The Herbal Compound Thymol Targets Multiple *Salmonella* Typhimurium Virulence Factors for Lon Protease Degradation

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Many important bacterial pathogens are using the type III secretion system to deliver effectors into host cells. *Salmonella* Typhimurium (*S*. Typhimurium) is a pathogenic Gram-negative bacterium with the type III secretion system as its major virulence factor. Our previous studies demonstrated that thymol, a monoterpenic phenol derivative of cymene, inhibited *S*. Typhimurium invasion into mammalian cells and protected mice from infection. However, the antibacterial mechanism of thymol is not clear. In this study, we revealed that thymol interferes with the abundance of about 100 bacterial proteins through proteomic analysis. Among the 42 proteins whose abundance was reduced, 11 were important virulence factors associated with T3SS-1. Further analyses with SipA revealed that thymol directly interacts with this protein to induce conformational changes, which makes it susceptible to the Lon protease. In agreement with this observation, thymol effectively blocks cell invasion by *S*. Typhimurium. Thus, thymol represents a class of anti-virulence compounds that function by targeting pathogenic factors for degradation.

**Keywords:** type III secretion, *Salmonella*, anti-virulence, natural compounds, thymol

**INTRODUCTION**

Bacterial pathogens actively modulate host defense systems for successful colonization; such modulation is often achieved by virulence factors delivered into host cells by specialized protein secretion machineries belonging to systems such as the type III, type IV, and type VI secretion apparatuses (Costa et al., 2015). As mutants lacking such systems often are completely defective in virulence, these systems are valuable targets for the development of anti-infection agents (Rasko and Sperandio, 2010). Because inhibition of secretion systems involved in virulence does not impose a survival pressure on either the pathogen or the normal microflora, such anti-virulence compounds will less likely cause the development of resistance, and thus have been viewed as an ideal alternative to traditional antibiotics (Rasko and Sperandio, 2010).

*Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) is an important foodborne pathogen. Recently, many antibiotic-resistant strains are constantly emerging worldwide. Therefore, it is urgent to develop new therapeutic methods to fight *Salmonella* infection. *S*. Typhimurium encoded two different machineries in T3SS, which is SPI-1 and SPI-2 (Agbor and McCormick, 2011). SPI-1 plays an important role in the process of bacterial invasion (Johnson et al., 2018). In the host cell, SPI-2 modulated the replication of bacteria (Johnson et al., 2018).
T3SS is a potent target for the development of therapeutic agents against microbes. Some compounds from natural products have been reported to target T3SS for the treatment of infection diseases (Pendergrass and May 2019; Lv et al., 2020; Sundin et al., 2020). Previous studies showed that thymol, a compound from traditional Chinese medicine (TCM), could reduce the biofilm formation (Cabarkapa et al., 2019) and disrupt the membrane integrity of S. Typhimurium in vitro (Chauhan and Kang, 2014). Studies based on proteomics and real-time PCR assay highlighted that thymol exposure significantly downregulated some main virulence genes of S. Typhimurium (Giovagnoni et al., 2020; Qi et al., 2020). Our previous study also confirmed the antibacterial effects of thymol in a mice model infected by S. Typhimurium (Zhang et al., 2018). However, the mechanism underlying the pharmacological activities of thymol is not clear. In this study, we reported that thymol strongly inhibited SipA delivered into HeLa cells but does not affect bacterial growth via using a reporter system of the activity of SPI-1. Further studies indicated that thymol functions by directly targeting multiple bacterial proteins, including important virulence factors for Lon protease degradation.

### MATERIALS AND METHODS

**Ethic Statements**

BALB/c mice were obtained from the Experimental Animal Center of Jilin University. All procedures involving animals...
## TABLE 2 | Primers used in this study.

| Primer name | Sequence (restriction enzyme sites are underlined) | Note |
|-------------|----------------------------------------------------|------|
| SL1101      | GTCGGAGCTGCTGGTGCTGACGAGAAGGGAACAGGCA            | sipA up Sac I |
| SL1102      | TCCCGCGGACGCTCAGTGGTAAGGGCACAGGCA               | Knock-in 3'flag |
| SL1103      | CGGCTCGAGTAATCCGCGGACAGATTGCAAGTGGTGAGTGA      | sipA down Sac I |
| SL1104      | TAGGATGACGTTGACGAGAGATAGGTAACGCTAA             | Knock-in 3'flag |
| SL1105      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA down Sac I |
| SL1106      | GTCGGAGCTGCTGGTGCTGACGAGAAGGGAACAGGCA          | Knock-in 3'flag |
| SL1107      | TCCCGCGGACGCTCAGTGGTAAGGGCACAGGCA               | sipA up Sma I |
| SL1108      | CGGCTCGAGTAATCCGCGGACAGATTGCAAGTGGTGAGTGA      | sipA down Xho I |
| SL1109      | TAGGATGACGTTGACGAGAGATAGGTAACGCTAA             | Knock-in 3'flag |
| SL1110      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 5 BamH I |
| SL1111      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 3 SaI |
| SL1112      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 3 SaI |
| SL1113      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 3 SaI |
| SL1114      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA (1–270) 5' BamH I |
| SL1115      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA (1–270) 5' BamH I |
| SL1116      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA (1–270) 3' SaI |
| SL1117      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 5 BamH I |
| SL1118      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | sipA 3 Xho I |
| SL1119      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | prgK 5 BamH I |
| SL1120      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | prgK 3 EcoR I |
| SL1121      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1122      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1123      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1124      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1125      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1126      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1127      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1128      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1129      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1130      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1131      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1132      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 3 EcoR I |
| SL1133      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | hiC 5 BamH I |
| SL1134      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1135      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1136      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1137      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1138      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1139      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1140      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1141      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1142      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1143      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1144      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1145      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1146      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1147      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1148      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1149      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1150      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1151      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1152      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1153      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1154      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1155      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1156      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1157      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
| SL1158      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 5 EcoR I |
| SL1159      | CGGCTCGAGTTGACGAGAGATAGGTAACGCTAA             | Lon 3 Xho I |
were carried out in accordance with the Guidelines for the Use of Animals in Research, issued by the Jilin University.

