Polish Journal of Veterinary Sciences Vol. 23, No. 3 (2020), 405–414

DOI 10.24425/pjvs.2020.134685

Original article

iTRAQ-based quantitative proteomics analysis reveals inhibitory mechanisms of the antimicrobial peptide MDAP-2 against Salmonella gallinarum

Y. Zhang[1], S. Yu[1], X. Ying[1], B. Jia[1], L. Liu[2], J. Liu[1], L. Kong[1], Z. Pei[1], H. Ma[1]

[1] College of Animal Science and Technology,
Jilin Agricultural University, Xincheng Street No. 2888, Changchun 130118, PR China
[2] Jilin Medical University, Jilin Street No. 5, Jilin 132013, PR China

Abstract

MDAP-2 is a new AMP with high inhibitory activity on Salmonella gallinarum, which may be developed as an antimicrobial agent in the agricultural industry and food preservation. To investigate the underlying the action mechanism of MDAP-2 on Salmonella gallinarum, impacts of MDAP-2 on the growth curve and bacterial morphology of Salmonella gallinarum were studied. iTRAQ-based proteomics analysis was also performed on proteins extracted from treated and untreated Salmonella gallinarum cells. The differentially expressed proteins were then analyzed using the KEGG and GO databases. Finally, the function of some differentially expressed proteins was verified. The results showed that 150 proteins (41 up-regulated and 109 down-regulated) were found differentially expressed (fold > 1.8, $p<0.05$). The results indicate that MDAP-2 kills Salmonella gallinarum mainly through two mechanisms: (i) direct inhibition of cell wall/ membrane/ envelope biogenesis, energy production/ conversion, carbohydrate transport/ metabolism, and DNA transcription/ translation through regulation of special protein levels; (ii) indirect effects on the same pathway through the accumulation of Reactive oxygen species ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet-}$).

Key words: Salmonella gallinarum, AMP, iTRAQ, inhibitory mechanism

Introduction

Salmonella is a Gram-negative (G-) bacterial pathogen with remarkable diversity in its host range and pathogenicity. In the United States (CDC, 2014) about 1.2 million people are infected every year, and treatment costs more than $365 million. It has been reported that cases of food poisoning caused by salmonella also top the list in China (Mezal et al. 2013). Salmonella outbreaks are usually associated with the consumption of contaminated eggs, poultry, meat, milk, fruit, and vegetables (Antunes et al. 2016). In recent years, salmonella has developed resistance to a variety of antibiotics creating a serious threat to human and

Correspondence to: Z. Pei, e-mail: peizhihua1978@126.com; H. Ma, e-mail: mahongxia@jlau.edu.cn

animal health. Therefore, it is of great importance to develop effective natural antimicrobial substances for the treatment of salmonellosis.

Antimicrobial peptides (AMPs) are short peptides with highly effective and broad-spectrum antimicrobial activities produced by almost all types of living organisms. Theories concerning the action mechanism of AMPs remain controversial. Bacteria death caused by AMPs is mainly divided into 3 categories: membrane dysfunction (Orioni et al. 2009), the inhibition of extracellular biopolymer synthesis (Fernandez et al. 2013), and the inhibition of intracellular functions (Hale and Hancock 2008).

MDAP-2 is an AMP mainly effective against G- bacteria, with the characteristics of high antimicrobial activity, lower hemolytic activity, and lower cytotoxicity. To better understand the action mechanism of MDAP-2 against Salmonella gallinarum, quantitative proteomics analysis was performed on proteins extracted from treated and untreated Salmonella_gallinarum cells by using isobaric tag for relative and absolute quantitation (iTRAQ) labeling and LC-MS/ /MS analysis to detect the changes in protein expression.

Materials and Methods

Bacterial strain and antimicrobial peptide

Salmonella gallinarum ATCC 9120 was stored at the basic Veterinary Medicine laboratory in Jilin agricultural university. AMP MDAP-2 was prepared according to the literature (Pei et al. 2014).

Inhibitory effects of MDAP-2 on the growth curve of Salmonella gallinarum

Salmonella gallinarum of 1×10^6 CFU/ml were mixed with nutrient broth either with or without MDAP-2 (50 μg/ml, 1×MIC) and incubated at 37°C. Bacterial samples were collected at 30 min, 50 min, 70 min, 90 min, 110 min, and 130 min, the value of OD_{600} was measured, and the growth curves of Salmonella gallinarum with or without MDAP-2 were recorded.

Observation of bacterial morphology

The Salmonella gallinarum were re-suspended in sodium-phosphate buffer (pH: 7.4) with 1×MIC (50 μg/ml) at 37°C for 2 h. The bacteria were centrifuged at 3500 rpm for 10 min, washed 2 times with the same buffer, and the pellets were fixed in 2.5% glutaraldehyde solution (v/v) for 3 h at 4°C. The samples were finally observed by SEM (Hitachi, S-3400N, JPN).

iTRAQ analysis of proteins

Protein preparation

Salmonella gallinarum cultures (OD_{600}=0.25) were incubated either with or without MDAP-2 (40 μg/ml, 0.8 MIC) at 37°C for 90 min. The bacteria protein was prepared according to the literature (Xu et al. 2018). Protein concentration was quantified using the Bradford method.

iTRAQ labeling

Total protein (100 μg/sample) were enzymatically hydrolyzed at 37°C for 4 h (Trypsin: protein = 1:40), vacuum dried, and removed in 0.5 M TEAB. Peptides from the treated and control cultures were labeled using iTRAQ reagents 114 and 116, respectively. The iTRAQ--labeled peptides were separated using an LC-20AB HPLC pump system (Shimadzu, Kyoto, Japan) with an Ultremex SCX column (Phenomenex, Torrance, CA, USA). Elution was carried out using a linear gradient of buffer A for 10 min, 5-35% buffer B for 40 min, 35-95% buffer B for 1 min, and 5% buffer B balance for 10 min. The flow rate was 1 ml/min, and the absorbance wavelength was 214 nm. The eluted peptides were harvested every minute, pooled into 20 fractions, desalted using a Strata X C18 column (Phenomenex), and dried using vacuum centrifugation.

