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EXPERIMENTAL WORKS

Comparative Proteomic Analysis of *Salmonella* Typhimurium LT2 and Its *his*G Gene Inactivated Mutant[1]

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Abstract—A *hisG* gene, encoding ATP phosphoribosyl phosphothansferase, insertion mutation was found to effected the production of four different proteins in *Salmonella* Typhimurium LT2 as a results of comparative analysis of two-dimensional analysis of hisG mutant (Δ*his*87) and the conrol strain (LT2). MALDI-TOF analysis of these protein spots which were expresed in *S.* Typhimurium LT2 but not its mutant Δ*his*87, showed that these proteins were; GMP synthase, arginine-tRNA ligase (Arginyl-tRNA synthetase), SopE and SifA. All of these proteins are virulence-associated proteins in *Salmonella*. Thus we have concluded that the proper function of histidine operon is very crucial for virulence of *S.* Typhimurium.

Keywords: *Salmonella*, proteomics, *hisG*

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INTRODUCTION

Infectious diseases represent a major worldwide threat to human health [8]. *Salmonella* species are enteric pathogens and a world leading cause of bacterial foodborne illness [21]. Essential virulence strategies of the enteric pathogen *S.* Typhimurium include survival and replication within the macrophage which are critical to defense against bacterial infection [35]. It has been reported that some mutants of *S.* Typhimurium such as purine, prymidine, histidine and aromatic amino acid auxotrophs, cannot survive within the macrophage, are avirulent [13, 27, 22]. These studies clearly showed that the metabolic flexibility and adaptation to intracellular environment were important for intracellular survival and replication of *S.* Typhimurium.

The histidine operon of *S.* Typhimurium is a cluster of nine genes that code for the ten enzymes that catalyze histidine biosynthesis. In the first step of this pathway, a phosphoribosyl transferase, encoding by *his*G gene, catalyses condensation of ATP and 5'-phosphoribosyl 1-pyrophosphate (PRPP) to form N'-5'-phosphoribosyl-ATP (PRATP) [25]. Although avirulent characteristics of *S.* Typhimurium mutants bearing nonfunctional activities which are required for histidine biosynthesis were concluded that due to auxotropy, and, polar and pleitropic effect of these mutations [4, 6, 10, 14, 21, 22], there is no direct impacts of these mutations on *Salmonella* pathogenicity.

The aim of this study was to determine the effect of *his*G gene on pathogenicity associated protein expression of *S.* Typhimurium LT2.

MATERIALS AND METHODS

Mutation Assay

HisG gene mutant (Δ*his*87) of *S.* Typhimurium LT2 was selected from a library of random T-POP insertion mutants (34) and T-POP insertion site was cloned by inverse PCR (23) using the primer pair 5'-GCACTTGTCTCCTGTTTACTCC-3' and 5'-CGCTTTTCCCGAGATCATATG-3' for amplification. PCR products were cloned into *Escherichia coli* DH5α using the vector PCR2.1 (Invitrogen) and the respective nucleotide sequences were determined.

Total Protein Extraction

S. Typhimurium strains was grown in 20 mL Luria Bertani (LB) broth medium at 37°C for 18 h with shaking (200 rpm). 500 μL of these fresh cells were inoculated to 50 mL Erlenmeyer flasks and incubated for 6 h with shaking (200 rpm). Growing cells were harvested by centrifugation (7500 rpm, 10 min). Cell residues was resuspended in 25 mL PBS (phosphate buffered saline) and this step repeated twice. After supernatants were removed, 1 mL of total protein extraction solution (Bio-Rad ReadyPrep Total Protein Extraction Kit, United States), 10 μL pH 3–10 ampholyte and 11 μL TBP (tributyl phosphate) was added on the tubes. In order to complete cell lysis, sonication process was per-

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formed (60 Amp, for 5 × 10 s) (Ultrasonic processor, Sonics, Vibra cell, Taiwan). At final stage, cell debris was precipitated by centrifugation (15000 rpm, 25 min) and supernatants was transferred to new tubes. For protein quantitation, RC DC protein assay was carried out (Bio-Rad RC DC Protein Assay, United States). BSA (bovine serum albumin) has been used as a standart protein to ensure equal protein loading. The experiments was carried out as triplicate.

