Whole-transcriptome profiling was performed on an *E. faecalis* strain P10748 with low-level linezolid resistance in comparison with a linezolid-susceptible strain 3138 and the standard control strain ATCC29212. The functions of differentially expressed genes (DEGs) were predicted, with some DEGs potentially involved in drug resistance were validated by PCR and quantitative PCR (qPCR). RNA-Seq on three *E. faecalis* strains generated 1920 unigenes, with 98% of them assigned to various function groups. A total of 150 DEGs were identified in the linezolid resistant strain P10748 compared to the linezolid susceptible strains 3138 and ATCC29212. Functional analysis indicated a significant transcriptomic shift to membrane transportation and biofilm formation in strain P10748, with three significantly up-regulated DEGs predicted to be associated with drug resistance through active efflux pumps and biofilm formation. The existence of these three DEGs was further confirmed by PCR and qPCR. The significant upregulation of genes associated with efflux pumps and biofilm formation in the linezolid resistant strain suggests their roles in low-level resistance to linezolid in *E. faecalis*.

1. Introduction

Linezolid is the first member of a new class of synthetic antibiotics oxazolidinones, and has been approved since 2000 for treatment of infections caused by resistant Gram-positive bacteria including streptococci, vancomycin-resistant enterococci, and methicillin-resistant *S. aureus* (Brickner et al., 2008). The mechanism of action of linezolid is believed to serve as a protein synthesis blocker by binding to the A-site of the ribosomal peptidyl transferase center within the 50S subunit of the ribosome (Long and Vester, 2011). Despite its proven efficiency *in vitro* and in clinical use, there have been increasing reports of resistance to linezolid in clinic settings over the past 15 years (Gales et al., 2006; Tsiodras et al., 2001; de Almeida et al., 2014; O'Driscoll et al., 2015).

The main mechanisms of linezolid resistance in enterococci known to date include point mutations in 23S rRNA and ribosomal proteins L3 and L4 as well as presence of plasmid-carried multi-drug resistance gene *cfr* (Wang et al., 2014; Bøsling et al., 2003; Liu et al., 2012). However, these mechanisms cannot explain the reduced susceptibility or low-level resistance to linezolid in some enterococci identified in various antimicrobial resistance surveillance programs (Jones et al., 2009; Ross et al., 2011; Chen et al., 2013; Patel et al., 2013), which exhibit a minimum inhibitory concentration (MIC) of 4–16 mg/L. These observations suggest the existence of mechanisms for the low-level linezolid resistance in enterococci. Low-level resistance is defined as an increase in MIC of linezolid above that of the average susceptible bacterial population but below the breakpoint for clinically relevant resistance (Baquero, 2001). It has been believed that low-level resistance can serve as stepping stones to develop high level, clinically relevant resistance (Baquero, 2001). Therefore, elucidating the mechanisms of low-level resistance is expected to help fight against bacterial infections and to predict the emergence of resistance to a new antibiotic before its clinical introduction (Martínez et al., 2007).

We have previously reported the identification of nine *E. faecalis* clinical isolates, all of which showed a low-level resistance to linezolid

Abbreviations: ABC, ATP-binding cassette; BHI, Brain Heart Infusion; CDSs, Coding sequences; COGs, Cluster of Orthologous Groups; DEG, Differentially expressed gene; FDR, False discovery rate; FPKM, Fragments per kilobase exon per million mapped fragments; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MIC, Minimum Inhibitory Concentration; NR, NCBI non-redundant protein database; NT, NCBI non-redundant nucleotide sequence database; RT-qPCR, Real time quantitative polymerase chain reaction; RNA-seq, RNA sequencing

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with MIC of 8–16 mg/L but without mutations in 23S rRNA or ribosomal proteins L3 and L4 or presence of the *cfr* gene (Wang et al., 2014). To investigate the mechanisms of this resistance, we performed a whole transcriptome analysis of *E. faecalis* P10748 with low-level linezolid resistance in comparison with linezolid-susceptible 3138 and the control strain ATCC 29212. We also carried out real-time quantitative PCR (RT-qPCR) to validate some highly differentially expressed genes. Our results show a significant transcriptomic shift to biofilm formation and membrane transportation in the linezolid-resistant strain compared to the linezolid-susceptible strain, suggesting new mechanisms of linezolid resistance in *E. faecalis*.

2. Materials and methods

2.1. Bacterial isolation and antibiotic susceptibility testing

This study involved two *E. facalis* strains (P10748 and 3138) previously isolated from necrotic tissues and secretions, respectively, from two patients hospitalized in the First Affiliated Hospital of Chongqing Medical University, Chongqing, China (Wang et al., 2014) (Table S1). In addition, the *E. faecalis* ATCC 29212 strain was obtained from American Type Culture Collection and used as the standard quality-control strain. All strains were cultured in the Brain Heart Infusion (BHI) broth (Solarbio, Beijing, China) at 37 °C to the mid-exponential phase. Susceptibility testing was conducted using the VITEK 2 system (bioMérieux) and clinical MIC breakpoints were determined according to the Clinical and Laboratory Standards Institute guidelines (Wayne, 2011). The MIC breakpoint for linezolid was found to be ≥ 4 mg/L (Table S1). The MIC value was further confirmed by broth microdilution method. The MIC for *E. faecalis* strains P10748, 3138 and ATCC 29212 was 8, 2 and 2 mg/L, respectively, with the former one considered as linezolid-resistant strain and the latter two as linezolid-susceptible strain.