LC-MS/ MS identification

Analytical separation was performed using an LC-20AD nanoHPLC (Shimadzu) coupled to a triple TOF 5600 system (AB SCIEX, Canada) fitted with a Nanospray III source (AB SCIEX, Canada) and a pulled quartz tip as the emitter (New Objectives, USA). The system was equipped with a 2 cm C18 trap column for online trapping and desalting, and a 10 cm C18 column for analytical separation. 10 ml of 0.5 μg/ml sample was loaded in buffer A (5% ACN, 0.1% FA). Separation was achieved using a gradient of 2% buffer B (95% ACN, 0.1% FA) at a flow rate of 300 μl/min for 1 min, followed by an 8-35% buffer B for 40 min, a 45 min linear gradient to 60% buffer B, a 35-60% gradient of buffer B for 45 min, 80% buffer B for 55 min, and finally 5% buffer B for 60 min. The nanoliter liquid phase separation ends are directly connected to the mass spectrometer. The first-order mass spectrum scanning range is 350-1600 m/z, the resolution is 60000. The resultant mass spectrum parameter was started from 100 m/z, 15000 (resolution). The ion fragmentation mode was HCD, and the fragment ions were measured in Orbitrap. Dynamic exclusion time was set at 30 s. AGC: level 1: 3E6, level 2: 1E5.

Table 1. Primers used for quantitative RT-PCR analysis for selected genes.

Protein name	Gene	Primers
MipA/OmpV family protein	MipA/OmpV	F-ggcgacagcgatgaccatcag R-gccagtgaggtacgcaggaatc
monofunctional biosynthetic peptidoglycan transglycosylase	MBPT	F-ttctggtcttccgccgcaattac R-tcggttatgtggcgcattctgac
TolC family protein	TolC	F-aatggtagaagcggcggaagaac R-gcactgtcgagattggcctgatac
oxidoreductase UcpA	UcpA	F-tgccgatccgctggaagtagg R-ggtactgccgccatcaatgacg
cytochrome-c peroxidase	CcP	F-gcgccgacggtattcaactcc R-tggtcctcctgcttgctcctg
superoxide dismutase [Fe]	SOD [Fe]	F-gcacttccgaaggcggcatc R-tgatagcggcatcggtgaattgc
universal stress protein E	Usp E	F-cgtgaacctggcaagcgaagag R-tggcgatattgattggcgtaacgg
DNA-binding response regulator	DNA-binding RR	F-cctggcatcaatggcctggatatc R-tgccgctaacagaacttgctgac
16S ribosomal RNA	16S rRNA	F-tggcaggctggagtcttgtagag R-ggcacaacctccaagtagacatcg

Data Analysis

MS/ MS data were matched using the Mascot search engine (Matrix Science, UK; version 2.3.02) against the Ens-Zea-0907 (98761 sequences) database. The parameters used potential variable modifications including: Gln- > pyro-Glu (N-term Q), oxidation (M), dominated (NQ), and carbamidomethyl (C). iTRAQ-8plex (N-term) and iTRAQ8plex (K) were used as fixed modifications. Results were considered statistically significant only if there was a 1.8-fold increase/decrease in protein expression levels ($P<0.05$). Functional annotation of the proteins was conducted using the Blast2GO program against the non redundant protein (NR; NCBI) database. Gene Ontology annotation (http://www.geneontology.org/) and the KEGG pathway (http://www.genome.jp/kegg/) enrichment analysis were both used to identify the functional subcategories and the metabolic pathways for the differentially expressed proteins.

Bacterial DNA gel blocking test

DNA was extracted from Salmonella gallinarum with a DNA extraction kit. 60 ng DNA was mixed with 20 µl different concentrations of MDAP-2 (1.0 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml, 0.016 mg/ml, 0 mg/ml), incubated at room temperature for 1 h. The above 10 µl samples were mixed with 2 µl 6 × loading buffer, and electrophoresis was performed in 0.8% agarose gel. Observation and photography were performed in the UVP gel imaging system, and the electrophoresis mobility was analyzed.

Real-time quantitative PCR (qPCR)

RNA was extracted from treated and untreated Salmonella gallinarum with MDAP-2 (40 µg/ml) and reverse-transcribed into cDNA. The primer sequences are shown in Table 1. The efficiency was confirmed using the standard curve method. qPCR reactions were carried out using an Applied Biosystems ABI 7500 Real-Time System in a total volume of 20 µl with 10 µl of SYBR qPCR Mix (TOYOBO), 1.0 µl of template cDNA, 1 µl of each primer, and PCR grade water to a final volume of 20 µl. The qPCR procedure was: 95 °C, 5 min; 40 cycles (95°C, 15 s; 60°C, 30 s). The fold change of selected genes was normalized to the fold change of the reference gene (16S rRNA). The relative gene expression analysis was conducted in triplicate. Significant differences in the data were determined using one-way ANOVA ($p<0.05$).

