Physiological significance of ClpP in Enterococcus faecalis and its regulating proteins were identified by Tandem Mass Tag Mass Spectrometry

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Enterococcus faecalis; ClpP; stress tolerance; biofilm formation; virulence; antimicrobials tolerance
Abstract

Background

ClpP is important for bacterial growth and plays an indispensable role in cellular protein quality control systems by refolding or degrading damaged proteins, but the physiological significance of ClpP in Enterococcus faecalis is still obscure. Thus a clpP deletion mutant (ΔclpP) was constructed in E. faecalis OG1RF strain to elucidate a more comprehensive picture of the effect of ClpP on E. faecalis. The global abundance of proteins was determined by a mass spectrometer with Tandem Mass Tags labeling.

Results

The ΔclpP mutant strain showed impaired growth at 20°C or 45°C, at 5% NaCl or 2 mM H2O2. The surviving bacteria of the ΔclpP mutant strain reduced after exposure to the high concentration (50 x MIC) of linezolid or minocycline for 96 h. The ΔclpP mutant strain also demonstrated decreased biofilm formation but increased virulence in a Galleria mellonella model. The mass spectrometry proteomics data indicated that the abundances of 135 proteins changed (111 proteins increased, 24 proteins decreased) in the ΔclpP mutant strain. Among those differential abundance proteins, the abundances of stress response or virulence relating proteins: FsrA response regulator, gelatinase GelE, regulatory protein Spx (spxA), heat-inducible transcription repressor HrcA, transcriptional regulator CtsR, ATPase/chaperone ClpC, acetyl esterase/lipase, and chaperonin GroEL increased in the ΔclpP mutant strain; however, the abundances of ribosomal protein L4/L1 family protein (rplD), ribosomal protein L7/L12 (rplL2), 50S ribosomal protein L13 (rplM), L18 (rplR), L20 (rplT), 30S ribosomal protein S14 (rpsN2) and S18 (rpsR) all reduced. The abundances of biofilm formation related adapter protein MecA increased, while the abundances of dihydroorotase (pyrC), orotate phosphoribosyltransferase (pyrE) and orotidine-5‘-phosphate decarboxylase (pyrF) all decreased in the ΔclpP mutant strain.
Conclusion

The present study demonstrates that ClpP participates in stress tolerance, biofilm formation, antimicrobials tolerance, and virulence of *E. faecalis*.

Background

*Enterococcus faecalis* has emerged as a significant cause of nosocomial infections resulting in urinary tract infections, bacteremia, prosthetic joint infection, abdominal-pelvic infections, and endocarditis in the last two decades [1]. *E. faecalis* has resistance to many commonly used antimicrobial agents, particularly vancomycin-resistant enterococci (VRE) which has emerged as a major cause of outbreaks of nosocomial infection in recent years [2]. In addition to drug resistance, *E. faecalis* carries a high capacity for biofilm formation; more than 40% of clinical *E. faecalis* isolates can form biofilms worldwide [3-7]. Several virulence factors have been found associated with the biofilm formation of *E. faecalis*. For example, the enterococcal surface protein (*esp*) was found to adhere to and colonize abiotic surfaces that participated in the biofilm formation of *E. faecalis*, and the gelatinase (*gelE*) which can hydrolyze gelatin, collagen and hemoglobin was also reported to be involved in the adherence and biofilm formation of *E. faecalis* [6, 8-10]. However, the *esp* or *gelE* were found to have no association with the biofilm formation of *E. faecalis* in other extensive collections of *E. faecalis* isolates [11-13]. Thus, the genes involved in the *E. faecalis* biofilm formation remain controversial and obscure. Other unknown factors may also participate in the biofilm formation of *E. faecalis*.

The Hsp100/Clp family protein, ClpP is important for bacterial growth and plays an indispensable role in cellular protein quality control systems by refolding or degrading damaged proteins in stressed cells[14]. ClpP was also found associated with the biofilm formation in some pathogenic species. For example, the biofilms of *S. mutans*, *S. epidermidis*, *P. aeruginosa*, and *A. pleuropneumoniae* decreased when the *clpP* was
mutated [15-18]. However, the capacities to form biofilms were enhanced when the \textit{clpP} was mutated in \textit{S. aureus}, \textit{H. parasuis}, and \textit{P. gingivalis} [19-21]. The roles of \textit{clpP} on the bacterial biofilm formation have not been fully understood. The RNA levels of \textit{clpP} of \textit{S. epidermidis} was decreased by the \textit{agr} quorum-sensing system, but in \textit{S. aureus} Newman and USA300 strains, the RNA levels of \textit{agrA} and \textit{agrC} were significantly reduced in the \textit{clpP} mutants [16, 21]. The \textit{clpP} affected the expression of transcriptional regulators \textit{csrA}, \textit{rpoD} and a possible biofilm repressor \textit{luxS} to enhance the biofilm formation of \textit{H. parasuis}, and negatively adjusted the surface exposure of the minor fimbrial (Mfa) protein that promoted the biofilm formation of \textit{P. gingivalis} [19, 20]. The role of \textit{clpP} on the biofilm formation of \textit{E. faecalis} remain unknown to date.

In addition to bacterial growth, stress response, and biofilm formation, ClpP also influences the virulence and antibacterial tolerance of several pathogenic organisms. The \textit{clpP} mutation significantly attenuated the virulence of \textit{S. pneumoniae} in a murine intraperitoneal infection model. The virulence-related pneumolysin and pneumococcal surface antigen A was regulated by ClpP proteases [22]. Michel A \textit{et al.} found that the abundance of the \textit{agr} system and \textit{agr}-dependent extracellular virulence factors was diminished in \textit{S. aureus} 8325 \textit{ΔclpP} strain [23]. In \textit{L. pneumophila}, the \textit{clpP} deficient mutant strain was unable to escape the endosome-lysosomal pathway in host cells [24].

The \textit{clpP} deletion mutation also causes the attenuation of \textit{S. Typhimurium} virulence through mis-regulation of RpoS and indirect control of CsrA and the SPI genes [25]. In \textit{S. aureus}, except for the stress response, biofilm formation, and virulence, the truncating mutation in \textit{clpP} is responsible for the raised vancomycin resistance in VISA strain LR5P1-V3 [26]. Bæk KT \textit{et al.} found that the inactivation of the components of the ClpXP protease substantially increased the β-lactam resistance level of the \textit{S. aureus} USA300 strain, and found the \textit{clpP} mutant strain displayed significantly thicker cell walls, increased
peptidoglycan cross-linking, and altered composition of monomeric muropeptide species compared to wild type [27]. As mentioned above, E. faecalis shows resistance to many antimicrobial agents, however, whether the clpP is involved in E. faecalis resistance to antimicrobials, especially resistance to vancomycin (VRE) is still unclear. Thus, in order to get a more comprehensive picture of the role of ClpP protease on the stress response, biofilm formation, virulence, and antimicrobials tolerance of E. faecalis, a △clpP strain was constructed in E. faecalis strain OG1RF. The global abundance of proteins was detected by Orbitrap Q Exactive HF-X mass spectrometer with tandem mass tags (TMT) labeling.