2D Gel Analysis

The first step of sodium dodecyl sulfate (SDS) two dimensional gel electrophoresis was rehydratation process. 24 cm pH 3–10 NL (non-linear) strips were used for that process (Bio-Rad Ready IPG Strip, United States). Samples which contain 150 μg/mL protein were mixed with rehydratation buffer including 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 0.5% IPG Buffer, 0.02% bromphenolblue, with DTT (2.8 mg/mL). The final volume was 450 μL. Following the at 15000 centrifugation rpm for 5 min, samples were loaded into wells. Mineral oil was added on the wells to prevent evaporation. Active rehydratation was performed for 15 h, at 20°C, 50 V (Protean IEF Cell, Bio-Rad, United States). Once rehydratation process was completed, IEF (Isoelectric focusing) was initiated at 80000 V-h. The focused IPG strips were equilibrated with equilibration buffer I (DTT, 0.5 M Tris-HCl (pH 6.8), 10% SDS (w/v), glycerol) and equilibration buffer II (Iodoacetamide, 0.5 M Tris-HCl (pH 6.8), 10% SDS (w/v), glycerol) for 10 min. Strips were loaded into dodeca electrophoresis apparatus (Protean Plus Dodeca Cell, Bio-Rad, United States) to seperate proteins according to their molecular weights. The running conditions for stacking gel were at 18°C, 125 V, and for seperating gel were at 18°C, 200 V (16). After running, the gels were stained with reference to silver nitrate staining method (30).

MALDI-TOF Analysis

Spot analysis were performed between mutant and wild type also replicates. The spots which was detected by using PD Quest programme was excised from the gels (The Proteome Works Spot Cutter, Bio-Rad, United States) and transferred to 96 microwell plates. Following the trypsinisation and lyophilization steps, samples were mixed with matrix CHCA (sinapinic acid) (1 : 1) and analysed by MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry). Five peptide mixture (20 pmol/μL ACTH 18-39, 1000 pmol/μL Renin 1–14, 10.000 pmol/μL Substance P, 20.000 pmol/μL Angiotensin and 1000 pmol/μL Glu-Fib) was used for calibration. The data obtained from MALDI-TOF-MS were analysed by MASCOT/Mass Finger Print search engine (32).

RESULTS AND DISCUSSION

Sequence comparision using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/) revealed that the T-POP insertion muation was occured at *hisG* gene of histidine operon of *S.* Typhimurium LT2 (Δ*his*87).

Protein patterns of 2-D gel electrophoresis showed that 4 different protein spots detected at *S.* Typhimurium LT2 extracts were not found at *S.* Typhimurium Δ*his*87 extracts. The spot numbers of these protein were; 7507, 6309, 2512 and 6308 (Figs. 1–4).

After protein spots were excised from the gels, they were identified by MALDI-TOF-MS. 9 common peptides were determined between protein spot 7507 and Arginine-tRNA ligase (913.6503, 2274.6172, 2274.9465, 2275.8030, 891.5310, 1343.2618, 1344.1228, 892.6037, 1117.9342 Dalton) (Fig. 5).

5 common peptides were detected between protein spot 6309 and secreted effector protein SifA (1060.5424, 1061.5332, 1304.9124, 1144.8882, 912.5651 Dalton) (Fig. 6).

6 common peptides were detected between protein spot 2512 and GMP synthase (glutamine-hydrolysing) (871.3944, 1020.8837, 1021.0162, 1060.6323, 1434.5200, 1305.3115 Dalton) (Fig. 7).

5 common peptides were detected between protein spot 6308 and Guanine nucleotide exchange factor SopE (1304.1641, 1143.9009, 928.7015, 1117.1028, 1898.6469 Dalton) (Fig. 8).

Histidine biosynthesis is an cross-road that plays important role in cellular metabolism being interconnected to both the de novo synthesis of purines and to nitrogen metabolism (15). Mutational analyses of the histidine operon showed that *hisG* gene, the first gene of this operon encoding ATP phosphpribosyl phosphptransferase enzyme, influenced related and unrelated traits in *Salmonella*. Polarity effects of *hisG* gene mutations are well documented in *Salmonella* and other bacteria [11, 24, 10, 2, 12, 15, 26] but there is a limited information about pleitropic effects of this gene [13, 21]. Here we first reported that the insertional inactivation of the *hisG* gene has resulted the repression of the proteins; ArgRS (arginine-tRNA synthetase, ArgRS), SopE (guanine nucleotide exchange factors for the RhoGTPase), SifA (secreted effector protein) and GuaA (guanosine monophosphate synthetase or GMP synthase).