**Chemicals**

Unless otherwise noted, all chemicals used in this study were from Sigma. Among these, the purity of thymol used in all experiments was higher than 98.5% (Sigma, cat# T0501-500G). DMSO was used to dissolve thymol; the stock concentration is 100 mM.

**Bacterial Strains and Plasmid Construction**

All strains of *S. Typhimurium* in this study were derived from the standard virulent strain SL1344 (Gulig and Curtiss, 1987). Bacteria were grown in the LB medium. Ampicillin (Amp, 100 μg/ml), kanamycin (Km, 50 μg/ml), or chloramphenicol (Cm, 30 μg/ml) was added into the medium for selection. The *lon* mutant derived from strain SL1344 (Boddicker and Jones, 2004) was a gift from Brady Jones (the University of Iowa), the strain harboring *hilA::lacZ* fusion (Lin et al., 2008) was provided by James Slauch (the University of Illinois at Urbana-Champaign). The lactamase reporter plasmid was constructed by inserting the fragment of *SipA*–lactamase fusion into pQE30 between *BamHI* and *SalI* sites. The EspG–lactamase fusion for testing protein translocation by T3SS in enterohemorrhagic *E. coli* was similarly constructed. All plasmids used in this study are listed in Table 1. To make strains that express *SipA* and *SipB*, the open reading frame (ORF) of the genes were, respectively, inserted into the pKS3xFlag plasmid that carried both N- and C-terminal 3xFlag tags. This cassette was then inserted into the *pir* protein-dependent R6K vector pSR47S (Luo and Isberg, 2004), and the resulting plasmid was introduced into SL1344 by triparental mating with the *E. coli* helper strain HB101 (pRK600) (Luo and Isberg, 2004). Transconjugants were streaked onto the LB plate containing 15% sucrose. Strains in which the tag was inserted properly into the chromosome were identified by diagnostic PCR with the appropriate primer sets (Table 2).

The deletion of *sipA* and *clpP* was performed with the λ Red and FLP-mediated site-specific recombination (Datsenko and Wanner, 2000). Primers containing 45-base corresponding to each end of the gene of interest linked to a 20-base of the FRT flanked the antibiotic resistance cassette on pKD3 were used to amplify the fragment containing chloramphenicol. A 100-ml culture was established from an overnight culture of SL1344 flanked the antibiotic resistance cassette on pKD3 were used to amplify the fragment containing chloramphenicol. A 100-ml culture was established from an overnight culture of SL1344, and the resulting plasmid was introduced into SL1344 by triparental mating with the *E. coli* helper strain HB101 (pRK600) (Luo and Isberg, 2004). Transconjugants were streaked onto the LB plate containing 15% sucrose. Strains in which the tag was inserted properly into the chromosome were identified by diagnostic PCR with the appropriate primer sets (Table 2). The chloramphenicol resistance cassette was eliminated by introducing pCP20, a temperature-sensitive plasmid that expresses the FLP recombinase (Cherepanov and Wackernagel, 1995).

**Experimental Design and Statistical Rationale for Proteomic Analysis**

Bacterial cells from 1 ml culture grown in the LB medium containing 300 mM NaCl to OD600 of 0.9 were used. Thymol was added to identical cultures at the concentration of 0.2 mM. Total bacterial lysates were run on a 10% SDS-PAGE and stopped once the bromophenol blue dye reached 1 cm below the stacking gel. The whole gel was equally cut into five horizontal slices and subjected to in-gel protein digestion with trypsin as described previously (Hu et al., 2014). Briefly, samples of digested peptides were dissolved in HPLC-grade water and were analyzed on a nanoflow reversed-phase liquid chromatography (EASY-nLC1000, Thermo Scientific) coupled with a linear ion trap mass spectrometer (LTQ Velos Pro, Thermo Scientific) in a data-dependent mode. The 75 μm × 150 mm capillary column was packed with 5 μm, 100 Å Magic C18 AQ silica-based particles (Michrom BioResources Inc., Auburn, CA). The column had an integrated electrospray tip that was made in-house with a laser-based puller (Model P-2000, Sutter Instruments). The LC separation was carried out with the following gradient: solvent B (100% acetonitrile and 0.1% formic acid) was started at 7% for 3 min, and then raised to 35% in 40 min; subsequently, solvent B was rapidly increased to 90% in 2 min and maintained for 10 min before 100% solvent A (97% H2O, 3% acetonitrile, and 0.1% formic acid) was used for column equilibration. One full MS scan (m/z 350–1,500) was followed by MS/MS analyses of the top 10 most intense ions. Dynamic exclusion was enabled with a repeat duration of 12 s and exclusion duration of 6 s. With three pairs of biological replicates (control and thymol-treated samples) and five gel fractions per sample, we carried out 30 (3 × 2×5) LC-MS/MS experiments.

**Proteomic Data Processing**

LC-MS/MS raw files were searched against *S. Typhimurium* LT2 protein database (strain LT2/SGSC1412/ATCC 700720, July 11, 2011, 5,199 sequences) downloaded from UniProt (http://www.uniprot.org/ with Mascot (version 2.3.02, Matrix Science Inc.). The following settings were used for database search: 1.5 Da precursor mass error tolerance and 0.8 Da fragment mass error tolerance. A maximum of two missed cleavage sites were allowed. Oxidation (M) was set as a variable modification. The peptide and protein identifications were filtered to achieve a false discovery rate (FDR) < 1%. Protein relative abundance between different samples was assessed using spectral counting (Liu et al., 2004), which represents the total number of repeated peptide identifications for a given protein and provides a semiquantitative assessment of protein abundance.

A protein fold change between paired samples was calculated by dividing the average spectral counts of triplicate measurements. Positive or negative values indicate higher or lower levels in the thymol-treated samples compared to untreated cells. Differences with average fold changes ≥ 1.5 or ≤ 0.67 and p value ≤ 0.05 were considered significant. Paired Student’s *t*-test was performed on data from three biological replicates.