Methods

Bacterial strains, plasmids, growth conditions, and chemicals

All of the bacterial strains and plasmids used in this study are shown in Table 3. The E. faecalis ATCC 47077 (OG1RF; GenBank accession number CP002621.1) and ATCC 29212 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The E. faecalis strains were cultured in tryptic soya broth (TSB; Oxoid, Basingstoke, UK) at 37℃ with shaking at 220 rpm. Glucose was added to the TSB medium at a concentration of 0.25% for the detection of biofilm formation. Electroporation was used for plasmid transformation, and B2 medium (1% casein hydrolysate, 2.5% yeast extract, 0.5% glucose, 2.5% NaCl, 0.1% K2HPO4, pH 7.5) was used for the recovery of bacteria. The antibiotics used in this study were purchased from Sigma Chemical Co. (Los Angeles, CA, USA) and used at concentrations of 20 mg/liter for chloramphenicol, 750 or 25 mg/liter for erythromycin.

Construction of △clpP mutants and complemented strains

The clpP deletion mutant of the OG1RF strain was constructed by in-frame deletion using the temperature-sensitive plasmid pJRS233 as previous described [49]. Briefly, the
upstream and downstream fragments of OG1RF_10505 (gene: clpP; product: ATP-dependent Clp protease proteolytic subunit), which highly homologous (86.8%) to SA0723 (product as the ClpP protease) of S. aureus N315 strain [23], were amplified from OG1RF by PCR and separately cloned into the pJRS233 vector, resulting in pJRS233-ΔclpP. The recombinant plasmid pJRS233-ΔclpP was successively transferred into E. coli DH5α and OG1RF by electroporation, and then the transformants selected at 30°C on Erm. Chromosomal integrants were selected by growth at 42°C in the presence of Erm. Selection for excision of the integrated plasmid by homologous recombination was accomplished by growing the bacteria at 30°C in the absence of Erm. The complemented ΔclpP mutant strain was constructed using an E. coli-Streptococcus shuttle vector pIB166. The clpP gene was amplified by PCR and cloned into the pIB166 vector, resulting in pIB166::clpP. The recombinant plasmid pIB166::clpP was transformed by electroporation into the ΔclpP mutant strain, forming the complemented ΔclpP/pIB166::clpP strain. The ΔclpP strain containing the empty vector pIB166 was designated the ΔclpP/pIB166 mutant. The ΔclpP mutant and the complemented ΔclpP mutant strain were identified by PCR, RT-qPCR, and direct sequencing. The primers used in this assay are listed in Table 4.

Growth analysis of the ΔclpP mutant strain

The OG1RF, ΔclpP, ΔclpP/pIB166::clpP and ΔclpP/pIB166 strains were cultured in TSB at 37°C with shaking for 12 h and diluted in the same medium to an OD600 value of 1.5, then 50 μL aliquot of the diluted suspension was inoculated into 100 mL fresh TSB and incubated at either 37°C, 45°C or 20 °C with circular agitation (220 rpm). The diluted suspension was also inoculated into fresh TSB with 5% NaCl pH5.5 or 2 mM H2O2 and incubated at 37°C with circular agitation (220 rpm). OD600 values for the cultures were determined using an Eppendorf Biospectrometer (Eppendorf, Hamburg, Germany) at one-hour intervals. Three independent experiments were performed.
The sensitivity of the \( △clpP \) mutant strain to sodium dodecyl sulphate (SDS)

Overnight cultures of the \( E. \) \( faecalis \) strains were diluted 1:200 in fresh TSB medium and incubated at 37°C for four hours until an OD600 of 1.0 was reached. After 10-fold serial dilution, 5 \( \mu \)L of the aliquot was spotted onto a TSB agar plate containing 0.008% SDS and incubated at 37°C for 24 h. The bacterial colonies on the plates were photographed and counted [28]. Three independent experiments were performed, and the representative results were shown.

Microtiter plate assay of biofilm formation

The biofilm-forming ability of the \( E. \) \( faecalis \) isolates was detected as previously described with modifications [50]. Overnight cultures were diluted 1:200 in 200 \( \mu \)l of TSBG (TSB with 0.25% glucose) and inoculated into 96 polystyrene microtiter plates. After 12, 24, or 48 h of static incubation at 37°C, the supernatant was discarded, and plates were washed thrice with deionized water to remove unattached cells, stained with 1% crystal violet (CV) for 20 min at room temperature, and rinsed with distilled water. Last, the CV was solubilized in ethanol-acetone (80:20, vol/vol) and optical density at 570 nm (OD\( _{570} \)) was determined. Three independent experiments were performed.

Quantification of extracellular DNA

Extracellular DNA (eDNA) was quantified, as described previously [51]. Overnight cultures of the \( E. \) \( faecalis \) strains were diluted to OD600 = 0.001 in AB medium supplemented with 0.5% glucose, 0.05 mM propidium iodide (PI) and 10% TSB. The diluted cultures were transferred to polystyrene microtitre plates (200 \( \mu \)L/well) and incubated for 24 h at 37°C. The cell density was measured at OD\( _{600} \) using a microtitre plate reader (BioRAD, United States). The fluorescence of PI-bound eDNA was measured by a Varioskan\textsuperscript{TM} LUX multimode microplate reader (Thermo Fisher, United States) with the excitation/emission
wavelength at 535/610 nm. Relative amounts of eDNA per OD\textsubscript{600} unit were determined.

Three independent experiments were performed.

Determination of MIC and antimicrobials tolerance of strains

The minimal inhibitory concentrations (MICs) of the antimicrobials against the \textit{E. faecalis} isolates were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines CLSI-M100-S26, with CLSI-recommended MIC breakpoints. The antimicrobials-tolerance of strains was detected as described previously with modifications [28]. Antimicrobials (at 50 x MIC) were added to the stationary-phase cultures (16 h) of the \textit{E. faecalis} strains, and then the cultures were incubated at 37°C for 120 h without shaking. At the time points of every 24 h, one-milliliter aliquots were sampled and washed twice with ice-cold saline, ten-fold dilutions were then plated on Muller-Hinton agar, and the numbers of CFU were determined. Three independent experiments were performed.

Virulence of \textit{E. faecalis} in \textit{Galleria mellonella}

Infection of \textit{G. mellonella} larvae with \textit{E. faecalis} strains was performed as described previously for other pathogens [52]. \textit{G. mellonella} larvae in groups of 40 were infected in the left posterior proleg with 20 μl inocula of \textit{E. faecalis} strains containing 5 × 10\textsuperscript{6} CFU/mL. Survival of \textit{G. mellonella} larvae was recorded at 12 h intervals for 72 h p.i.. Every trial included a group of 20 \textit{G. mellonella} larvae injected with saline as a method control. Experiments were performed in at least three independent tests, and the representative results were shown.

Protein extraction and detected by a mass spectrometer with Tandem Mass Tags (TMT) labeling

The \textit{E. faecalis} strains OG1RF and the ΔclpP mutant were inoculated into TSB and cultured
at 37°C for 4 h to logarithmic phase or for 12 h to stationary phase. The cells were harvested at 4°C centrifugation and minced individually with liquid nitrogen and lysed in lysis buffer, followed by 5 min of ultrasonication on ice. Protein concentration was determined again by the Bradford protein assay. The supernatant from each sample, containing precisely 0.1 mg of protein, was digested with Trypsin Gold (Promega) at 1:50 enzyme-to-substrate ratio. After 16 h of digestion at 37°C, peptides were desalted with C18 cartridge to remove the high urea, and desalted peptides were dried by vacuum centrifugation. Desalted peptides were labeled with TMT6/10-plex reagents (TMT6/10plex™ Isobaric Label Reagent Set, Thermo Fisher), following the manufacturer’s instructions. For 0.1 mg of the peptide, 1 unit of labeling reagent was used. Peptides were dissolved in 100 μL of 0.1 M TEAB, and the labeling reagent was dissolved in 41 μL of acetonitrile. After incubation for 1 h, the reaction was stopped with ammonium hydroxide. Differently labeled peptides were mixed equally and then desalted by peptide desalting spin columns (Thermo Fisher, 89852). TMT-labeled peptide mix was fractionated using a C18 column (Waters BEH C18 4.6×250 mm, 5 μm) on a Rigol L3000 HPLC operating at 1 mL/min, and the column oven was set at 50°C. Shotgun proteomics analyses were performed using an EASY-nLCTM 1200 UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher) operated in the data-dependent acquisition (DDA) mode. Q Exactive HF-X mass spectrometer was operated in positive polarity mode with a spray voltage of 2.3 kV and capillary temperature of 320°C. Two independent experiments were performed.