GuaA is a glutamine amido transferase that catalyses the glutamine or NH_3 dependent synthesis of GMP from xanthosine 5'-monophosphate. De novo synthesis of guanine nucleotides is essential for DNA and RNA synthesis. It also provides GTP, which is a key regulator and energy source in many cellular processes [29, 35]. SifA protein that encoded in *Salmonella* pathogenicity island-2 (SPI-2) is an effector of the type three secretion system (TTSS). After the infection of non-phagocytic host cells, *Salmonella enterica* serovars build up *Salmonella* containing vacu-

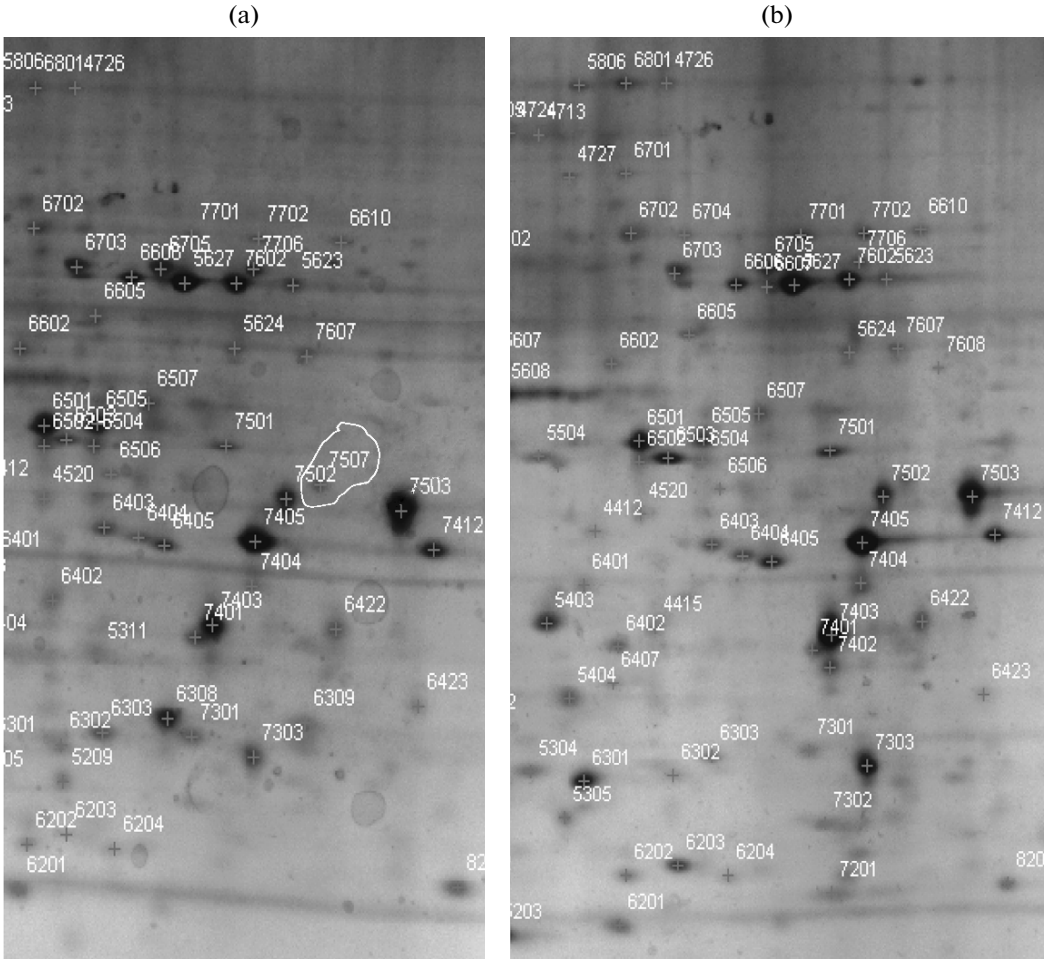

Fig. 1. (a) *S.* Typhimurium LT2, (b) *S.* Typhimurium Δ*his87* (7507).