**Immunoblotting**

The HilA-specific antibody (Sang et al., 2016) is a generous gift from Yufeng Yao (Shanghai Jiao Tong University). Mouse
### TABLE 3 | Protein with decreased abundance in thymol-treated samples.

| Gene   | Protein description | Untreated | Thymol-treated | Fold change | p-value |
|--------|---------------------|-----------|----------------|-------------|---------|
| sipA   | Cell invasion protein sipA OS = Salmonella Typhimurium GN = sipA PE = 1 SV = 2 | 95 | 4 | 16.4 | 0.001 |
| sipB   | Cell invasion protein sipB OS = Salmonella Typhimurium GN = sipB PE = 1 SV = 1 | 123 | 5 | 12.6 | 0.003 |
| sipD   | Cell invasion protein sipD OS = Salmonella Typhimurium GN = sipD PE = 2 SV = 1 | 37 | 2.5 | 9.1 | 0.002 |
| hiA    | Transcriptional regulator hiA OS = Salmonella Typhimurium GN = hiA PE = 2 SV = 2 | 29 | 3 | 8.7 | 0.001 |
| tdcB   | Threonine dehydratase catalytic OS = Salmonella Typhimurium GN = tdcB PE = 1 | 45 | 5 | 8.7 | 0.002 |
| sicA   | Chaperone protein sicA OS = Salmonella Typhimurium GN = sicA PE = 1 SV = 1 | 20 | 2 | 8.6 | 0.002 |
| tdcC   | Threonine/serine transporter tdcC OS = Salmonella Typhimurium GN = tdcC PE = 3 | 28 | 3.5 | 7.7 | 0.003 |
| sopB   | Inositol phosphate phosphatase sopB OS = Salmonella Typhimurium GN = sopB PE = 1 SV = 2 | 93 | 3 | 7.1 | 0.002 |
| sipP   | Effector protein sipP OS = Salmonella Typhimurium GN = sipP PE = 1 SV = 1 | 22 | 2.5 | 6.5 | 0.005 |
| tdcG   | L-serine deaminase OS = Salmonella Typhimurium GN = tdcG PE = 4 SV = 1 | 18 | 3.5 | 5.2 | 0.003 |
| STM4498 | Putative inner membrane protein OS = Salmonella Typhimurium GN = STM4498 PE = 4 SV = 1 | 12 | 2.5 | 4.8 | 0.003 |
| mbiB   | Putative inner membrane protein OS = Salmonella Typhimurium GN = mbiB PE = 1 SV = 2 | 9 | 2 | 4.5 | 0.001 |
| prgH   | Protein prgH OS = Salmonella Typhimurium GN = prgH PE = 3 SV = 1 | 14 | 2 | 4.5 | 0.001 |
| hsdR   | Endonuclease R OS = Salmonella Typhimurium GN = hsdR PE = 4 SV = 1 | 34 | 6 | 4.2 | 0.029 |
| invG   | Protein invG OS = Salmonella Typhimurium GN = invG PE = 3 SV = 3 | 108 | 26 | 3.8 | 0.008 |
| hID    | Transcriptional regulator hID OS = Salmonella Typhimurium GN = hID PE = 4 SV = 2 | 12 | 1.5 | 3.7 | 0.001 |
| meIA   | Alpha-galactosidase OS = Salmonella Typhimurium GN = meIA PE = 3 SV = 2 | 9 | 2.5 | 3.6 | 0.001 |
| ydJ    | Putative oxidase OS = Salmonella Typhimurium GN = ydJ PE = 4 SV = 1 | 15 | 4.5 | 3.3 | 0.005 |
| Rob    | Transcriptional regulator OS = Salmonella Typhimurium GN = rob PE = 4 SV = 1 | 24 | 7 | 3.3 | 0.008 |
| pduJ   | Propanediol utilization protein OS = Salmonella typhimurium GN = pduJ PE = 4 SV = 1 | 7 | 2 | 3.3 | 0.002 |
| yiaO   | Putative dicarboxylate-binding periplasmic protein OS = Salmonella Typhimurium GN = yiaO PE = 4 SV = 1 | 5 | 1.5 | 3.0 | 0.015 |
| STM2504 | Putative cytoplasmic protein OS = Salmonella Typhimurium GN = STM2504 PE = 4 SV = 1 | 6 | 2 | 3.0 | 0.015 |
| mreC   | Rod shape-determining protein OS = Salmonella Typhimurium GN = mreC PE = 4 SV = 1 | 5 | 1.5 | 3.0 | 0.015 |
| prgI   | Protein prgI OS = Salmonella Typhimurium GN = prgI PE = 1 SV = 1 | 7 | 2 | 3.0 | 0.020 |
| glnE   | Glutamate-ammonia-ligase adenyltransferase OS = Salmonella Typhimurium GN = glnE PE = 3 SV = 1 | 10 | 4 | 3.0 | 0.038 |
| STM2529 | Putative anaerobic dimethylsulfoxide reductase OS = Salmonella Typhimurium GN = STM2529 PE = 4 SV = 1 | 7 | 2.5 | 2.8 | 0.013 |
| cheR   | Chemotaxis protein methyltransferase OS = Salmonella Typhimurium GN = cheR PE = 1 SV = 1 | 7 | 2.5 | 2.8 | 0.013 |
| siaA   | Phosphohydrolase phosphatase OS = Salmonella Typhimurium GN = siaA PE = 4 SV = 1 | 7 | 2.5 | 2.8 | 0.013 |
| Cta    | Cyclopropane fatty acyl phospholipid synthase OS = Salmonella Typhimurium GN = cta PE = 4 SV = 1 | 8 | 2.5 | 2.7 | 0.038 |
| trnA   | TnpA OS = Salmonella Typhimurium GN = trnA PE = 4 SV = 1 | 5 | 1.5 | 2.6 | 0.032 |
| yjY    | Putative carbon starvation protein OS = Salmonella Typhimurium GN = yjY PE = 4 | 13 | 5 | 2.5 | 0.015 |
| citE   | Citrate lyase beta chain (Acyl lyase subunit) OS = Salmonella Typhimurium GN = citEPE = 4 SV = 1 | 15 | 6 | 2.4 | 0.007 |
| degS   | Periplasmic serine endopeptidase OS = Salmonella Typhimurium GN = degS PE = 4 SV = 1 | 12 | 5 | 2.4 | 0.007 |
| Bla    | Beta-lactamase SHV-2 OS = Salmonella Typhimurium GN = bla PE = 3 SV = 1 | 5 | 2 | 2.4 | 0.020 |
| exoX   | DNA exonuclease X OS = Salmonella Typhimurium GN = exoX PE = 4 SV = 1 | 4 | 1.5 | 2.4 | 0.020 |
| hybF   | Probable hydrogenase nickel incorporation protein hybF OS = Salmonella Typhimurium GN = hybF PE = 3 SV = 1 | 4 | 1.5 | 2.4 | 0.020 |
| potE   | APC family, putrescine/ornithine antiporter OS = Salmonella Typhimurium GN = potEPE = 4 SV = 1 | 6 | 2 | 2.4 | 0.020 |
| waaK   | Lipo polysaccharide 1,2-N-acetylglycosaminotransferase OS = Salmonella Typhimurium GN = waaK PE = 4 SV = 1 | 8 | 3.5 | 2.4 | 0.020 |
| yhE    | Putative MFS family transport protein OS = Salmonella Typhimurium GN = yhE PE = 4 SV = 1 | 4 | 1.5 | 2.4 | 0.020 |
| ytbK   | Putative inner membrane protein OS = Salmonella Typhimurium GN = ytbK PE = 4 | 5 | 2 | 2.4 | 0.020 |
| rbf    | Putative rbf protein OS = Salmonella Typhimurium GN = rbf PE = 4 SV = 1 | 29 | 11 | 2.3 | 0.035 |
| srfB   | SrfB (SsrAB activated gene) OS = Salmonella Typhimurium GN = srfB PE = 4 SV = 1 | 63 | 27 | 2.2 | 0.017 |
| argH   | Argininosuccinate lyase OS = Salmonella Typhimurium GN = argH PE = 3 SV = 1 | 9 | 4 | 2.1 | 0.004 |
| ygR    | Putative dehydrogenase OS = Salmonella Typhimurium GN = ygR PE = 4 SV = 1 | 10 | 5.5 | 2.1 | 0.049 |
| ybB    | Putative transferase OS = Salmonella Typhimurium GN = ybB PE = 4 SV = 1 | 10 | 4.5 | 2.1 | 0.002 |