Cellular antioxidant activity/capacity

Bacteria cells were collected at 0, 30, 60, 90, 120,150 min after incubation with or without MDAP-2 (40 µg/ml), then centrifuged at 8000×g for 5 min. The Superoxide dismutase [Fe] and cytochrome-c peroxidase activities of the bacteria were determined using SOD and CCP determination kits. To determine

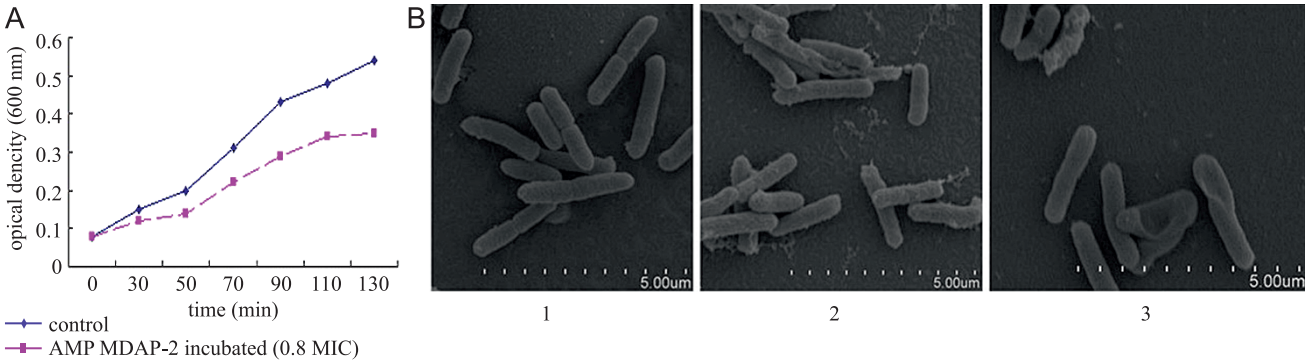

Fig. 1. Time-kill curves of MDAP-2 against Salmonella gallinarum and bacterial morphology of Salmonella gallinarum treated with MDAP-2 under SEM and TEM

A: Time-kill curves of MDAP-2 against Salmonella gallinarum.

B: Bacterial morphology of Salmonella gallinarum under scanning electron microscope. 1: Normal bacterial morphology (negative control); 2, 3: Bacterial morphology of Salmonella gallinarum treated with MDAP-2 at 1×MIC for 60 min under SEM.

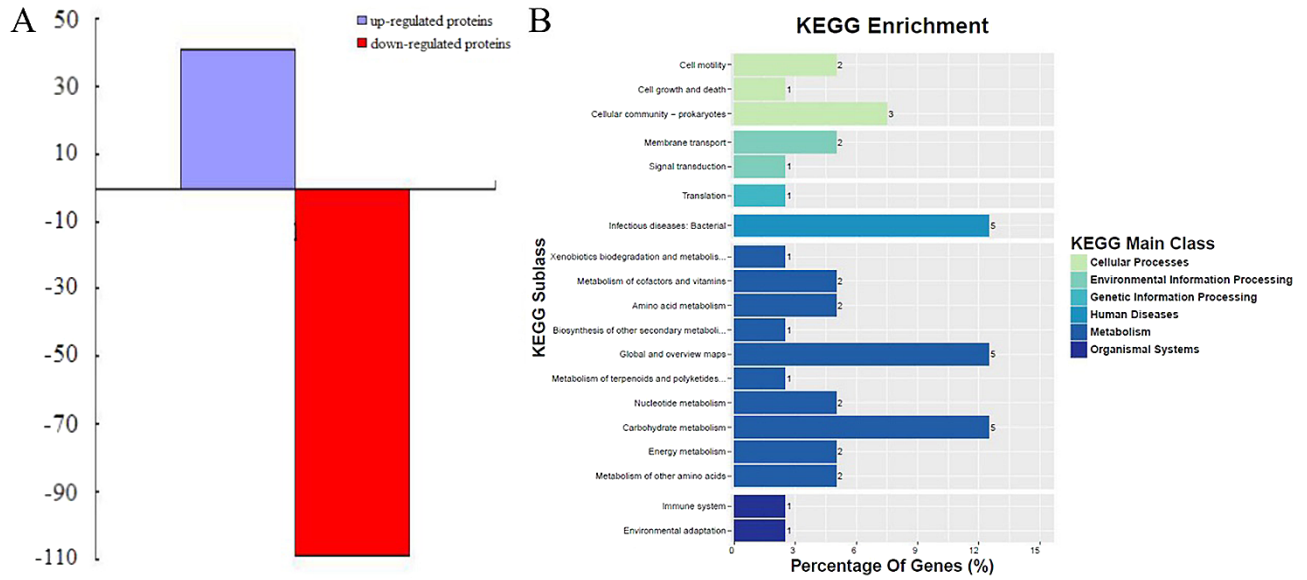

Fig. 2. Statistical summary of iTRAQ results: (A) results of differentially expressed proteins: up-regulated proteins (41), down-regulated proteins (109); (B) KEGG pathway analysis of differentially expressed proteins.

reactive oxygen species, the bacteria cells treated with the above methods were incubated with 2, 7-dichloro-fluorescindiacetate (DCFH-DA) (excitation/ emission: 502/530 nm) at 37°C for 30 min, and fluorescence was measured using a fluorescence spectrophotometer.

Results

Inhibition of MDAP-2 on the growth of Salmonella gallinarum

The inhibitory curves showed that MDAP-2 had inhibitory effects on the growth of Salmonella gallinarum. After only 30 min, the optical density of the treated bacteria was less than the control bacteria. After 90 min, the optical density of the treated bacteria became relatively stable (Fig. 1A). Thus, bacterial cells used to isolate cellular proteins for proteomics analysis were treated with MDAP-2 at 0.8 MIC for 90 min.

Bacterial morphology changes after treatment with MDAP-2

Salmonella gallinarum cells are treated without any antibacterial peptides displayed a normal, smooth membrane surface (Fig. 1B (1)). Compared to the above results, when the Salmonella gallinarum cells were incubated with MDAP-2 at 50 μg/ml for 1 h, the bacterial cells appeared shriveled became flat (Fig. 1B (2) and Fig. 1B (3)).