Global protein abundance analysis

The resulting spectra from each fraction were searched separately against NCBI E. faecalis strains OG1RF (CP002621.1) database (https://www.ncbi.nlm.nih.gov/nuccore/CP002621.1) by the search engines: Proteome Discoverer 2.2 (PD 2.2, Thermo). The searched
parameters as follows: A mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.02 Da for the production scans were used. Carbamidomethyl was specified in PD 2.2 as fixed modifications. Oxidation of methionine, acetylation of the N-terminus and TMT of lysine were specified in PD 2.2 as variable modifications. A maximum of 2 miscleavage sites was allowed. For protein identification, a protein with at least one unique peptide was identified at FDR less than 1.0% on peptide and protein level, respectively. Proteins containing similar peptides and could not be distinguished based on MS/MS analysis were grouped separately as protein groups. Reporter Quantification (TMT) was used for TMT quantification. The protein quantitation results were statistically analyzed by Mann-Whitney Test, the significant ratios, defined as p < 0.05 and ratio > 1.2 or ratio < 0.83 [fold change, FC], were used to screen the differential abundance proteins (DAPs). Gene Ontology (GO) and InterPro (IPR) analysis were conducted using the interproscan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER), and the databases COG (Clusters of Orthologous Groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to analyze the protein family and pathway. The probable interacting partners were predicted using the STRING-db server (http://string.embl.de/) based on the related species. STRING is a database of both known and predicted protein-protein interactions. The enrichment pipeline was used to perform the enrichment analysis of GO, IPR, and KEGG, respectively.

RNA isolation and RT-qPCR
The RNA isolation of E. faecalis strains were performed as described previously with some modifications [28]. The E. faecalis strains OG1RF and the ΔclpP mutant were inoculated into TSB and cultured at 37°C for 4 h to logarithmic phase or for 12 h to stationary phase, the following operations were performed at 4°C centrifugation or on ice: bacterial cultures were centrifuged at 5,000 x g for 5 min, and then the pellets were washed twice with 0.9%
saline; the culture was homogenized 5 times using 0.1-mm zirconia-silica beads in a mini-BeadBeater (Biospec, Bartlesville, OK) at 5,000 rpm for 60 s at 1-min intervals; the samples were centrifuged at 15,000 rpm and the bacterial RNA in the supernatant was purified using an RNeasy minikit (Qiagen, Hilden, Germany) and quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA samples that had a 260/280 ratio between 2.0 and 2.2 were used for the RT-qPCR.

Total RNA extracted from strains OG1RF and the ΔclpP mutant were reverse transcribed with the PrimeScript RT Reagent Kit (TaKaRa Biotechnology, Dalian, China) and RT-qPCR was performed with the SYBR Premix Ex Taq II Kit (TaKaRa Biotechnology, Dalian, China) on the Mastercycler ep realplex system (Eppendorf), with an initial incubation at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Each sample was analyzed in triplicate. For all samples, the internal control gene recA was used to normalize the abundance of *E. faecalis* strains OG1RF genes [53]. The threshold cycle (Ct) numbers were confirmed by the detection system software, and the data were analyzed based on the $2^{-\Delta\Delta Ct}$ method. The primers used for the RT-qPCR are listed in Table S2.

Statistical analysis

Experimental data were analyzed with SPSS software (version 16.0; SPSS, Chicago, IL, USA) and compared using the Student’s t test, one-way analysis of variance, Mann-Whitney test, or the log-rank test. Differences with a P value of <0.05 were considered statistically significant.

Results

Construction of *clpP* deletion mutant and the complemented strain

To explore the role of ClpP in *E. faecalis*, we constructed the *clpP* deletion mutant in the *E. faecalis* OG1RF strain using the temperature-sensitive plasmid pJRS233. The deletion
mutant strain was verified by PCR and direct sequencing and was referred to as the OG1RF ΔclpP mutant strain. The complemented ΔclpP strain was constructed using shuttle vector pIB166, which named OG1RF ΔclpP/pIB166::clpP and also verified by PCR and direct sequencing. The ΔclpP strain containing the empty vector pIB166 was designated as OG1RF ΔclpP/pIB166. The RNA levels of the clpP gene of all the above four E. faecalis OG1RF strains were determined by RT-qPCR and shown in Fig. S1. 

ΔclpP mutant strain showed impaired growth at 20°C, 45°C, 5%NaCl or 2 mM H2O2

Previous research indicated that ClpP participated in the stress of low or high temperature and the oxidative stress response in S. aureus [23], however, these issues are still unknown in E. faecalis. Thus we first investigated the effects of clpP deletion on the growth of E. faecalis under the stress of low or high temperature, hyperosmotic pressure, low pH, and oxidative stress. At 37°C, there were no significant growth differences between the E. faecalis OG1RF parent strain and its ΔclpP mutant. However, under the 20°C or 45°C, the ΔclpP mutant strain showed a lower OD₆₀₀ than was observed for the wild-type after entering logarithmic phase growth (Fig.1). As Fig.2 indicated, the growth of ΔclpP mutant strain was also impaired under 5%NaCl (logarithmic phase) or 2 mM H2O2 (later logarithmic phase or stationary phase).

Deletion of clpP leads to decreased biofilm formation

The polystyrene microtiter plate assay was performed to evaluate the role of clpP in the biofilm formation of E. faecalis under static conditions. The biofilm formation of E. faecalis OG1RF parent strain and its ΔclpP mutant was monitored at 12, 24, and 48 h on microtiter plates stained with crystal violet, and the OD₅₇₀ were determined. The biofilms of the ΔclpP mutant strain (OD₅₇₀, 0.835 ± 0.091) were significantly decreased compared with that of the parent strain (OD₅₇₀, 2.247 ± 0.138, P<0.001, student’s t test) after incubation
for 48 h, and this issue was also observed after incubation for 12 or 24 h (Fig. 3A). We further investigated the eDNA release during the biofilm formation of E. faecalis, but found no differences between the ΔclpP mutant and its parent strain (Fig. 3B).

Antimicrobials tolerance of the ΔclpP mutant strain

The minimum inhibitory concentrations (MICs) of eight antimicrobials for E. faecalis were detected by broth microdilution method and the MICs for the ΔclpP mutant strain were found to be similar to those of the parent strain (Table S1). To explore the antimicrobial concentrations that ensured only drug-tolerant bacterial cells survived, we did the time-killing assays for six antimicrobials used in the present study. Based on the previous research [28] and our preliminary experimental results, the concentrations of six antimicrobials were used at 50 x MIC in the present study. As Fig. 4 indicated, the surviving bacteria of the ΔclpP mutant strain (log_{10} CFU/ml, under the detection limit) were significantly decreased compared with those of the parent strain (log_{10} CFU/ml, 2.873 ± 0.243, P<0.001, student’s t test) after exposure to the linezolid for 96 h. After exposure to the minocycline for 96 h, the surviving bacteria of the ΔclpP mutant strain (log_{10} CFU/ml, 1.477 ± 0.171) were also decreased compared with those of the parent strain (log_{10} CFU/ml, 3.078 ± 0.303, P<0.01, student’s t test).