*Average spectral counts from three biological replicates.

Fold change in protein abundance; positive or negative values indicate higher or lower levels in the thymol-treated samples compared to untreated cells.

*p-values were calculated using paired Student’s t-test.

Anti-SipA antibodies were produced in our animal facility. Briefly, 1 mg of His<sub>6</sub>-SipA was emulsified with complete volume of Freund’s adjuvant and was injected to five mice intracutaneously 4 times a month at 7 day intervals. The presence of SipA-specific antibodies in the sera of the immunized mice was tested, and those that were reactive were used for affinity IgG purification. To test protein stability, overnight cultures of wild-type S. Typhimurium and the relevant mutants expressing the indicated recombinant or tagged proteins were diluted at 1:20 in 2 ml LB and grown for 4 h at 37°C in a shaker (200 rpm);
Thymol was added to identical cultures at indicated concentrations. OD$_{600}$ values were used to estimate the number of bacterial cells collected for subsequent experiments. Cells pelleted by centrifugation at 12,000 g for 20 min were resuspended with the 100 μl 1x SDS sample buffer. The samples were boiled for 5 min prior to SDS-PAGE. Proteins transferred onto nitrocellulose membranes were incubated in 5% BSA supplemented with the appropriate antibodies, including anti-SipA antibody (1:500), anti-HilA (1:1,000), anti-β-lactamase (Abcam) (1:170), and antibody for metabolic enzyme isocitrate dehydrogenase (ICDH, 1:20,000) (Xu et al., 2010). In all cases, the signals were detected with a secondary antibody conjugated to horseradish peroxidase by the enhanced chemiluminescence (ECL) method.

### In Vitro Protease Degradation Assay

To analyze the degradation of SipA and its derivatives in reactions with defined components, 400 μg His$_6$-Lon SipA or equivalent volumes of buffer were added into reactions containing SipA or its derivatives purified from cells in cultures with or without thymol. The reaction was initiated by 1 mM ATP followed by incubation in 37°C for 30 min. Identical reactions terminated by SDS sample buffers immediately after adding ATP were used as input controls. Proteins of interest in the reactions were detected by Coomassie bright blue staining or by immunoblotting. For cell-free assays, total protein lysates of S. Typhimurium were prepared from the 100 ml cultures grown at the exponential phase (OD$_{600}$ = 0.8) by sonication followed by centrifugation at 10,000 g for 15 min. 400 μg purified GST-SipA expressed in cultures with or without thymol was added to the lysates. After incubation for the indicated time duration, the protein was detected by immunoblotting with specific antibodies.

### β-galactosidase Assay

β-galactosidase activity was determined as described by Luo et al. before (Luo and Farrand, 1999). Briefly, JS749 strain was cultured overnight (Lin et al., 2008) and diluted 1:20 in LB broth with or without thymol at the indicated concentrations. Bacteria density was determined by OD$_{600}$ measurement, and the bacteria were harvested by centrifugation (12,000 g, 10 min). Cells resuspended in Z-buffer were mixed with 20 μl 0.1% freshly configured SDS solution and 40 μl chloroform. 100 μl cells lysed on a vortex for 10 s were transferred to a 96-well plate, and 20 μl of ONPG (4 mg/ml) in Z-buffer was then added into the lysates to initiate the reaction. The mixtures were incubated at room temperature for 10 min before the reactions were terminated with 50 μl of 1 M Na$_2$CO$_3$. A$_{420}$ values were measured by a plate reader.