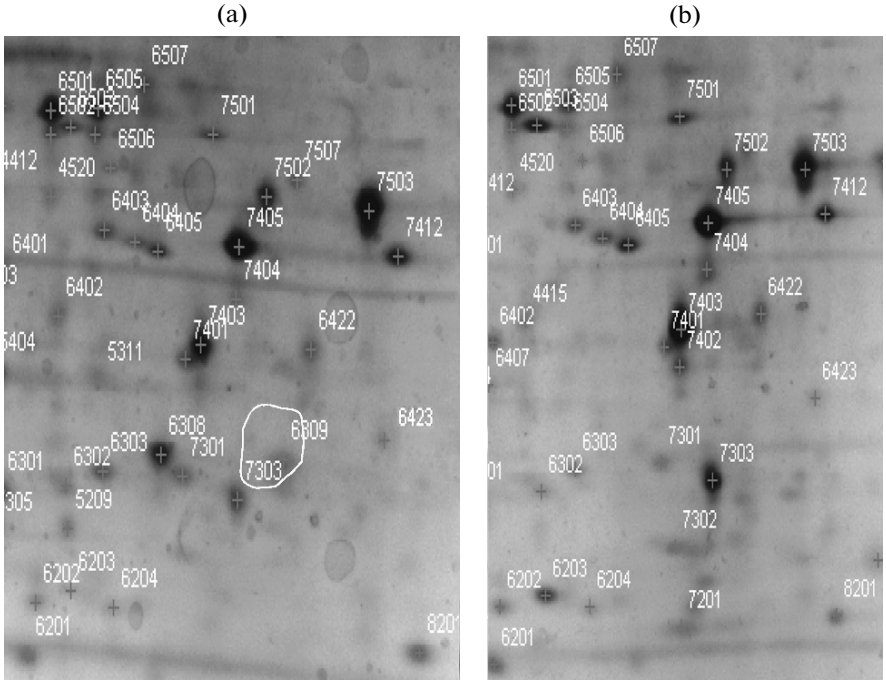

Fig. 2. Comparative protein profile analysis (a) *S.* Typhimurium LT2, (b) *S.* Typhimurium Δ*his*87 (6309).

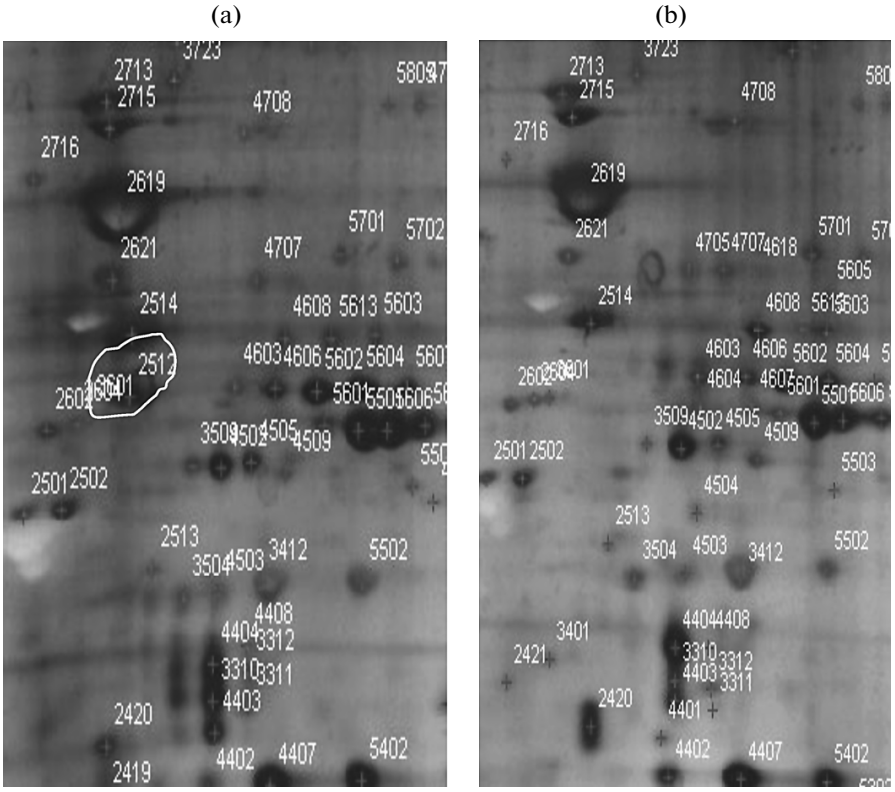

Fig. 3. Comparative protein profile analysis (a) *S.* Typhimurium LT2, (b) *S.* Typhimurium Δ*his*87 (2512).