ΔclpP mutant leads to increased virulence of E. faecalis

The virulence of E. faecalis strains was detected by the infection of G. mellonella larvae. The survival of G. mellonella larvae infected with the ΔclpP mutant strain (15/40, 37.5%) significantly decreased compared with the parent strain (28/40, 70.0%, P<0.01, log-rank test) after 72 h p.i (Fig. 5). The complemented ΔclpP/pIB166::clpP strain (23/40, 57.5%) showed a partially restored survival ability.

Comparison of the global protein abundances of the ΔclpP mutant and the parent strain
We compared the global protein abundance of the ΔclpP mutant with the parent strain. The total proteins were extracted from logarithmic-phase (4 h), and stationary phase (12 h) bacteria and their abundances were determined by Q Exactive HF-X mass spectrometer with Tandem Mass Tags (TMT) labeling. The protein quantitation results were statistically analyzed by Mann-Whitney Test, the significant ratios, defined as p < 0.05 and ratio > 1.2 or ratio < 0.83 [fold change, FC], were used to screen the differential abundance proteins (DAPs). The protein quantitation results were given as the means from two independent experiments, the repeatability of the two independent experiments were evaluated by the coefficient of variation (CV), and as Fig. S2 indicated that the CV of the two independent experiments was very low. All the DAPs were summarized in Table 1, the abundances of 135 proteins changed in the ΔclpP mutant strain, of which 111 proteins increased, while 24 proteins decreased.

Gene Ontology (GO) and KEGG analysis of DAPs

All the DAPs between the ΔclpP mutant and the parent strain were analyzed by the Gene Ontology (GO) and KEGG analysis. As Fig.6 GO analysis showed, the increased DAPs in the ΔclpP mutant strain (logarithmic phase) were mainly concentrated in the following molecular functions: N-acetyltransferase activity, coenzyme binding, cofactor binding, ATPase activity, nucleoside-triphosphatase activity, hydrolase activity, ATP binding, kinase activity, nucleotide binding, organic cyclic compound binding, heterocyclic compound binding, DNA binding and nucleic acid binding. However, the decreased DAPs were mainly included in the following molecular functions: structural constituent of ribosome, rRNA binding, orotidine-5'-phosphate decarboxylase activity, hydrolase activity, organic cyclic compound binding, heterocyclic compound binding, and nucleic acid binding. The KEGG analysis demonstrated that functions of most of these DAPs in the ΔclpP mutant (logarithmic phase) belonged to the ribosome, fructose and mannose metabolism,
pyrimidine metabolism, purine metabolism, pentose phosphate pathway, glycolysis/gluconeogenesis, ABC transporters (Fig. 7). The functions of DAPs in the stationary phase of ΔclpP mutant strain were similar to that in the logarithmic phase (Fig. S3).

DAPs associated with the stress response or virulence, biofilm formation of *E. faecalis*

Based on the literatures, we selected the DAPs which may associate with stress response or virulence, biofilm formation of *E. faecalis* for further analyzed. The abundance of DAPs which associated with the stress response or virulence of *E. faecalis*, including the FsrA response regulator and gelatinase GelE, ATPase/chaperone ClpC, chaperonin GroEL, acetyl esterase/lipase, and transcriptional regulator proteins, HrcA, CtsR, and Spx increased in the ΔclpP mutant strain (Table 2). However, the abundances of ribosomal proteins L4/L1, L7/L12, L13, L18, L20, S14, and S18 decreased in the ΔclpP mutant strain. The abundance of the biofilm formation of *E. faecalis* associated DAPs, adapter protein MecA increased in the ΔclpP mutant strain, while the abundances of orotate phosphoribosyltransferase, orotidine-5'-phosphate decarboxylase and dihydroorotase reduced (Table 2). The RNA levels of all the above DAPs which may associate with stress response, virulence or biofilm formation of *E. faecalis*, were verified by RT-qPCR and were consistent with protein abundance changes in the ΔclpP mutant strain.

Discussion

ClpP as a protease of Hsp100/Clp family is very important for bacterial growth and plays an irreplaceable role in cellular protein quality control systems by refolding or degrading damaged proteins in stressed cells [14]. Up to now, ClpP has been found participating in many essential activities of bacteria, such as stress response, which including abnormal temperature stress, hyperosmotic pressure, low pH, and oxidative stress response, virulence, biofilm formation and so on. However, the global abundance of proteins
affected by ClpP in bacteria is still little known for us. Feng J et al. found that the
abundance of transcriptional regulators CtsR and Spx, the ClpC adaptor proteins McsB and
MecA, the cell division protein FtsZ and so on, were clearly affected by ClpP in S. aureus
strains NCTC8325-4, COL, SA564 and Newman by 2-D DIGE technique [29, 30]. However,
the abundance of not more than 80 proteins changed in their studies, and it may be due
to the low sensitivity of 2-D DIGE technique. In the present study, we exacted the total
proteins from E. faecalis OG1RF and its ΔclpP mutant strains, digested and then all the
peptides labeled with Tandem Mass Tags (TMT, a new fluorescent dyes similar with
iTRAQ), finally detected by Orbitrap Q Exactive HF-X mass spectrometer and found 135
differential abundance proteins (DAPs) in the ΔclpP mutant strain. Among those DAPs, we
also found the transcriptional regulators CtsR and Spx, the ClpC adaptor proteins MecA
and FtsZ-interacting cell division protein YlmF, which were similar to that in S. aureus
strains, and interestingly we also found other new proteins, such as Acetyl
esterase/lipase, ribosomal protein, orotidine-5'-phosphate decarboxylase and so on.
The ClpP has been shown to participate in stress tolerance by refolding or degrading
damaged proteins during the growth of bacteria, and several studies have indicated that
the ΔclpP mutant strain showed a growth defect over a broad range of temperatures,
which included high (40, 42, 45°C) or low (20, 30°C) temperatures, even at the 37°C
condition [19, 23, 31, 32]. However, this study only found the growth of E. faecalis OG1RF
ΔclpP mutant strain to be impaired under the 45°C or 20°C temperatures, but not
observed at 37°C. Previous studies also demonstrated the ΔclpP strain was more
vulnerable to oxidative stress, osmotic stress, acid, or sodium dodecyl sulfate [19, 33-35],
this study found the growth of OG1RF ΔclpP strain was impaired under the osmotic stress
or oxidative stress conditions. The heat shock protein heat-inducible transcription
repressor HrcA and chaperonin GroEL were found associated with the temperature stress
response [36]. However, the increased abundances of HrcA and GroEL may not contribute to the impaired growth of the ΔclpP mutant strain under the 45°C or 20°C temperatures in this study. As the increased abundances of HrcA and GroEL were in the stationary phase, not in the logarithmic phase growth of the ΔclpP mutant strain, while the impaired growth of the ΔclpP mutant strain mainly occurred in logarithmic phase growth under the 45°C or 20°C temperatures. The ribosomal protein L9 was found played a significant role in the E. coli response to starvation stress [37]. The present study found that when the clpP was deleted in E. faecalis OG1RF, the abundance of many ribosomal proteins, including both 50S and 30S ribosomal proteins decreased in the ΔclpP mutant strain. Thus, ClpP may through affecting the abundance of ribosomal proteins participates in the stress response of E. faecalis.