### The Binding Between SipA and Thymol

5 ml cultures of BL21 (DE3) derivatives harboring the plasmids directing the expression of GST-SipA$_{27-270}$ or GST-SipA$_{497-669}$ were induced with IPTG. Alkyne thymol was added to identical cultures, and the cultures were incubated at 20°C for 16 h. The bacterial cells were suspended in 1 ml 0.1% SDS solution containing 50 mM triethanolamine (TEA, Sigma), 150 mM NaCl, 1xEDTA-free protease inhibitor cocktail (Roche), 5 mM PMSF (Sigma), and 0.1 μl benzonase (Sigma). Cells were lysed by sonication for 3 min, and the lysates were then incubated on ice for 30 min. After removing the debris by centrifugation at 12,000 g for 10 min, the supernatant was loaded onto a glutathione-Sepharose column for protein purification. The proteins of interest were eluted with the elution buffer (50 mM NaCl, 1xEDTA-free protease inhibitor cocktail (Roche), 5 mM PMSF (Sigma), and 0.1 μl benzonase (Sigma)). Cells were lysed by sonication for 3 min, and the lysates were then incubated on ice for 30 min. After removing the debris by centrifugation at 12,000 g for 10 min, the supernatant was loaded onto a glutathione-Sepharose column for protein purification. The proteins of interest were eluted with the elution buffer (50 mM Tris-Cl pH 8.0, 0.4 M NaCl, 25 mM reduced glutathione, and 0.1% Triton X-100, 1 mM DTT). 400 μg of protein in the elution buffer was used to detect the interactions. 12% SDS buffer (12% w/v in 50 mM TEA and 150 mM NaCl) was added to the protein solution to bring the final SDS concentration to 4% in a total volume of 44.5 μl. Click chemistry reaction was performed as described (Speers et al., 2003; Tsou et al., 2016). Briefly, a master mixture of click chemistry reagents that contains 1 μl 5 mM Azide-fluor 488 (Sigma), 1 μl 50 mM TCEP (Tris(2-carboxyethyl)phosphine), 2.5 μl 2 mM TBTA Tris(benzyltriazolylmethyl) amine, and 50 mM CuSO$_4$ was prepared. 5.5 μl of the master mixture was added to each protein sample for a final volume of 50 μl. After vigorous mixing on a vortex, the reactions were incubated at room temperature for 1 h. Finally, unreacted molecules were removed by

### Table 4

| Gene | Protein description | Abundance | Fold change | p-value |
|------|---------------------|-----------|-------------|---------|
| sipA | Effector protein    | 95        | 4           | 16.4    | 0.001  |
| sipB | Effector protein    | 123       | 5           | 12.6    | 0.003  |
| sipD | Effector protein    | 37        | 2.3         | 9.1     | 0.002  |
| hilA | Transcriptional regulator HilA | 29 | 3         | 8.7     | 0.001  |
| sicA | Chaperone protein SicA | 20 | 2         | 8.6     | 0.002  |
| sopB | Effector protein    | 93        | 3           | 7.1     | 0.022  |
| sipP | Effector protein    | 22        | 2.5         | 6.5     | 0.006  |
| prgH | Needle complex proteins PrgH | 34 | 6         | 4.2     | 0.029  |
| invG | Invasion protein    | 108       | 26          | 3.8     | 0.017  |
| hilD | Transcriptional regulator HilA | 12 | 1.5       | 3.7     | 0.030  |
| prgl | Needle complex proteins Prgl | 7  | 2         | 3       | 0.020  |

*aAverage of spectral counts from three biological replicates.

*bFold change in protein abundance: the average of fold change of three independent experiments.

*p-values were calculated using paired Student’s t-test.

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chloroform/methanol extraction, and the samples were resolved by SDS-PAGE. Proteins were detected by Coomassie bright blue staining or immunoblotting. Azido-rhodamine bound to alkyne-thymol was detected by fluorescence scanning with a Typhoon 7,000 imager (Amersham Biosciences).

**Circular Dichroism Spectroscopy**

GST-SipA\(_{27-270}\) and GST-SipA\(_{497-669}\) purified from cells or lysates with or without thymol were analyzed by circular dichroism spectroscopy for structural changes. CD spectroscopy analysis of GST-SipA\(_{27-270}\) and GST-SipA\(_{497-669}\) was performed with protein concentrations of 7.9 and 7.3 \(\mu\)M, respectively, in phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), and 8 mM Na\(_2\)HPO\(_4\)). The spectrum was obtained at 25°C by averaging over five scans with a step size of 1 nm and an averaging time of 2.5 s on a Chirascan-plus CD spectrometer. Measurements were performed in a Hellma quartz cuvette of path length 0.1 cm. Helix content was calculated from the molar ellipticity at 208 nm.

**RESULTS**

**Thymol Treatment Alters the Level of About One Hundred Proteins in S. Typhimurium**

To determine the mechanism underlying the inhibition of T3SS-1 by thymol, we performed proteomic analyses of S. Typhimurium cells by liquid chromatography–tandem mass spectrometry (LC-MS/MS). A total of 2,875 proteins were detected in all three independent experiments in which at least two tryptic peptides were scored for each protein (Lau et al., 2014). Consistent with the observation that thymol used at less than 0.4 mM did not detectably affect bacterial viability, the level of most of the identified proteins (2,764 or >96%) remained unchanged in cells grown in the presence of 0.2 mM thymol. The abundance of 65 proteins, including many predicted to be involved in metabolism, was significantly increased upon thymol treatment. For example, TorA and TorC, two enzymes involved in trimethylamine-N-oxide metabolism, exhibited the highest induction, at 72- and 13-fold, respectively. It is possible that thymol triggers the expression of some regulatory proteins, but the mechanism underlying such induction of these proteins needs further investigation.