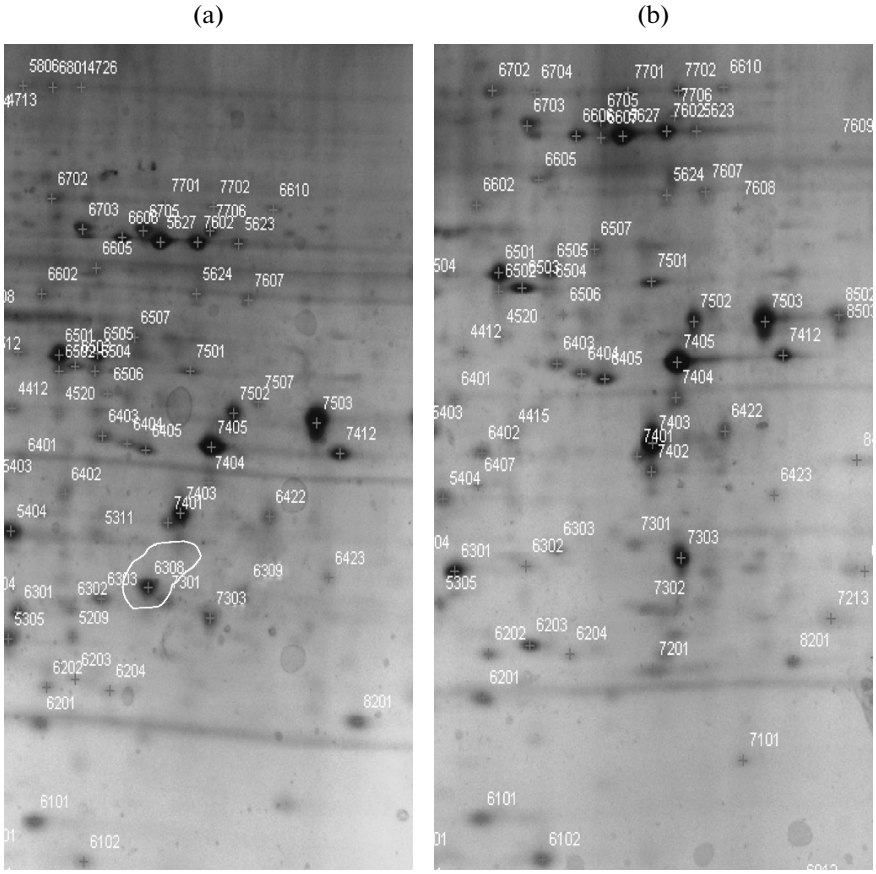

Fig. 4. Comparative protein profile analysis (a) *S.* Typhimurium LT2, (b) *S.* Typhimurium Δ*his*87 (6308).