Primary Data Analysis of Protein Profiles

In this study, 2588 proteins were identified by iTRAQ analysis. Among these proteins, 150 proteins

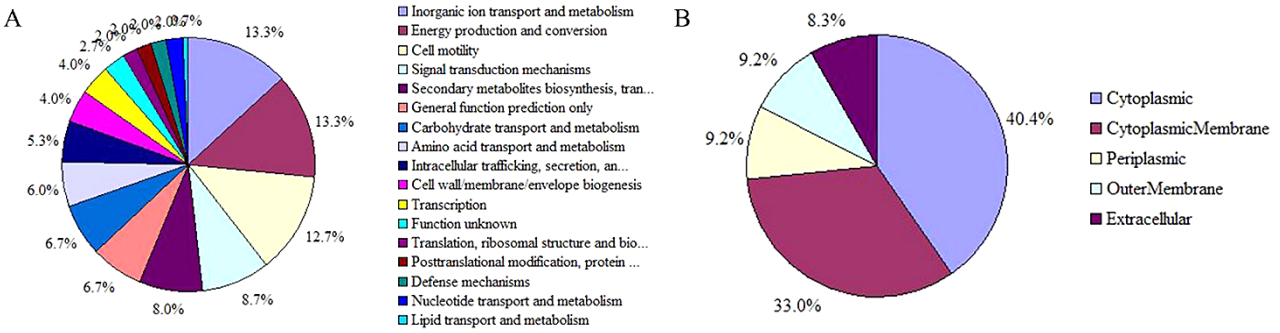

Fig. 3. Classification of the 150 differentially expressed proteins: (A) Enrichment results based on the COG Function-Categories; (B) Enrichment results of the differentially expressed proteins in the cellular components category.

(41 up-regulated and 109 down-regulated) were found differentially expressed (fold > 1.8, $p<0.05$) (Fig. 2A). KEGG pathway analysis revealed that 150 differentially expressed proteins were mainly involved in 6 classes (metabolism, cellular processes, human diseases, environmental information processing, immune system/ /environmental adaptation, genetic information processing) and 19 pathways (Fig. 2B).

Classification of differentially expressed proteins

Based on the COG Function-Categories, the differentially expressed proteins were classified into 17 categories: inorganic ion transport/metabolism (13.3%), energy production and conversion(13.3%), cell motility (12.7%), signal transduction mechanisms (8.7%), secondary metabolites biosynthesis (8.0%), general function prediction only (6.7%), carbohydrate transport/metabolism (6.7%), amino acid transport/metabolism (6.0%), intracellular trafficking/secretion (5.5%), cell wall/membrane/envelope biogenesis (4.0%), transcription (4.0%), translation/ribosomal structure, bio posttranslational modification, defense mechanisms, nucleotide transport/metabolism, and lipid transport and metabolism (Fig. 3A). In the cellular components category, as shown in Fig. 3B, these differentially expressed proteins were mainly classed as Cytoplasmic (40.4%), Cytoplasmic Membrane (33.0%), Periplasmic (9.2%), Outer Membrane (9.2%), and Extracellular (8.3%).

Cell wall/membrane biogenesis

6 proteins involved in cell wall/membrane biogenesis (5 down-regulated and 1 up-regulated) were detected among the differentially expressed proteins (Table 2). N-acetylneuraminate epimerase, monofunctional biosynthetic peptidoglycan, L-Ala-D/L-Glu epimerase, TolC family protein, and chaperone protein Skp are involved in bacteria cell wall/ membrane biogenesis. MipA/OmpV family protein is associated with the outer membrane scaffolding protein for murein synthesis, which is one of the integral components of the membrane. The down-regulation of proteins mostly associated with cell wall/membrane biogenesis implied that MDAP-2 may kill the bacteria by inhibiting the synthesis of the cell wall/membrane.

Cellular antioxidant activity/capacity

As shown in Table 2, 4 proteins involved in cellular antioxidant activity were found down regulated. These were superoxide dismutase (SOD) [Fe], oxidoreductase UcpA, dimethyl sulfoxide (DMSO) reductase and cytochrome-c peroxidase (CCP). UcpA belongs to the short-chain dehydrogenase/reductase family, which are involved in lipid transport/metabolism, secondary metabolite biosynthesis, transport and catabolism. CCP is a heme-containing enzyme of the peroxidase family that takes reducing equivalents from cytochrome c and reduces hydrogen peroxide to water. Both SOD [Fe] and DMSO reductase are closely related to the degradation and scavenging of oxoanions produced in biological processes (McEwan AG 2002, 2004). The results suggest that MDAP-2 inhibited the expression of antioxidant proteins. Four antioxidant proteins involved in cell antioxidant activity/capacity were found down-regulated, including SOD [Fe] UcpA, DMSOR and CCP. Both SOD [Fe] and CCP play important roles in scavenging peroxides produced during biological processes. Both SOD [Fe] and CCP activities were reduced substantially after 2.5 hours exposure of Salmonella gallinarum cells to MDAP-2 (Fig. 4A, B). Meanwhile ROS accumulated in Salmonella gallinarum cells, as shown in Fig. 4C. The results were basically consistent with those of proteomics analysis.

Carbohydrate/amino acid transport and metabolism

In this study, some proteins involved in carbohydrate/amino acid transport and metabolism were also detected based on the iTRAQ analysis. These were N-acetylmannosamine-6-phosphate 2-epimerase, anion

Table 2. Part of the differentially abundant proteins of Salmonella gallinarum after treatment of MDAP-2.