We also observed significant reduction of 46 proteins in cells grown in 0.2 mM thymol (Table 3). Importantly, 11 of these 46 proteins were virulence factors associated with the SPI-1, including its effectors SipA, SipB, SipD, and SopB whose reduction was more than 7-fold (Table 4). The level of HilA and HilD, regulatory proteins that directly control the expression of the invasion genes, including many effectors (Bajaj et al., 1995; Thijs et al., 2007), was reduced by about 9- and 3.7-fold, respectively, suggesting that the reduction associated with the effectors can be caused by lower transcription or lower protein stability, or a combination of both.
Thymol Reduced the Cellular Levels of Several Proteins Associated With T3SS-1

To distinguish whether thymol directly affects the stability of these SPI-1 effectors, we focused on SipA by first examining levels of the SipA-lactamase fusion expressed from the synthetic tac promoter (Luo and Farrand, 1999). Whereas untreated cells expressed readily detectable fusion protein; inclusion of 0.1 mM thymol in the bacterial cultures drastically reduced the protein level of the fusion and 0.2 mM rendered the protein completely undetectable; thymol at concentrations as high as 0.4 mM did not detectably affect the level of the β-lactamase expressed from the plasmid backbone (Figure 1A), suggesting that thymol targets SipA but not β-lactamase. Similar results were obtained when a Flag–SipA fusion was expressed from the tac promoter from a multi-copy plasmid or when the level of SipA was probed from a strain in which this protein was tagged with the Flag epitope on the chromosome (Figures 1B,C). Further, direct probing of the endogenous protein with a SipA-specific antibody revealed that treatment with as low as 25 μM caused a clear reduction and 0.1 mM made the protein completely undetectable under our experimental conditions (Figure 1D).

Thymol-induced protein reduction was also observed for SipB under two experimental conditions in which the protein was expressed ectopically on a plasmid or from its cognate promoter on the chromosome (Figures 1E,F). We also examined the levels of HilA by measuring the activity of a HilA–LacZ fusion (Lin et al., 2008) and by detecting endogenous protein in wild-type S. Typhimurium. Treatment with 0.1 mM thymol caused severe reduction of both the HilA–LacZ fusion and the endogenous protein (Figure 1G). Taken together, these results demonstrate that thymol causes the reduction of a number of virulence proteins associated with the SPI-1.

To determine the extent to which thymol affects the stability of S. Typhimurium virulence proteins, we further examined other important components of SPI-1. When expressed from an artificial promoter, thymol did not affect the stability of Prgk or PrgH, two proteins involved in the formation of the needle, nor did it affect the transcriptional factor HilC or HilD, which functions upstream of HilA (Ellermeier and Slauch, 2007) (Figure 2A). These results indicate that thymol does not indiscriminately reduce protein stability in S. Typhimurium.

The observation that thymol destabilizes a set of virulence proteins associated with SPI-1 prompted us to examine its effect on the activity of SPI-2, which codes for T3SS-2 that is required for intracellular replication, particularly in macrophages (Figueira and Holden, 2012). Because the expression of SPI-2 is induced by intracellular environment, which is more difficult to be mimicked in bacteriological media than SPI-1, we determined whether thymol affects the stability of two SPI-2 effectors, SifA and SseG (Figueira and Holden, 2012). S. Typhimurium strains expressing β-lactamase fusion to each of these proteins were constructed, and protein levels of the fusions were examined upon thymol treatment. The addition of 0.2 mM thymol led to the reduction of the SifA–lactamase but not the SseG-lactamase fusion (Figure 2B), indicating that thymol affects the stability of a subset of SPI-2 effectors.

**FIGURE 2 |** Thymol does not affect the stability of all proteins associated with SPI-1. (A) GST fusion of the indicated proteins was expressed in S. Typhimurium with or without thymol for 4 h, and the protein levels were evaluated by immunoblotting with a GST-specific antibody. ICDH was probed as a loading control. Note that only the protein level of GST-SipA decreased in cultures that received thymol. (B) The effects of thymol on the stability of SifA and SseG, two SPI-2 effectors. β-lactamase fused to SPI-2 effector SifA (left panel), or SseG (right panel) was expressed from a plasmid in wild-type S. Typhimurium in the presence of indicated concentrations of thymol for 4 h, and the protein was detected by immunoblotting with an antibody specific for β-lactamase. β-lactamase expressed from the plasmid backbone was used as a loading control. The experiments were repeated at least three times each with independent biological samples.
Thymol Induces Conformational Changes in SipA by Directly Interacting With Two Regions of This Effector

To further probe the mechanism responsible for thymol-induced reduction of proteins associated with the SPI-1, we synthesized an alkyne-thymol derivative for click chemistry protein labeling experiments (Speers et al., 2003) (Figure 3A); this modification did not alter the effectiveness of thymol in the induction of SipA instability (Figure 3B). To simplify the study of the potential interactions between thymol and SipA, we first determined the responsiveness of regions of this effector to this compound. To this end, we expressed Flag-SipA1-270 and Flag-SipA497-669, two soluble domains of this protein involved in chaperone-binding (Lilic et al., 2006) and actin nucleation (Galkin et al., 2002), respectively, in S. Typhimurium. Thymol was able to cause degradation of both fragments (Figure 3C). The removal of the first 27 residues that contain the signal necessary for T3SS-1-mediated secretion did not alter the responsiveness of SipA1-270 to thymol (Figure 3C). Thus, thymol interacts with at least two regions of SipA.