Previous studies have found that ClpP can significantly affect the biofilm formation of bacteria, but the effects of clpP on the biofilm formation in different genera of bacteria were obviously different, or even opposite [15, 16, 18, 19, 21]. This study first found that biofilm formation decreased when the clpP of OG1RF strain was deleted. The adapter protein MecA was found decreased the RNA level of eps, which encodes synthesis of the biofilm matrix exopolysaccharide, thus inhibited the biofilm formation of B. subtilis [38]. The present study indicated that the abundance of MecA increased in the ΔclpP mutant strain, and it may lead to the decreased biofilm formation of the clpP deleted strain.

Another reason for the reduced biofilm formation of the ΔclpP mutant strain may be the reduced abundances of orotate phosphoribosyltransferase (pyrE) and orotidine-5′-phosphate decarboxylase (pyrF), the two proteins which have been found promoted the biofilm formation of S. sanguinis and E. faecalis respectively [39, 40].

ClpP was found also participated in the bacterial virulence; the virulence of S. pneumoniae, S. aureus and L. pneumophila attenuated in the clpP mutation strains [22-
Recently, Liu Q et al. reported that in *S. aureus*, the *clpP* mutant strain showed increased biofilm formation and reduced virulence [21]. However, this study found the Δ*clpP* mutant strain with the decreased biofilm formation and increased virulence in a *G. mellonella* model. The previous study explored that the CtsR regulator controlled the expression of *clpC*, *clpE*, and *clpP*, and was required for the virulence of *E. faecalis V583*, but the role of *clpP* in the virulence of *E. faecalis* was still unclear [41]. The FsrABDC signal transduction system and the GelE were major virulence factors in *E. faecalis* [42, 43]. Thus it may be that the increased abundance of FsrA and GelE lead to the increased virulence of the Δ*clpP* mutant strain. The abundance of acetyl esterase/lipase, another virulence factor of *E. faecalis*, also was found increased in the Δ*clpP* mutant strain, this suggests that it may also cause the increased virulence of the Δ*clpP* mutant strain [44].

This study also found that the sensitivities to linezolid or minocycline of the Δ*clpP* mutant strain increased. As we knew, linezolid as an inhibitor of bacterial protein synthesis which acts on the 50S ribosome subunit of gram-positive bacteria, and minocycline is a synthetic tetracycline derivative which acts on the 30S ribosome subunit of gram-positive or gram-negative bacteria [45, 46]. The present study indicated that the abundance of 50S ribosomal proteins L13, L18, L20, the 30S ribosomal proteins S14, S18, all reduced in the Δ*clpP* mutant strain, thus might lead to the increased sensitivities of the Δ*clpP* mutant strain to linezolid or minocycline.

In the *B. subtilis*, Spx plays a significant role in the protection system against oxidative stresses [47]. Recently Rojas-Tapias DF and Helmann JD found that the Spx was itself a regulator of the ctsR operon, and the ctsR operon was found regulated the expression of *clpC* and *clpP* [48]. The present study indicated that when the *clpP* was deleted in *E. faecalis* OG1RF, the abundance of ClpC, CtsR, and Spx all increased, this was similar with that observed in *S. aureus* [30]. In *S. aureus*, the RNA levels of the *clpC* operon (*ctsR-
mcsA-mcsB-clpC), groE, and dnaK were induced in response to an accumulation of misfolded proteins, which supported the notion that ClpP proteases served to degrade misfolded proteins [30]. Our study found the abundances of ClpC, GroEL, and the DnaB, but not the DnaK, increased in the $\Delta clpP$ mutant strain, may also due to the accumulation of misfolded proteins.

It is easy to understand that ClpP, as a protease, has a significant effect on the abundance of proteins, but not on the RNA level of genes. In the present study, the abundance of many transcription regulation related proteins changed in the $\Delta clpP$ mutant strain, such as regulatory protein Spx (spxA), heat-inducible transcription repressor HrcA, transcriptional regulator CtsR and so on, and this issue was also observed in other studies [29, 30]. As we knew, the transcriptional regulators usually control the transcription and RNA levels of their functional genes. So the ClpP may through affecting the abundance of transcriptional regulators to alter the RNA levels of other genes, and the RNA levels of many genes changed in the $\Delta clpP$ mutant strain in this study, and the similar results were also observed in other studies [23, 30]. Since ClpP was a protease which involved in protein degradation, thus its absence should provoke the accumulation of proteins, and this was consistent with our result that the abundance of most of the DAPs increased in the $\Delta clpP$ mutant strain. However, the abundance of some proteins and their corresponding RNA levels of genes decreased in the $\Delta clpP$ mutant strain in the present study, and the similar results were also found in another study [30]. The reason for this issue may be that as mentioned above, ClpP reduced the transcription and expression of those genes by regulating the abundance of transcriptional regulators.

Conclusions

The present study indicates that ClpP may through affecting the abundance of ribosomal proteins L4/L1, L7/L12, L13, L18, L20, S14, and S18 participates in the stress response,
and the linezolid or minocycline tolerance of *E. faecalis*. ClpP participates in the biofilm formation of *E. faecalis* may by affecting the abundances of adapter protein MecA, orotate phosphoribosyltransferase (*pyrE*), orotidine-5′-phosphate decarboxylase (*pyrF*). Our results also suggest that ClpP may modulate the abundances of FsrA, GelE, and acetyl esterase/lipase participates in the virulence of *E. faecalis*.

**Abbreviations**

CV: crystal violet; DAPs: differential abundance proteins; eDNA: Extracellular DNA; MIC: minimal inhibitory concentration; TMT: Tandem Mass Tags; VRE: vancomycin-resistant enterococci;

**Declarations**

Ethics approval and consent to participate Not applicable. Consent for publication Not applicable. Availability of data and materials All data generated or analysed during this study are included in this published article and its supplementary information files The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014211. Conflict of Interest The authors declare that they have no competing interests Funding This work was supported by the following grants: the Sanming Project of Medicine in Shenzhen (grant number SMGC201705029); Science, Technology and Innovation Commission of Shenzhen Municipality of key funds (JCYJ20170412143551332; JCYJ20180508162403996) and basic research funds (JCYJ20180302144721183; JCYJ20180302144431923). Author’s contributions Jinxin Zheng participated in the design of the study, carried out the gene manipulation, biofilm and eDNA assay, analyzed and interpreted the proteomic data, and drafted the manuscript. Yang Wu participated in the gene manipulation, RT-qPCR, and proteomic data. Zhiwei Lin conducted the RNA extraction
and RT-qPCR. Guangfu Wang performed antibiotic-susceptibility testing, antimicrobials tolerance experiments and protein extraction. Sibo Jiang, Xiang Sun and Haopeng Tu performed the gene manipulation, stress tolerance experiments, biofilm and eDNA test, G.mellonella trials and RNA extraction and RT-qPCR. Zhijian Yu and Di Qu designed the study, participated in the data analysis, and provided critical revisions of the manuscript for valuable intellectual content. Acknowledgments The authors thank Prof. Michael G. Caparon (Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, Missouri, USA) for providing generously plasmid pJRS233, thank Prof. Jingren Zhang (Center for Infectious Disease Research, School of Medicine, Tsinghua University, Beijing, China) for providing generously the plasmid pIB166. We also thank Ms. Cynthia Brast (University of Florida, Gainesville, FL, USA) for her review on this manuscript.