Protein_ID	Protein name / Description	Mean_Ratio	P-value
Cell wall/membrane biogenesis			
WP_001258065.1	L-Ala-D/L-Glu epimerase	2.9	< 0.05
WP_000163762.1	MipA/OmpV family protein	0.38	< 0.05
WP_000525764.1	N-acetylneuraminate epimerase	0.47	< 0.05
WP_000044648.1	monofunctional biosynthetic peptidoglycan transglycosylase	0.48	< 0.05
WP_001746915.1	TolC family protein	0.55	< 0.05
WP_000758966.1	chaperone protein Skp	0.62	< 0.05
cellular antioxidant activity/capacity			
WP_000007299.1	superoxide dismutase [Fe]	0.21	< 0.05
WP_000517461.1	oxidoreductase UcpA	0.32	< 0.05
WP_000724474.1	cytochrome-c peroxidase	0.54	< 0.05
WP_020976729.1	dimethyl sulfoxide reductase	0.55	< 0.05
Signal transduction mechanisms			
WP_001262139.1	universal stress protein E	2.62	< 0.05
WP_000697899.1	two-component system response regulator BasR	1.94	< 0.05
WP_000870073.1	two-component system sensor histidine kinase BaeA	1.81	< 0.05
WP_001031690.1	two-component system sensor histidine kinase PhoQ	1.8	< 0.05
WP_000875811.1	hypothetical protein YebF	1.8	< 0.05
WP_001115499.1	DNA-binding response regulator	0.39	< 0.05
WP_001575963.1	phosphate starvation protein PhoH	0.4	< 0.05
WP_001128499.1	Phosphoenolpyruvate protein phosphotransferase	0.32	< 0.05
WP_000508940.1	envelope stress response membrane protein PspC	0.43	< 0.05
WP_000061317.1	chemotaxis protein CheA	0.55	< 0.05
WP_000147295.1	chemotaxis protein CheW	0.55	< 0.05
transcriptional regulator/activitor			
WP_000019953.1	transcriptional regulator	0.29	< 0.05
WP_000062872.1	transcriptional activator NhaR	0.39	< 0.05
WP_000176719.1	LysR family transcriptional regulator	0.5	< 0.05
WP_000205982.1	TetR/AcrR family transcriptional regulator	0.52	< 0.05
Carbohydrate transport and metabolism			
WP_000054439.1	N-acetylmannosamine-6-phosphate 2-epimerase	0.35	< 0.05
WP_000059042.1	anion permease	0.35	< 0.05
WP_000073069.1	PTS N,N'-diacetylchitobiose transporter subunit IIC	0.41	< 0.05
WP_000078780.1	6-phospho-beta-glucosidase	0.41	< 0.05
WP_000108071.1	MFS transporter	0.45	< 0.05
WP_000152558.1	NAD(P)-dependent alcohol dehydrogenase	0.49	< 0.05
Amino acid transport and metabolism			
WP_000042658.1	putrescine-ornithine antiporter	0.32	< 0.05
WP_000068095.1	thymidine kinase	0.4	< 0.05
WP_000068731.1	aminomethyltransferase	0.4	< 0.05
WP_000097497.1	propanediol utilization microcompartment protein PduB	0.44	< 0.05
Translation, ribosomal structure and biogenesis			
WP_000059075.1	transporter, partial	0.37	< 0.05
WP_000084480.1	endoribonuclease YbeY	0.43	< 0.05
WP_000093987.1	tRNA [N6-threonylcarbamoyladenosine(37)-N6] methyltransferase TrmO	0.44	< 0.05
WP_000132295.1	tRNA glutamyl-Q(34) synthetase GluQRS	0.47	< 0.05