2.2. RNA and DNA extraction and illumina sequencing

Bacterial cells were harvested from culture grown to mid-exponential phase. Total RNA and genomic DNA was extracted using the Trizol reagent (Life Technologies) and HiPure Bacterial DNA Kits (Magen, Guangzhou, China) following the manufacturer's protocols, respectively. The quantity and quality of RNA extracts were assessed by Nanodrop (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples displayed a 23S/16S rRNA ratio > 1.0, RNA integrity number ≥ 7.0, OD260/280 > 1.9, and OD260/230 ≥ 1.8 (Table S1). Total RNA samples were sent to Beijing Genomics Institute (BGI) (Shenzhen, China), where the samples were subjected to poly (A) + RNA isolation, cDNA synthesis, and construction of Illumina sequencing libraries with a mean size of 225 bases, and sequencing using 100 base paired-end reads on the Illumina HiSeq 4000 platform.

2.3. Sequence data analysis and functional annotation

Raw-reads were pre-processed to generate clean reads by filtering low quality reads, trimming low quality nucleotides of both ends, and trimming Illumina adaptors and poly-A/T tails following the protocols of BGI (www.bgi.com). The *de novo* transcriptome assembly using clean reads from all samples was performed using Trinity (Grabherr et al., 2011). The assembly of each transcriptome used all the reads of the three strains. The resulting contigs were processed for read alignment and abundance estimation using Bowtie 2.0 (Langmead and Salzberg, 2012) and RSEM (Li and Dewey, 2011), respectively. Fragments per kilobase exon per million mapped fragments (FPKM) method was used to estimate unigene expression levels (Mortazavi et al., 2008). All unigenes were annotated by homology and searched against the NCBI non-redundant protein (Nr), NCBI non-redundant nucleotide sequence (Nt), Swiss-Prot, Cluster of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) database using algorithm blastx with *E*-value cut-off of 10^{-5}. Gene ontology (GO) functional categories were determined using the BLAST 2 GO software (Conesa et al., 2005). Interpro annotations were assigned by InterProScan5 (Quevillon et al., 2005). Unigenes unable to match in above databases were tested by EST-Scan (http://myhits.isb-sib.ch/cgi-bin/estscan) to predict the coding regions and identify sequence orientations.

2.4. Detection and clustering analysis of differentially expressed genes (DEGs)

DEGs between the linezolid resistant and sensitive strains of *E. faecalis* were determined by using the DESeq2 package ($P < 0.05$) with PoissonDis (Audic and Claverie, 1997; Anders and Huber, 2010; Love et al., 2014). *P* values used to identify the significance of DEGs were estimated according to the hyper geometric test (Audic and Claverie, 1997). The false discovery rate (FDR) was calculated based on the Benjamini & Hochberg algorithm (Hochberg, 2010). The significance threshold of the *P* value in multiple tests was set based on the FDR (FDR ≤ 0.001 and Fold Change ≥2.00). Hierarchical clustering and heatmap production for the expression profiles were performed using the Java Treeview software (Saldanha, 2004). The distributions of DEGs were illustrated by the MA plot and volcano plot (Dudoit et al., 2002; Li, 2012).

2.5. GO and KEGG analysis of DEGs

To have an overview on the functions and pathways of DEGs, DEGs were mapped to terms in GO database (Ashburner et al., 2000) and KEGG database (Kanehisa and Goto, 2000). GO analysis was implemented by the GOseq R packages based on Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can be adjusted for gene length bias in DEGs. With the GO annotation result, we classified DEGs according to official classification and calculated the gene numbers each GO term had. GO enrichment analysis applied a hypergeometric test to map all DEGs to terms in the GO database and identifies significantly enriched GO terms in DEGs compared to the transcriptome background. GO terms with corrected FDR < 0.001 were considered significantly enriched. With the KEGG annotation result, we classified DEGs according to official classification and calculated the gene numbers each pathway had. Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs compared against the whole genome background. KEGG pathways with a FDR ≤ 0.001 were considered significantly enriched pathways. All heat maps for gene clustering in the present study were depicted by using R program (http://www.R-project.org/).

2.6. Identification and expression level of differentially expressed genes (DEGs)

To validate the DEGs identified by transcriptome analysis described above, PCR and RT-qPCR were performed on selected DEGs using primers listed in Table S2. The PCR reaction was conducted using the following conditions: 94 °C for 10 min, followed by 35 cycles of 94 °C for 60 s, 55 °C for 20 s and 72 °C for 60 s. Aliquots of the total RNA extracts were subjected to cDNA synthesis with the Superscript PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). RT-qPCR was carried out in a 20 μL reaction volume containing 80 ng of cDNA using the SYBR Premix Ex TaqII Kit (Takara, Japan) on the Light Cycler 480 System (Applied Biosystems, USA) following the manufacturer's protocols. Each sample was tested in triplicate in three independent experiments with negative controls (no template). The 16S rRNA was used as an internal reference for normalization (Nadkarni et al., 2002). The relative gene expression was calculated with the