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**Tables**

**Table 1** Global differential abundance of proteins between the ΔclpP mutant and its parent strains

| Protein ID | Description or predicted function | Protein abundance ratio of |
|------------|-----------------------------------|---------------------------|
| (locus_tag, gene name) |                                      |                           |
| Accession       | Description                                                                 | Abundance |
|-----------------|-----------------------------------------------------------------------------|-----------|
| AEA95010.1      | hypothetical protein                                                         | 2.953     |
| AEA94213.1      | gelatinase GelE                                                              | -         |
| AEA93457.1      | aspartate 4-decarboxylase                                                    | -         |
| AEA95218.1      | transcriptional regulator CtsR                                               | 2.165     |
| AEA93816.1      | hypothetical protein                                                         | 2.086     |
| AEA95217.1      | ATPase/chaperone ClpC, probable specificity factor for ClpP protease         | 1.906     |
| AEA94588.1      | HD domain protein                                                            | -         |
| AEA94933.1      | Acetyl esterase/lipase                                                       | -         |
| AEA94972.1      | putative penicillin amidase                                                  | -         |
| AEA94928.1      | LysR family transcriptional regulator                                         | 1.799     |
| AEA93656.1      | beta-lactamase                                                              | -         |
| AEA92985.1      | mannitol-1-phosphate 5-dehydrogenase                                         | 1.711     |
| AEA93763.1      | heat-inducible transcription repressor HrcA                                  | -         |
| AEA93514.1      | hypothetical protein                                                         | 1.655     |
| AEA94757.1      | hypothetical protein                                                         | 1.651     |
| AEA95193.1      | cell wall surface anchor family protein                                       | -         |
| AEA95024.1      | hydrolase                                                                   | 1.585     |
| AEA94737.1      | HAD-superfamily hydrolase                                                    | -         |
| AEA94733.1      | adapter protein MecA                                                         | 1.560     |
| AEA93419.1      | FtsZ-interacting cell division protein YlmF                                  | 1.560     |
| AEA94308.1      | NgoFVII restriction endonuclease superfamily protein                         | -         |
| AEA94061.1      | butyrate kinase                                                             | 1.544     |
| AEA94734.1      | regulatory protein Spx                                                       | -         |
| AEA94693.1      | chaperonin GroEL                                                            | -         |
| AEA93199.1      | MutT/NUDIX family protein                                                    | 1.524     |
| AEA94670.1      | UDP-N-acetylgalcosamine 1-carboxyvinyltransferase                            | 1.520     |
| AEA93452.1      | daunorubicin resistance protein                                              | 1.507     |
| AEA94047.1      | tyrosine recombinase XerC                                                    | 1.500     |
| AEA94288.1      | GNAT family acetyltransferase                                                | 1.497     |
| AEA94227.1      | PP-loop family protein                                                       | 1.496     |
| AEA93700.1      | GntR family transcriptional regulator                                        | 1.478     |
| AEA93154.1      | putative thioredoxin                                                         | -         |
| AEA94591.1      | ribosomal-protein-alanine acetyltransferase                                  | -         |
| AEA93907.1      | fructose-1,6-bisphosphatase                                                  | 1.455     |
| AEA93067.1      | nitroreductase                                                               | -         |
| AEA94513.1      | NifU family SUF system FeS assembly protein                                  | 1.422     |
| AEA93553.1      | hypothetical protein/Thioredoxin_like                                        | -         |
| AEA95234.1      | myosin-cross-reactive antigen                                                | 1.414     |
| Accession Number | Description                                                                 | Conf. (Outlier) |
|------------------|-----------------------------------------------------------------------------|----------------|
| AEA94616.1       | hypothetical protein                                                         | -              |
| AEA94405.1       | hypothetical protein                                                         | 1.409          |
| AEA93483.1       | hypothetical protein                                                         | 1.405          |
| AEA93230.1       | hypothetical protein                                                         | 1.395          |
| AEA95035.1       | GNAT family acetyltransferase                                                 | 1.394          |
| AEA95136.1       | M protein trans-acting positive regulator                                    | 1.392          |
| AEA94760.1       | recombination regulator RecX                                                  | 1.389          |
| AEA94771.1       | ABC superfamily ATP binding cassette transporter, ABC oxidoreductase          | 1.385          |
| AEA92793.1       | hypothetical protein                                                         | 1.384          |
| AEA93715.1       | haloacid dehalogenase family hydrolase                                       | -              |
| AEA95260.1       | GntR family transcriptional regulator                                        | 1.362          |
| AEA93441.1       | GNAT family acetyltransferase                                                 | 1.361          |
| AEA94573.1       | hypothetical protein                                                         | 1.357          |
| AEA93214.1       | ABC superfamily ATP binding cassette transporter, ABC protein                 | 1.353          |
| AEA92756.1       | hypothetical protein                                                         | 1.350          |
| AEA93410.1       | cell division protein                                                        | 1.327          |
| AEA94176.1       | phosphoribosylamine-glycine ligase                                           | 1.316          |
| AEA93340.1       | response regulator                                                           | 1.316          |
| AEA93256.1       | L-seryl-tRNA(Sec) selenium transferase                                       | 1.314          |
| AEA94441.1       | lactoylglutathione lyase                                                     | 1.300          |
| AEA94128.1       | 3-dehydroquinate dehydratase                                                 | -              |
| AEA94544.1       | GntR family transcriptional regulator                                        | 1.296          |
| AEA94273.1       | transcriptional regulator                                                    | 1.294          |
| AEA92934.1       | lactoylglutathione lyase                                                     | 1.290          |
| AEA95077.1       | DNA-directed RNA polymerase subunit omega                                     | -              |
| AEA92957.1       | hypothetical protein                                                         | 1.276          |
| AEA94962.1       | aminotransferase                                                            | 1.276          |
| AEA94536.1       | Fur family transcriptional regulator ZurR                                    | 1.271          |
| AEA93513.1       | hypothetical protein                                                         | 1.268          |
| AEA93713.1       | protein of hypothetical function DUF1212                                     | 1.263          |
| AEA94801.1       | ABC superfamily ATP binding cassette transporter, ABC protein                 | 1.261          |
| AEA93430.1       | sigma-54 interaction domain protein                                          | -              |
| AEA94727.1       | NAD(+) kinase                                                                | 1.261          |
| AEA94097.1       | MerR family transcriptional regulator                                        | 1.258          |
| AEA93950.1       | hypothetical protein                                                         | 1.257          |
| AEA94980.1       | M protein trans-acting positive regulator                                    | -              |
| AEA92806.1       | adenosine deaminase                                                          | 1.255          |
| AEA93450.1       | endonuclease/exonuclease/phosphatase                                          | -              |
| AEA94787.1       | exonuclease                                                                  | -              |
| Accession   | Description                                                                 | Abundance   |
|-------------|-----------------------------------------------------------------------------|-------------|
| AEA94745.1  | (OG1RF_12058, sbcC) exonuclease SbcC                                        | 1.253       |
| AEA95129.1  | (OG1RF_12442) FMN reductase                                                 | -           |
| AEA93282.1  | (OG1RF_10595, opuAA) glycine betaine/L-proline ABC superfamily ATP binding | -           |
| AEA93239.1  | (OG1RF_10552, rplY) 50S ribosomal protein L25                               | 1.246       |
| AEA93267.1  | (OG1RF_10580) PemK family transcriptional regulator                         | -           |
| AEA95167.1  | (OG1RF_12480) DEAH-box family ATP-dependent helicase                        | 1.245       |
| AEA94265.1  | (OG1RF_11578) alpha-hemolysin-like protein                                  | -           |
| AEA92804.1  | (OG1RF_10117) lipase/acylhydrolase                                          | -           |
| AEA93429.1  | (OG1RF_10742) DEAD/DEAH box family ATP-dependent RNA helicase               | 1.243       |
| AEA92904.1  | (OG1RF_10217) phosphoglycerate mutase                                       | 1.241       |
| AEA93066.1  | (OG1RF_10379) phage integrase family site-specific recombinase              | -           |
| AEA94914.1  | (OG1RF_12227) HAD superfamily hydrolase                                     | 1.237       |
| AEA95081.1  | (OG1RF_12394) YicC like protein                                             | 1.236       |
| AEA94216.1  | (OG1RF_11529, fsrA) FsrA response regulator                                 | -           |
| AEA93148.1  | (OG1RF_10461, gatC) glutamyl-tRNA(Gln) amidotransferase subunit C           | -           |
| AEA94809.1  | (OG1RF_12122, yaaT) stage 0 sporulation protein YaaT                         | -           |
| AEA94698.1  | (OG1RF_12011) ABC superfamily ATP binding cassette transporter, ABC protein| 1.231       |
| AEA94715.1  | (OG1RF_12028, coaC) phosphopantothenoylcysteine decarboxylase               | 1.230       |
| AEA93059.1  | (OG1RF_10372, pgpA) phosphatidylglycerophosphatase A                        | 1.229       |
| AEA94099.1  | (OG1RF_11412) transcriptional regulator                                     | 1.223       |
| AEA93605.1  | (OG1RF_10918) MutT/NUDIX family protein                                     | -           |
| AEA93586.1  | (OG1RF_10899, murl) glutamate racemase                                      | 1.217       |
| AEA92699.1  | (OG1RF_10012, dnaB) replicative DNA helicase DnaB                            | 1.216       |
| AEA94183.1  | (OG1RF_11496, purS) phosphoribosylformylglycinamidine synthase subunit PurS | 1.215       |
| AEA94204.1  | (OG1RF_11517, agaS) sugar isomerase protein AgaS                            | 1.215       |
| AEA94381.1  | (OG1RF_11694) ABC superfamily ATP binding cassette transporter, ABC protein| 1.213       |
| AEA92898.1  | (OG1RF_10211, dus) tRNA-dihydouridine synthase                              | -           |
| AEA93779.1  | (OG1RF_11092) ABC superfamily ATP binding cassette transporter, membrane protein | 1.212 |
| AEA94888.1  | (OG1RF_12201) D-isomer specific 2-hydroxyacid dehydrogenase                | 1.212       |
| AEA93602.1  | (OG1RF_10915) 2,5-diketo-D-gluconate reductase                              | 1.212       |
| AEA94423.1  | (OG1RF_11736) group 2 glycosyl transferase                                  | 1.211       |
| AEA94516.1  | (OG1RF_11829, sufC) ABC superfamily ATP binding cassette transporter, ABC protein | - |
| AEA93609.1  | (OG1RF_10922) hypothetical protein                                          | -           |
| AEA93584.1  | (OG1RF_10897) glutamine ABC superfamily ATP binding cassette transporter, binding protein | 1.200 |