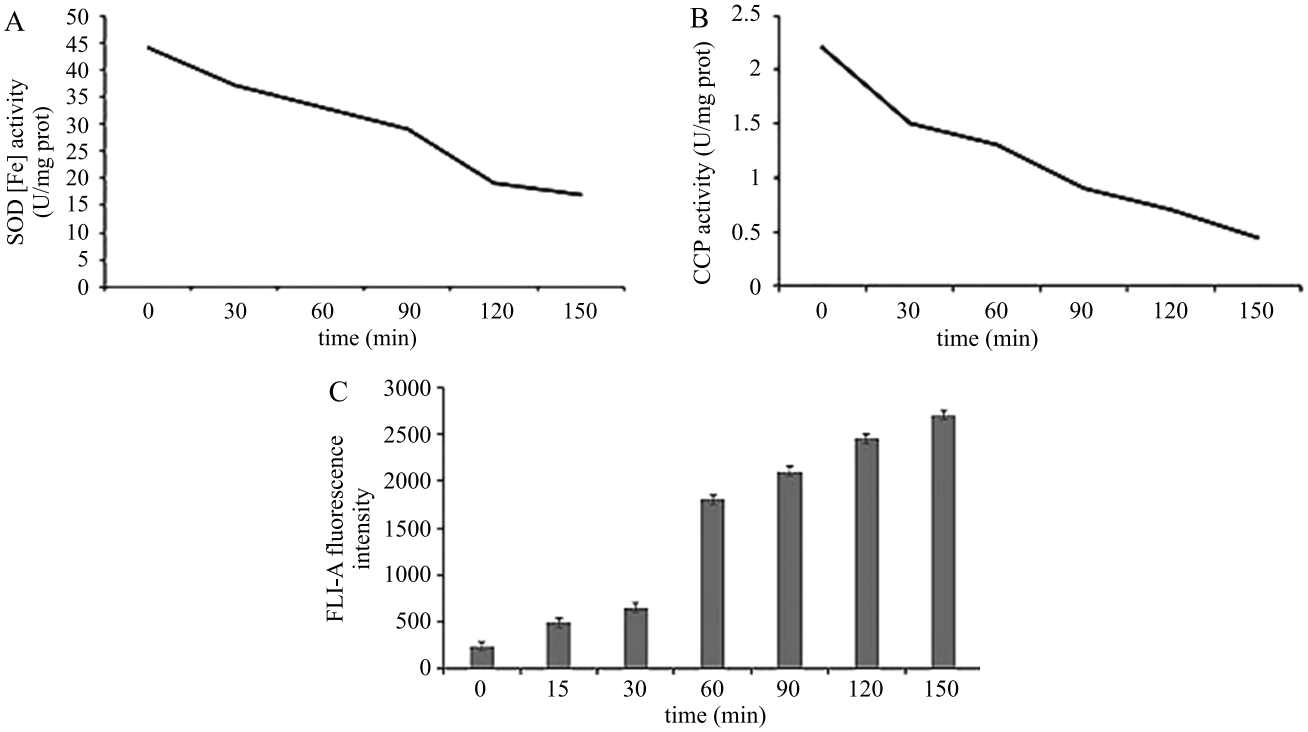

Fig. 4. Antioxidant activity and reactive oxygen species (ROS) determination result: (A) SOD(Fe) activity of bacteria cells, treated with MDAP-2 at 40 μg/mL for 0, 30, 60, 90, 120 and 150 min; (B) CCP activity of bacteria cells, treated with MDAP-2 at 40 μg/mL for 0, 30, 60, 90, 120 and 150 min. Both SOD(Fe) and CCP activity were found continuously decreasing following MDAP-2 exposure; (C) FL1-Afluorescence intensity changes of Salmonella gallinarum cells exposed to MDAP-2 at 40 μg/mL for 0, 30, 60, 90, 120 and 150 min. The ROS of bacteria cells were found continuously increasing following MDAP-2 exposure.

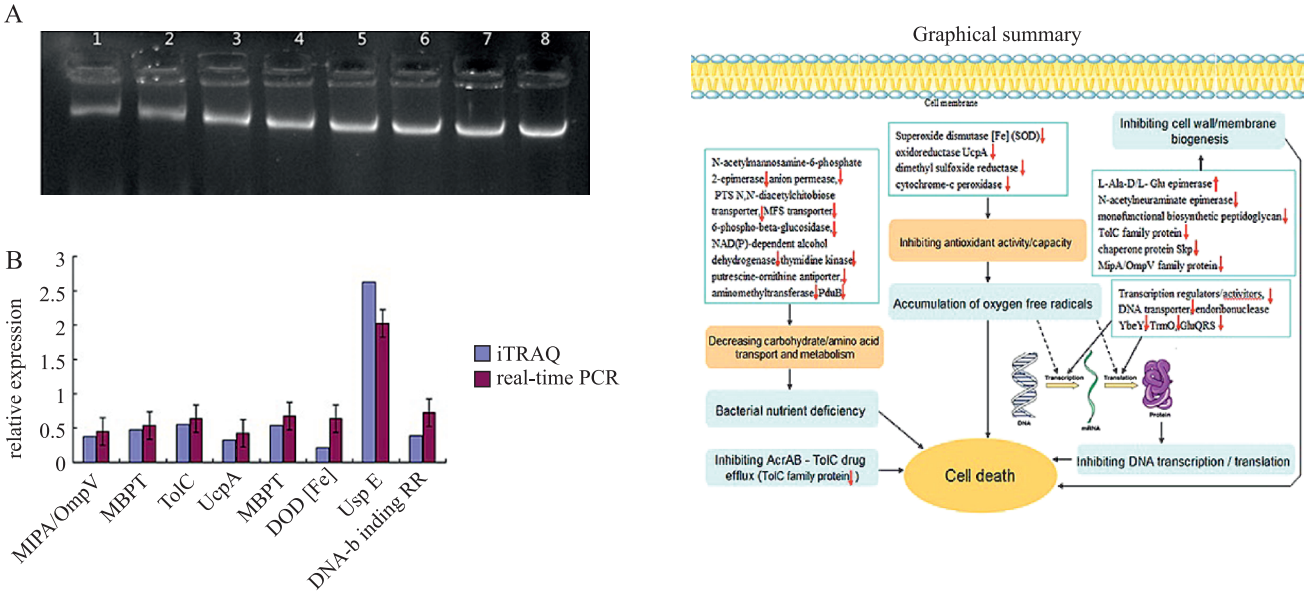

Fig. 5. Bacterial DNA gel blocking test result and Real-time quantitative PCR results: (A)1-8: Concentrations of MDAP-2 were 1.0mg/mL, 0.5 mg/mL, 0.25mg/mL, 0.125mg/mL. 0.0625 mg/mL, 0.03125 mg/mL. 0.016mg/mL and 0 mg/mL, respectively, the bacterial DNA was 30 ng in each lane; (B) Validation of proteomics results at mRNA levels.

permease, PTS N, N'-diacetyl chitobiose transporter, 6-phospho-beta-glucosidase, MFS transporter, NAD (P)-dependent alcohol dehydrogenase, putrescine-ornithine antiporter, thymidine kinase, aminomethyl transferase, PduB etc. Most of these were down-regulated, and the results indicate that MDAP-2 can decrease the transport and metabolism of carbohydrate/amino acid, resulting in bacterial nutrient deficiency, and eventually causing bacterial death (Table 2).

DNA transcription and translation

Several proteins related to DNA transcription and translation are further identified by iTRAQ analysis. These are transcription regulators/ activitors, DNA transporter, endoribonuclease YbeY, TrmO, GluQRS etc. Most of these proteins are down-regulated (Table 2). The results indicate that MDAP-2 inhibited DNA transcription and translation of Salmonella gallinarum. Bacterial DNA gel blocking test results showed that MDAP-2 can bind to the DNA of Salmonella gallinarum and inhibit the electrophoretic mobility 0.8% in agarose gel. Migration of MDAP-2-DNA complexes in agarose gel was significantly delayed (Fig. 5A). This result indicates that MDAP-2 may kill the bacterium by inhibiting DNA transcription and translation. The results were consistent with those of proteomics analysis.