To detect the interactions between thymol and these two fragments of SipA, alkyne-thymol was added to cultures of cells expressing the indicated SipA fragments fused to a Flag tag, and cell lysates resolved by SDS-PAGE were probed by immunoblotting with the Flag-specific antibody. ICDH was probed as a loading control. (D,E) The binding between alkyne-thymol and two fragments of SipA. GST fusion of SipA27-270 and SipA497-669 expressed in cells exposed to alkyne-thymol or solvent control reacted with azido-conjugated rhodamine was probed by fluorescence scanning. Note that in each case only protein from cells grown in the presence of alkyne-thymol retained the fluorescence signals. (F) Thymol induces conformational changes in SipA fragments. GST-SipA27-270 and GST-SipA497-669 purified from E. coli or its lysates exposed to thymol or solvent were analyzed by circular dichroism (CD) spectroscopy. Note that proteins from cells treated with thymol (green curve: thymol-induction) assume structures that differ greatly from those not exposed to thymol (red curve: untreated) or exposed to the compound (blue curve: thymol-post) after protein translation, untreated, thymol-post, and thymol-induction twisted antiparallel β-structures. The experiments were repeated at least three times each with independent biological samples.
Next, we determined the consequence of thymol binding to the structure of SipA by circular dichroism (CD) spectroscopy analysis. GST-SipA purified from cultures containing thymol or its solvent was added to total cell lysates (TCL) of S. Typhimurium. Reactions terminated at the indicated time by SDS sample buffer were probed for the fusion protein by immunoblotting. ICDH in the reactions was probed to indicate the presence of equal amounts of TCL in relevant reactions. (C–E) The Lon but not the CtpP protease is required for thymol-induced degradation of SipA in S. Typhimurium. Total proteins of wild type, the lon (C,D), or the ctpP mutant (E) of S. Typhimurium grown in the presence of thymol or its solvent resolved by SDS-PAGE were probed for SipA or HilA by immunoblotting. Note that in the ctpP mutant, thymol still caused robust reduction in SipA. The arrows indicate the bands nonspecifically recognized by the antibodies that serve as loading controls. (F,G) Lon only degrades GST-SipA from cells that have been exposed to thymol. GST-SipA purified from cultures supplemented with thymol (F) or its solvent (G) was mixed with His6-Lon, and ATP was added to a subset of samples to initiate the reactions. Reactions terminated at the indicated time points resolved by SDS-PAGE were probed for SipA or HilA by immunoblotting. Note that in the clpP mutant, thymol still caused robust reduction in SipA. The arrows indicate the bands nonspecifically recognized by the antibodies that serve as loading controls. (H–K) The degradation of GST-SipA27-270 and GST-SipA497-669 by Lon. GST-SipA27-270 and GST-SipA497-669 purified from cultures grown in the presence of thymol were mixed with His6-Lon, and ATP was added to a subset of reactions to initiate the activity. Proteins of interest in reactions terminated at the indicated time points resolved by SDS-PAGE were probed by immunoblotting (H,J) or by Coomassie bright blue staining (I,K). The experiments were repeated at least three times each with independent biological samples.

The Protease Lon Is Responsible for Thymol-Induced Virulence Protein Degradation

The observation that thymol treatment lowered the cellular level of SipA suggests that the binding leads to protein degradation. We tested such degradation in a cell-free system by adding GST-SipA purified from E. coli into total cell lysates of S. Typhimurium. Whereas the protein level of GST-SipA from untreated cells remained constant throughout the entire experimental duration (Figure 4A), the abundance of GST-SipA from thymol-treated cells became markedly lower after 1 h incubation and became undetectable when the incubation time was extended to 4 h (Figure 4B). Thus, SipA in complex with thymol is actively degraded by one or more protease systems. These results also indicate that the degradation machinery only recognizes SipA obtained from thymol-treated cells.

In bacteria, Lon and the ClpP are the two major protease systems responsible for energy-dependent degradation of most misfolded proteins (Gur and Sauer, 2008; Olivares et al., 2016). To determine whether any of them is involved in thymol-induced protein degradation, we obtained mutants defective in each of these protease systems and examined the level of SipA after thymol treatment. In the mutant lacking the Lon protease, even 0.2 mM thymol failed to induce SipA degradation (Figure 4C). This mutant is also defective in the thymol-induced degradation of HilA (Figure 4D). In contrast, SipA in
the ΔcLP mutant was still robustly degraded in the presence of 0.1 mM thymol (Figure 4E), indicating that the Lon protease is responsible for degrading the proteins whose conformation has been altered by binding to thymol.

We further examined Lon-mediated degradation of SipA and its fragments in reactions containing purified proteins. In reactions containing recombinant Lon and a GST fusion of full-length SipA purified from cells treated with thymol, the

TABLE 5 | Protein with increased abundance in thymol-treated samples.