Abundance-decreased

| Accession   | Description                                                                 | Abundance   |
|-------------|-----------------------------------------------------------------------------|-------------|
| AEA92854.1  | (OG1RF_10167, rplR) 50S ribosomal protein L18                                | 0.828       |
| AEA93526.1  | (OG1RF_10839) universal stress protein                                       | -           |
| AEA92839.1  | (OG1RF_10152, rplD) ribosomal protein L4/L1 family protein                  | 0.825       |

30
| Accession     | Gene Name            | Description                                                                 | Ratio  |
|--------------|----------------------|-----------------------------------------------------------------------------|--------|
| AEA95174.1   | (OG1RF_12487, rplM) | 50S ribosomal protein L13                                                   | 0.816  |
| AEA95058.1   | (OG1RF_12371, acpP2)| acyl carrier protein                                                          | 0.797  |
| AEA93354.1   | (OG1RF_10667)       | hypothetical protein                                                          | 0.794  |
| AEA93187.1   | (OG1RF_10500)       | ATP-binding protein                                                          | -      |
| AEA93783.1   | (OG1RF_11096)       | hypothetical protein                                                          | 0.787  |
| AEA93274.1   | (OG1RF_10587)       | hypothetical protein                                                          | -      |
| AEA94998.1   | (OG1RF_12311, traC2)| peptide ABC superfamily ATP binding cassette transporter, binding protein   | 0.776  |
| AEA93330.1   | (OG1RF_10643, rplT) | 50S ribosomal protein L20                                                    | 0.773  |
| AEA94110.1   | (OG1RF_11423, pyrE) | orotate phosphoribosyltransferase                                            | -      |
| AEA94119.1   | (OG1RF_11432, upp)  | uracil phosphoribosyltransferase                                            | -      |
| AEA94115.1   | (OG1RF_11428, carA) | carbamoyl-phosphate synthase, small subunit                                  | -      |
| AEA92893.1   | (OG1RF_10206)       | S1 RNA-binding domain protein                                                | 0.705  |
| AEA92696.1   | (OG1RF_10009, rpsR) | 30S ribosomal protein S18                                                    | 0.671  |
| AEA95155.1   | (OG1RF_12468, rpsN2)| 30S ribosomal protein S14                                                    | -      |
| AEA94111.1   | (OG1RF_11424, pyrF) | orotidine-5'-phosphate decarboxylase                                         | 0.670  |
| AEA94116.1   | (OG1RF_11429, pyrC) | dihydroorotase                                                               | 0.650  |
| AEA94767.1   | (OG1RF_12080, rplL2)| ribosomal protein L7/L12                                                      | 0.649  |
| AEA93420.1   | (OG1RF_10733, ylmG) | YlmG protein                                                                 | 0.579  |
| AEA94625.1   | (OG1RF_11938)       | fumarate reductase                                                            | 0.565  |
| AEA93191.1   | (OG1RF_10504)       | thioredoxin superfamily protein                                              | 0.493  |
| AEA93192.1   | (OG1RF_10505, clpP) | ATP-dependent Clp protease proteolytic subunit                               | 0.385  |

The data were given as the means of the results from two independent experiment. **WT**, wildtype/parent strain; -, 0.83 ≤ ratio of ΔclpP/WT ≤ 1.2.