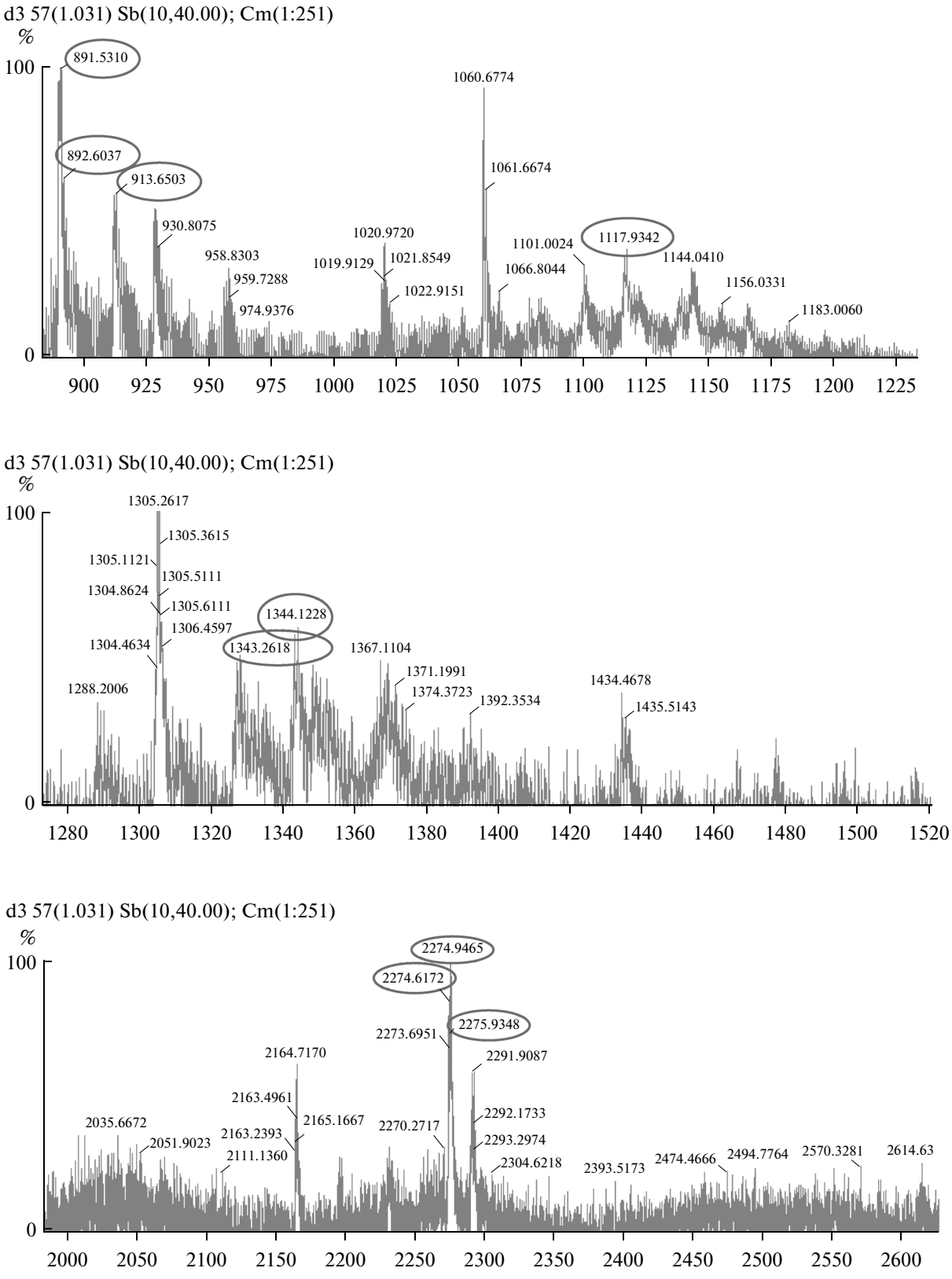

Fig. 5. Common peptides with Arginine-tRNA ligase in protein spot 7507.

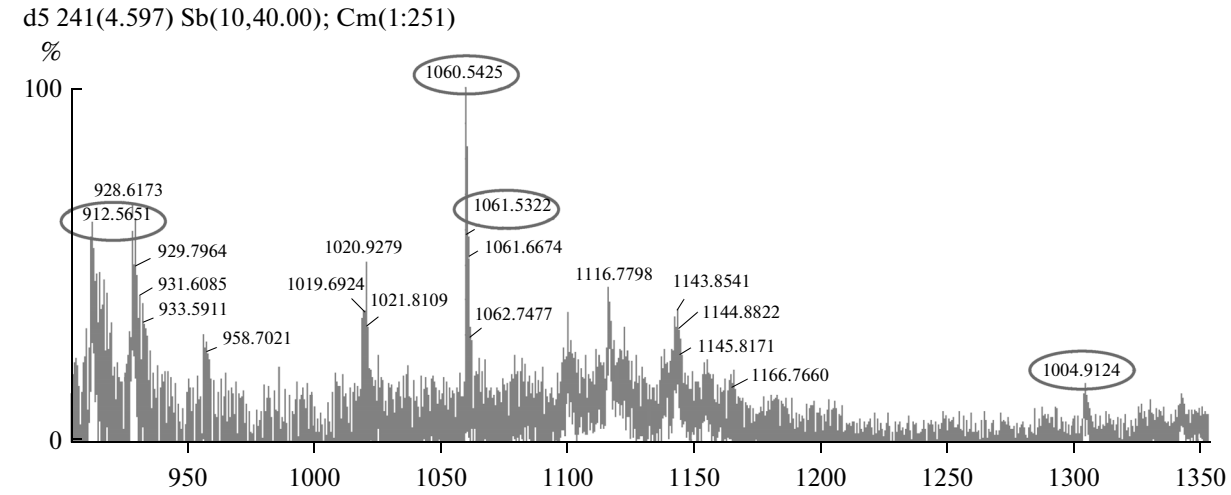

Fig. 6. Common peptides with secreted effector protein SifA in protein spot 6309.