Signal transduction mechanisms

In this study, 12 differentially expressed proteins involved in signal transduction mechanisms were also identified (Table 2). Among the 5 up-regulated proteins, USP E belongs to universal stress proteins (USPs), which play an important role in Salmonella growth arrest, stress, and virulence. The response regulator BasR, sensor histidine kinase BaeA and PhoQ belong to a two-component signal system (TCSs), which is involved in a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. Among the 7 down-regulated proteins, the DNA-binding response regulator belongs to the phosphorelay signal transduction system, which regulates the DNA-templated transcription. Phosphate starvation protein PhoH is involved in ATP binding. Phosphoenolpyruvate-protein phosphotransferase participates in the phosphotransferase system (PTS), specifically those parts of the system transferring phosphorus-containing groups with a nitrogenous group as acceptor. Envelope stress response protein PspC is the major component inhibiting the phage shock protein (Psp) response. Chemotaxis protein CheA and CheW are associated with the transmission of sensory signals from the chemoreceptors to the flagellar motors.

Real-time quantitative PCR results

QPCR reactions were carried out to validate the proteomics results at mRNA levels, as shown in Fig. 5B; the alterations of all the selected genes were basically consistent with these proteins in expression levels. The mRNA expression of MipA/OmpV, MBPT, TolC, UcpA, CcP, SOD [Fe], DNA-binding RR was down-regulated with a range from 0.45 to 0.73 -fold and mRNA expression of Usp E was up-regulated 2.02-fold (Fig. 5B). The results were basically consistent with those of proteomics analysis.

Discussion

In recent years, proteomics tools have been developed greatly to reveal microbial pathogenicity, microbial metabolism, bio-marker discovery and drug mechanism. As a mainstream MS-based proteomics technology, iTRAQ could be used to analyze multiple samples in a single assay. Therefore, iTRAQ-based quantitative proteomics study has been extensively applied to study several kinds of bacteria adaptation to various stress situations, such as nutrient starvation, oxidants, antibacterial agent, and DNA-damaging agents (Walsh 2000). Han et al. (2017) first reported the antibacterial mechanism of LI-Fs against B.cereus at proteome level. In our study, MDAP-2 of 0.8 MIC affected the expression level of many functional bacteria proteins; a total of 150 differentially expressed proteins were detected, based on KEGG analysis, and these proteins were mainly classified into 6 categories, involved in 19 pathways. The results indicate that MDAP-2 has multiple modes of action on Salmonella gallinarum.

Previous studies on the antimicrobial mechanisms of AMPs mainly indicated the inhibition of cell wall/ /membrane biogenesis (Shabir et al. 2018), changes in membrane permeability (Rathinakumar et al. 2009), and protein biosynthesis (Guilhelmelli et al. 2013, Chakchouk-Mtibaa et al. 2014). In this study, many proteins involved in cell wall/membrane biogenesis were detected down-regulated, and the results indicate that MDAP-2 can directly or indirectly interact with bacterial cell membranes through inhibiting the synthesis of the cell wall/ membrane, damaging the integrity of the cell membrane, and eventually killing bacterial cells; this is consistent with the SEM and TEM results. ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet-}$), are produced as by-products of the cell metabolism. The literature (Imlay 2003) shows that the overproduced ROS have numerous adverse effects on bacterial DNA damage, protein degradation and the peroxidation of the cell membrane lipids. In this study, proteomics analysis also detected that MDAP-2 can inhibit the expression of mostly antioxidant proteins, such as SOD, UcpA, and CCP, resulting in the accumulation of ROS in cells, and the accelerated death of bacteria. In addition to the above two main mechanisms, it also inhibited carbohydrate/amino acid transport/metabolism, DNA transcription/translation, and the AcrAB-TolC

drug efflux system by down-regulation of specific proteins.

When Salmonella gallinarum was exposed to AMP MDAP-2, some proteins were up-regulated. This means that MDAP-2 also activated the stimulus-response coupling mechanism to allow Salmonella gallinarum to sense and respond to changes in many different environmental conditions. This notion is in agreement with previous reports with elucidated the drug resistance response of bacteria to AMPs (Yu et al. 2012, Han et al. 2017). TCSs is a basic stimulus-response coupling mechanism allowing G^- bacteria to sense and respond to changes in many different environmental conditions (Stock et al. 2000, Sperandio et al. 2002). TCSs generally consist of a membrane-bound histidine kinase (HK) that senses a specific environmental stimulus and a corresponding response regulator (RR) which mediate the cellular response through differential expression of target genes (Mascher et al. 2006). In this study, the expression of sensor HK BaeA, HK PhoQ and RR BasR was identified as up-regulated. This result indicates that MDAP-2 had activated the TCSS in Salmonella gallinarum.

Some studies (Jovanovic et al. 2010, Flores-Kim and Darwin 2016) have shown that the Psp system plays an important role in the competition for survival under nutrient or energy-limited conditions. In Salmonella gallinarum, the PspB-PspC complex has been indicated to be a possible stress-responsive switch (Tkaczuk et al. 2013, Flores and Darwin 2015). In this study, the expression of envelope stress response membrane protein PspC was detected down-regulated, and resulted in the disruption of the PspB-PspC regulatory complex, reduction of the inhibition on the Psp system; the switch changed from OFF to ON the resulling Psp response was excited, and broad regulon gene-encoding heat shock proteins, ABC transporters, and several putative membrane-associated or secreted proteins were up-regulated to counteract the extra cytoplasmic stresses. USPs are widely spread proteins in archaea, bacteria, fungi and plants. In stress conditions (heat shock, nutrient starvation, oxidants, DNA-damaging agents and other stress agents), USPs are overproduced and, through a variety of mechanisms, aid the organism in surviving in such uncomfortable conditions (Hensel 2009). It has also been shown that USPs are helping pathogens during the invasion of the host organisms (Tkaczuk et al. 2013), which presents potential new opportunities for the pathogenic infection treatment. Bangera et al. (2015) have recently shown that USPs play a significant role in Salmonella growth arrest, stress, and virulence. In this study, proteomics analysis showed that the expression of Usp E was up-regulated (fold = 2.62), and might participate in cellular protection, but the mechanism has not been clearly elucidated.