**Table 2** Differential abundance proteins associated with the stress response or virulence, biofilm formation of *E. faecalis*
| Protein ID (locus_tag, gene name) | Description or predicted function | Protein abundance ratio of ΔclpP/WT<sup>a</sup> |
|----------------------------------|-----------------------------------|-----------------------------------------------|
|                                 |                                   | **4 h** | **12 h** |
| **Stress response or virulence** |                                   |        |        |
| AEA94216.1 (OG1RF_11529, fsrA)  | FsrA response regulator           | -       | 1.235   |
| AEA94213.1 (OG1RF_11526, gelE)  | gelatinase GelE                   | -       | 2.577   |
| AEA95218.1 (OG1RF_12531, ctsR)  | transcriptional regulator CtsR    | 2.165   | 1.894   |
| AEA95217.1 (OG1RF_12530, clpC)  | ATPase/chaperone ClpC             | 1.906   | 1.802   |
| AEA94933.1 (OG1RF_12246)        | Acetyl esterase/lipase            | -       | 1.873   |
| AEA94734.1 (OG1RF_12047, spxA)  | regulatory protein Spx             | -       | 1.524   |
| AEA93763.1 (OG1RF_11076, hrcA)  | heat-inducible transcription repressor HrcA | -       | 1.692   |
| AEA94693.1 (OG1RF_12006, groEL) | chaperonin GroEL                  | -       | 1.490   |
| AEA92854.1 (OG1RF_10167, rplR)  | 50S ribosomal protein L18         | 0.828   | -       |
| AEA92839.1 (OG1RF_10152, rplD)  | ribosomal protein L4/L1 family protein | 0.825   | -       |
| AEA95174.1 (OG1RF_12487, rplM)  | 50S ribosomal protein L13         | 0.816   | -       |
| AEA93330.1 (OG1RF_10643, rplT)  | 50S ribosomal protein L20         | 0.773   | -       |
| AEA92696.1 (OG1RF_10009, rpsR)  | 30S ribosomal protein S18         | 0.671   | -       |
| AEA95155.1 (OG1RF_12468, rpsN2) | 30S ribosomal protein S14         | -       | 0.671   |
| AEA94767.1 (OG1RF_12080, rplL2) | ribosomal protein L7/L12          | 0.649   | -       |
| **Biofilm formation**           |                                   |        |        |
| AEA94733.1 (OG1RF_12046, mecA)  | adapter protein MecA              | 1.560   | -       |
| AEA94110.1 (OG1RF_11423, pyrE)  | orotate phosphoribosyltransferase | -       | 0.763   |
| AEA94111.1 (OG1RF_11424, pyrF)  | orotidine-5'-phosphate decarboxylase | 0.670   | -       |
| AEA94116.1 (OG1RF_11429, pyrC)  | dihydroorotase                    | 0.650   | -       |

<sup>a</sup>The data were given as the means of the results from two independent experiment. <sup>b</sup>The data were given as the means of the results from three independent experiment. **WT**, wildtype/parent strain; -, 0.83 ≤ ratio of ΔclpP/WT ≤ 1.2.

**Table 3** Bacterial strains and plasmids used in the present study
Bacterial strain or plasmid | Description
--- | ---
**Bacterial strains**

*E. faecalis* OG1RF | Rifampin- and fusidic acid-resistant derivative of human oral cavity isolate

Δ*clpP* mutant | *clpP* deletion mutant obtained using OG1RF as the parent strain

Δ*clpP/pIB166::clpP* | *clpP* deletion mutant complemented with plasmid pIB166 harboring the *clpP* gene

Δ*clpP/pIB 166* | *clpP* deletion mutant complemented with plasmid pIB166

*E. faecalis* ATCC29212 | Standard strain for the MIC detection

**Plasmids**

pJRS233 | Temperature-sensitive *E. coli* (Erm<sup>750</sup>)-*Enterococcus* (Erm<sup>25</sup>) shuttle vector

pJRS233-Δ*clpP* | Temperature-sensitive plasmid for generation of in-frame deletion of *clpP*

pIB166 | *E. coli* (Cm<sup>20</sup>)-*Streptococcus* (Cm<sup>20</sup>) shuttle vector

pIB166::*clpP* | Used for complementation of *clpP* deletion

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**Table 4 Primers used for the construction of Δ*clpP* mutant and complemented strain**

| Primers<sup>a</sup> | Sequences (5' → 3') | Location<sup>b</sup> | PCR product size (bp)
| --- | --- | --- | ---
| **Construction of Δ*clpP* mutant** | | | |
| *clpP* us-F | GCTCTAGATTGGGGGTGTTGGTTTAGCAG | 530796-530816 | 1059 |
| *clpP* us-R | CGGGATCCCAGCGACACTCG | 529747-529794 |
| *clpP* ds-F | CGGGATCCGGAAGTAAATCC | 529139-529159 |
| *clpP* ds-R | TCTTTATATAAG | 528132-528152 |
| **Construction of Δ*clpP* complemented strain** | | | |
| HB*clpP*-F | CCGGATCCAGGGCATTTCAAGTGGCTTTGTG | 529816-529836 | 714 |
| HB*clpP*-R | CGGAACCACGGACAGAGGACGACG | 529139-529159 |
| **RT-qPCR** | | | |
| *recA*-F | CGACTAATGTCTCAAGCATACTCC | 2574587-2574608 | 106 |
| *recA*-R | CGAACATCACGCCAACTT | 2574503-2574520 |
| *clpP*-F | TTAATTCCAACAGTTTATTGAA | 529746-529766 |
| *clpP*-R | ACCAGGAGAGTATAGTA | 529569-529586 |

<sup>a</sup>Primers were designed according to the genomic sequence of *E. faecalis* OG1RF (GenBank accession number CP002621.1). F, forward primer; R, reverse primer. <sup>b</sup>Location
of the primer in the genomic sequence of *E. faecalis* OG1RF (GenBank accession number CP002621.1). The underlined sequences represent the restriction enzyme sites.

**Figures**

**Figure 1**

Effect of clpP deletion on the growth of *E. faecalis* at 37°C, 20°C and 45°C. Three independent experiments were performed and the data represent as means ± SD.
Sensitivity of the ΔclpP mutant to hyperosmotic pressure, low pH, oxidative stress and SDS. (A) Overnight cultures of the E. faecalis strains were diluted in TSB containing 5% NaCl or with pH 5.5 and then incubated at 37°C for 24 h, or in TSB containing 2 mM H2O2 incubated at 37°C for 10 h. Three independent experiments were performed and the data represent as means ± SD. (B) The E. faecalis strains were spotted onto TSB agar plates containing 0.008% SDS and incubated for 24h at 37°C. Three independent experiments were performed and the representative results were shown.
Figure 3

Effects of the ΔclpP mutant on the biofilm formation and eDNA release of E. faecalis. (A) The biofilms of E. faecalis strains were stained with crystal violet, and the OD570 was determined. *: P < 0.05; **: P<0.01; ***: P<0.001; (Student’s t test). (B) The PI-bound eDNA of E. faecalis strains was measured by a VarioskanTM LUX multimode microplate reader. Three independent experiments were performed and the data represent as means ± SD.
Figure 4

Survival of the ΔclpP mutant and the parent strain with antimicrobials exposure over time. Three independent experiments were performed and the data represent as means ± SD. The dashed line indicates the detection limit of this assay.
Deletion of clpP leads to increased virulence of E. faecalis. G. mellonella were infected with 20 μl inocula of E. faecalis strains containing 5 x 10^6 CFU/mL, and the survival of G. mellonella larvae was recorded at 12 h intervals for 72 h p.i..

The data were collected from three independent experiments, and the representative results were shown. **P < 0.01 (log-rank test)
Gene Ontology (GO) analysis of differential abundance proteins (DAPs). (A) The biological process of DAPs was classified by GO analysis. (B) The molecular function of DAPs was classified by GO analysis.
Figure 7

KEGG analysis of differential abundance proteins (DAPs) (logarithmic phase). The protein family and pathway were analyzed using KEGG (Kyoto Encyclopedia of Genes and Genomes)

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.