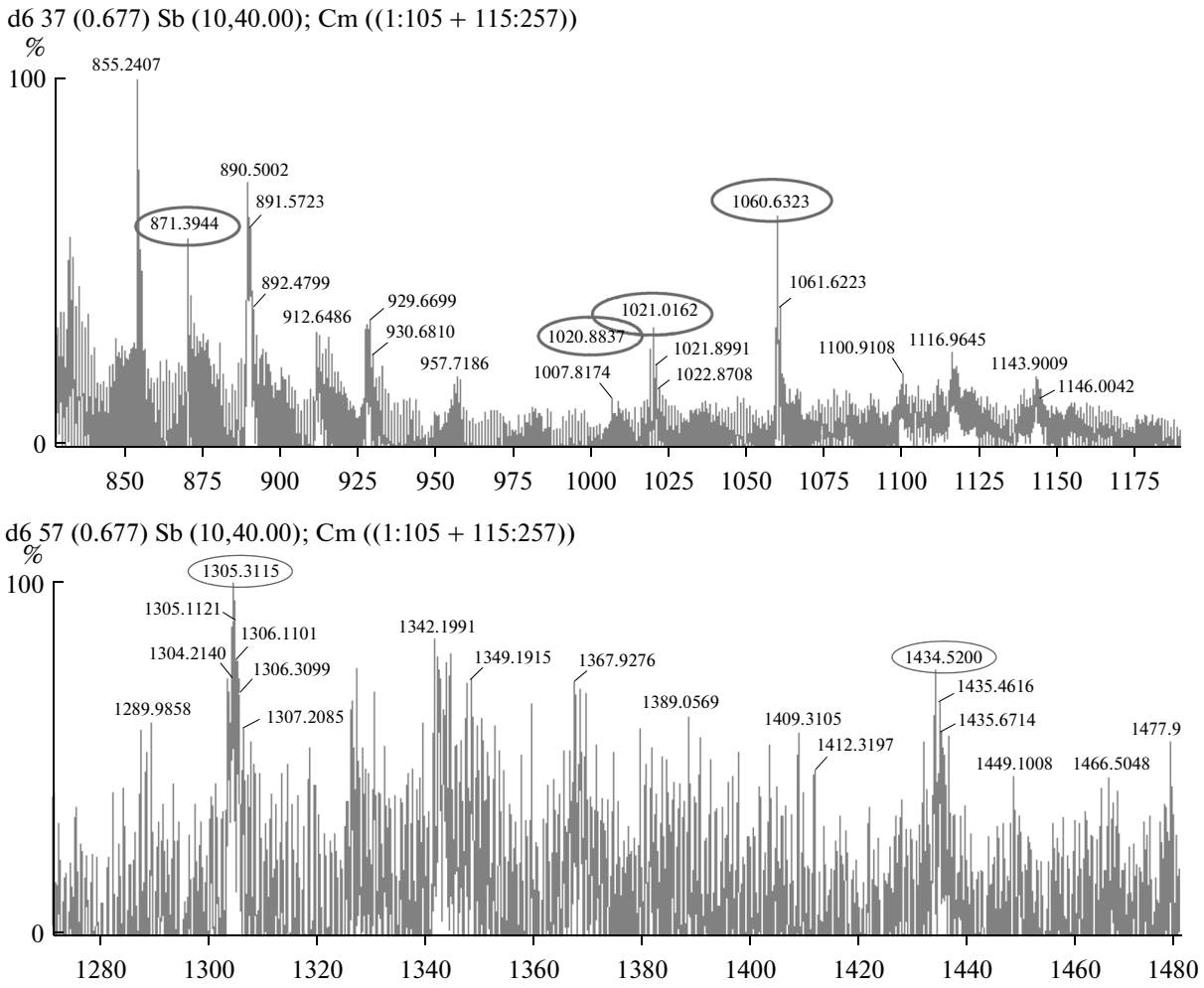

Fig. 7. Common peptides with GMP synthase in protein spot 2512.