Acknowledgements

This research was funded by the science and technology project of the 13th five-year plan of Jilin province department of education (Grant number: JJKH20200354KJ), The National Natural Science Foundation of China (Grant number: 31872519), The National Key Research and Development Plan (Grant number: 2017YFD0501401), and The Natural Science Foundation of Jilin Province (Grant number: 20170101021JC, 20180101003JC).

References

Antibiotic resistance threats in the United States (**2013**) Salmonella Homepage, Centers for Disease Control and Prevention reat-report.

Antunes P, Mourão J, Campos J, Peixe L (**2016**) Salmonellosis: the role of poultry meat. Clin Microbiol Infect 22: 110-121.

Bangera M, Panigrahi R, Sagurthi SR, Savithri HS, Murthy MR (**2015**) Structural and functional analysis of two universal stress proteins YdaA and YnaF from Salmonella typhimurium: possible roles in microbial stress tolerance. J Struct Boil 189: 238-250.

Chakchouk-Mtibaa A, Elleuch L, Smaoui S, Najah S, Sellem I, Abdelkafi S, Mellouli L (**2014**) An antilisterial bacteriocin BacFL31 produced by Enterococcus faecium FL31 with a novel structure containing hydroxyproline residues. Anaerobe 27: 1-6.

Flores-Kim J, Darwin AJ (**2015**) Activity of a bacterial cell envelope stress response is controlled by the interaction of a protein binding domain with different partners. J Biol Chem 290: 11417-11430.

Flores-Kim J, Darwin AJ (**2016**) The phage-shock-protein response. Annu Rev Microbiol 70: 83-101.

Fernandez DI, Sani MA, Miles AJ, Wallace BA, Separovic F (**2013**) Membrane defects enhance the interaction of antimicrobial peptides, aurein1.2 versus caerin 1.1. Biochim Biophys Acta 1828: 1863-1872.

Guilhelmelli F, Vilela N, Albuquerque P, Derengowski LS, Silva-Pereira I, Kyaw CM (**2013**) Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Front Microbiol 4: 353.

Hale JD, Hancock RE (**2007**) Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. Expert Rev Anti Infect Ther 5: 951-959.

Han JZ, Gao P, Zhao SM, Bie XM, Lu ZX, Zhang C, Lv FX (**2017**) iTRAQ-based proteomics analysis of LI-F type peptides produced by Paenibacillus polymyxa JSa-9 mode of action against Bacillus cereus. J Proteomics 150: 130-140.

Hensel M (**2009**) Secreted proteins and virulence in salmonella enterica. In: Bacterial secreted proteins: secretary mechanisms and role in pathogenesis. Caister Academic Press, Nottingham, UK.

Imlay JA (**2003**) Pathways of oxidative damage. Annu Rev Microbiol 57: 395-418.

Jovanovic G, Engl C, Mayhew AJ, Burrows PC, Buck M (**2010**) Properties of the phage-shock-protein (Psp) regulatory complex that govern signal transduction and induction of the Psp response in Escherichia coli. Microbiology 156: 2920-2932.

Mascher T, Helmann JD, Unden G (**2006**) Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol Mol Biol Rev 70: 910-938.

McEwan AG, Kappler U (**2004**) The DMSO reductase family of microbial molybdenum enzymes. Australian Biochemist 35: 17-20.

McEwan AG, Ridge JP, McDevitt CA, Hugenholtz P (**2002**) The DMSO Reductase Family of Microbial Molybdenum Enzymes; Molecular Properties and Role in the Dissimilatory Reduction of Toxic Elements. Geomicrobiology 19: 3-21.

Mezal EH, Stefanova R, Khan AA (**2013**) Isolation and molecular characterization of salmonella enterica serovar javiana from food, environmental and clinical samples. Int J Food Microbiol 164: 113-118.

Orioni B, Bocchinfuso G, Kim JY, Palleschi A, Grande G, Bobone S, Park Y, Kim JI, Hahm Ks, Stella L (**2009**) Membrane perturbation by the antimicrobial peptide PMAP-23: a fluorescence and molecular dynamics study. Biochim Biophys Acta 1788: 1523-1533.

Pei Z, Sun X, Tang Y, Wang K, Gao YH, Ma HX (**2014**) Cloning, expression, and purification of a new antimicrobial peptide gene from Musca domestica larva. Gene 549: 41-45.

Rathinakumar R, Walkenhorst WF, Wimley WC (**2009**) Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: the importance of interfacial activity. J Am Chem Soc 131: 7609-7617.

Shabir U, Ali S, Magray AR, Ganai BA, Firdous P, Hassan T, Nazir R (**2018**) Fish antimicrobial peptides (AMP's) as essential and promising molecular therapeutic agents: A review, Microb Pathog 114: 50-56.

Sperandio V, Torres AG, Kaper JB (**2002**) Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli. Mol Microbiol 43: 809-821.

Stock AM, Robinson VL, Goudreau PN (**2000**) Two--component signal transduction. Ann Rev of Biochem 69: 183-215.

Tkaczuk LA, Shumilin I, Chruszcz M, Evdokimova E, Savchenko A, Minor W (**2013**) Structural and functional insight into the universal stress protein family. Evol Appl 6: 434-449.

Walsh C (**2000**) Molecular mechanisms that confer antibacterial drug resistance. Nature 406: 775-781.

Xu Y, Shi J, Hao W, Xiang T, Zhou H, Wang W, Meng Q, Ding Z (**2018**) iTRAQ-based quantitative proteomic analysis of Procambarus clakii hemocytes during Spiroplasma eriocheiris infection. Fish Shellfish Immunol 77: 438-444.

Yu W, Yin C, Zhou Y, Ye B (**2012**) Prediction of the mechanism of action of fusaricidin on Bacillus subtilis. PLoS One 7: e50003.