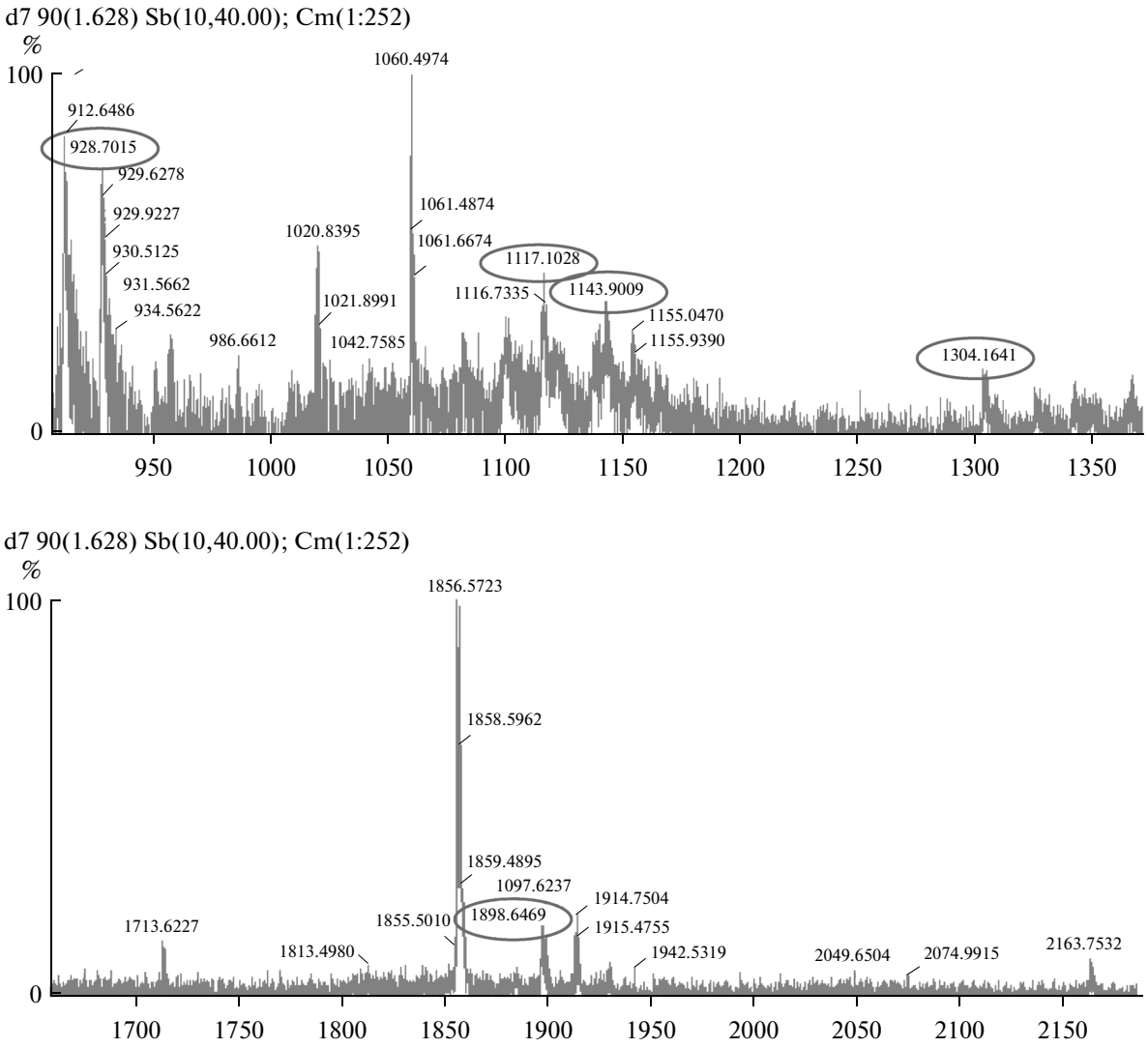

Fig. 8. Common peptides with Guanine nucleotide exchange factor SopE in protein spot 2512.

ole (SCV) to survive and replicate itself in the host. Formation of *Salmonella*-induced filament (Sif) provides an appropriate niche for replication of *Salmonella*. SifA shows its function by activating RhoA family GTPases [1, 17, 18]. Guanine nucleotide exchange factor SopE is encoded in *Salmonella* pathogenicity island-1 (SPI-1) and one of the effectors of type III secretion system. It is essential for membrane ruffling in the host. SopE induces membrane ruffling by stimulating GDP/GTP nucleotide exchanging in Rho GTPases and activates Rac-1 and Cdc42 cell signal transduction cascade. SopE plays a crucial role both rearrangement of cytoslceloton and production of proinflammatory cytokines [5, 19]. GMP synthase, SopE and SifA production regulation is mainly subject to stringent control, growth-phase dependent regulation, purine repression and their activities coupled to the DNA replication cycle [7, 3, 33, 22, 31]. According to our results purine auxotrophic character of *hisG* mutant, resulted from direct repression of GMP synthetase in the absence of ATP phospho-ribosyl phosphotransferase, directly effected the production of the *Salmonella* virulence factors SopE and SifA.

ArgRS catalyses ATP + L-arginine + tRNA(Arg) = AMP + diphosphate + L-arginyl-tRNA(Arg) reaction (28). Regulation of ArgRS synthesis seems to be important in histidine auxotrophy to prevent waste of cellular resources by synthesis of components at a level beyond what is required for efficient growth [20]. It has been determined that histidine-transporter encoded by *hisJOMP* operon, induced by histidine auxotrophy, is a member of arginine regulon. The corresponding gene products are part of the lysine, arginine and ornithine amino acids uptake system is strongly repressed by arginine. Therefore a stronger arginine-dependent repression is incompatible with histidine uptake (9). Our

results shows that argRS regulation is related with the production of ATP phosphpribosyl phosphptransferase enzyme production to cope with this problem under histidine auxotrophic conditions.

This study shows that the first enzyme of histidine biosynthetic pathway is very important for *Salmonella* Typhimurium pathogenicity. Determination of the regulatory roles of *his*G gene mutations at molecular level will play a crucial role for understanding the global regulation of *Salmonella* infection.

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