| Gene | Protein description | Untreated | Thymol-treated | Fold change | p-valuea |
|------|---------------------|-----------|----------------|-------------|----------|
| torA | Trimethylamine-N-oxide reductase OS = Salmonella Typhimurium GN = torA PE = 3 | 2 | 124 | 72.3 | 0.000 |
| torC | Trimethylamine N-oxide reductase OS = Salmonella Typhimurium GN = torC PE = 4 | 1.5 | 20 | 13.3 | 0.000 |
| anE2 | Putative N-acetylmuramic acid-N-acetylglucosamine-2-epimerase 2 OS = Salmonella Typhimurium GN = nanE2 PE = 3 SV = 1 | 3.5 | 24 | 9.5 | 0.003 |
| Rna | tRNA synthetase cleaves phosphodiester bond between any two nucleotides OS = Salmonella Typhimurium GN = rna PE = 4 SV = 1 | 2 | 14 | 8.7 | 0.030 |
| STM1540 | Putative hydroxase OS = Salmonella Typhimurium GN = STM1540 PE = 4 SV = 1 | 4 | 28 | 7.7 | 0.004 |
| fadA | 3-ketoadip-CoA thiolase OS = Salmonella Typhimurium GN = fadA PE = 3 SV = 1 | 2 | 12 | 6.7 | 0.002 |
| gpmB | Probable phosphoglycerate mutase gpmB OS = Salmonella Typhimurium GN = gpmBPE = 3 SV = 1 | 7 | 28 | 6.3 | 0.030 |
| fadD | Long-chain-fatty-acid-CoA ligase OS = Salmonella Typhimurium GN = fadD PE = 3 | 1.5 | 9 | 6.0 | 0.004 |
| proW | Glycine betaine/L-proline transport system permease protein proW OS = Salmonella Typhimurium GN = proW PE = 3 SV = 2 | 2.5 | 14 | 6.0 | 0.001 |
| A0SXM1 | Beta-lactamase TEM-63 (Fragment) OS = Salmonella Typhimurium GN = proW PE = 4 SV = 1 | 2 | 11 | 6.0 | 0.004 |
| STM2006 | Putative branched chain amino acid transport protein OS = Salmonella Typhimurium GN = STM2006 PE = 4 SV = 1 | 3.5 | 14 | 5.3 | 0.025 |
| rhaD | Rhamnollose-1-phosphate aldolase OS = Salmonella Typhimurium GN = rhaD PE = 3 | 3.5 | 12 | 5.0 | 0.027 |
| yfbF | Putative enzyme enzyme OS = Salmonella Typhimurium GN = yfbF PE = 4 SV = 1 | 3 | 15 | 5.0 | 0.024 |
| STM1123 | Putative periplasmic protein OS = Salmonella Typhimurium GN = STM1123 PE = 4 | 2 | 9 | 4.7 | 0.023 |
| pta | Low-affinity phosphate transporter OS = Salmonella Typhimurium GN = pta PE = 4 SV = 1 | 1.5 | 7 | 4.7 | 0.023 |
| yhbH | Probable sigma (54) modulator protein OS = Salmonella Typhimurium GN = yhbH PE = 3 SV = 1 | 3 | 10 | 4.7 | 0.023 |
| yeIT | Uncharacterized oxidoreductase yeIT OS = Salmonella Typhimurium GN = yeIT PE = 4 SV = 1 | 11 | 33 | 4.2 | 0.017 |
| Crl | Sigma factor-binding protein crl OS = Salmonella Typhimurium GN = crl PE = 2 SV = 1 | 3 | 12 | 4.0 | 0.020 |
| pyrL | Aspartate carbamoyltransferase regulatory chain OS = Salmonella Typhimurium GN = pyrL PE = 3 SV = 3 | 1.5 | 4 | 2.7 | 0.020 |
| T059 | Putative adenine-specific DNA methylase OS = Salmonella Typhimurium GN = PSLT059 PE = 4 SV = 1 | 2 | 6 | 2.7 | 0.020 |
| traD | Conjugative transfer: DNA transport OS = Salmonella Typhimurium GN = traD PE = 4SV = 1 | 1.5 | 4 | 2.7 | 0.020 |
| trec | Conjugative transfer: DNA transport OS = Salmonella Typhimurium GN = trec PE = 4 SV = 1 | 4.5 | 10 | 2.7 | 0.020 |
| trnC | Threonine/2,3-deoxyosonate OS = Salmonella Typhimurium GN = trnC PE = 4 SV = 1 | 2.5 | 7 | 2.7 | 0.020 |
| yehS | Putative cytoplasmic protein OS = Salmonella Typhimurium GN = yehS PE = 4 SV = 1 | 1.5 | 4 | 2.7 | 0.020 |
| yraL | Putative methyltransferase OS = Salmonella Typhimurium GN = yral PE = 4 SV = 1 | 2.5 | 7 | 2.7 | 0.020 |
| folE | GTP cyclohydrolase 1 OS = Salmonella Typhimurium GN = folE PE = 3 SV = 2 | 11 | 28 | 2.6 | 0.040 |
| odc1 | Superoxide dismutase [Cu-Zn] 1 OS = Salmonella Typhimurium GN = odc1 PE = 1 | 9 | 23 | 2.6 | 0.017 |
| hutH | Histidine ammonia-lyase OS = Salmonella Typhimurium GN = hutH PE = 3 SV = 1 | 21 | 51 | 2.5 | 0.017 |
| spoT | Pseudomonas type II OS = Salmonella Typhimurium GN = spoT PE = 3 SV = 1 | 3.5 | 7 | 2.5 | 0.049 |
| ynhA | Suppressor of ompf assembly mutants OS = Salmonella Typhimurium GN = ynhA PE = 4 SV = 1 | 6 | 16 | 2.4 | 0.014 |
| STM3780 | Fructose-bisphosphate aldolase OS = Salmonella Typhimurium GN = STM3780PE = 3 SV = 1 | 3 | 7 | 2.3 | 0.010 |
| recR | Recombination protein recR OS = Salmonella Typhimurium GN = recR PE = 3 SV = 1 | 3 | 7 | 2.3 | 0.010 |
| sufC | Putative transport protein OS = Salmonella Typhimurium GN = sufC PE = 3 SV = 1 | 3 | 7 | 2.3 | 0.010 |
| yfbF | Putative ABC-type multidrug transport system, ATPase component OS = Salmonella Typhimurium GN = yfbF PE = 4 SV = 1 | 7 | 17 | 2.3 | 0.040 |
| ybeB | Putative ACR, homolog of plant lipoxygenase protein OS = Salmonella Typhimurium GN = ybeB PE = 4 SV = 1 | 18 | 35 | 2.3 | 0.047 |
| mobA | Molybdopterin-guanine dinucleotide biosynthesis protein A OS = Salmonella Typhimurium GN = mobA PE = 3 SV = 1 | 5 | 10 | 2.2 | 0.035 |
| nuoL | NADH:quinone oxidoreductase subunit 1 OS = Salmonella Typhimurium GN = nuoL PE = 3 SV = 1 | 13 | 28 | 2.2 | 0.030 |
| iolD | Lipoprotein-releasing ATP-binding protein iolD OS = Salmonella Typhimurium GN = iolD PE = 3 SV = 1 | 8 | 17 | 2.1 | 0.002 |
| nagD | Putative phosphatase in N-acetylgalactosamine metabolism OS = Salmonella Typhimurium GN = nagD PE = 4 SV = 1 | 13 | 26 | 2.1 | 0.027 |
| pdxY | Pyridoxamine kinase OS = Salmonella Typhimurium GN = pdxY PE = 3 SV = 1 | 7 | 14 | 2.1 | 0.044 |
| rpmH | 5S ribosomal protein L34 OS = Salmonella Typhimurium GN = rpmH PE = 3 SV = 1 | 9 | 17 | 2.1 | 0.044 |

*aAveraged spectral counts from three biological replicates.

bFold change in protein abundance; positive or negative values indicate higher or lower levels in the thymol-treated samples compared to untreated cells.

cp-values were calculated using paired Student’s t-test.
degradation was evident after 10 min incubation, and the level of GST–SipA became almost undetectable when the reaction was allowed to proceed for 30 min (Figure 4F). Furthermore, in agreement with the fact that the activity of Lon requires energy (Gur and Sauer, 2008), no degradation occurred in reactions that did not receive ATP (Figure 4F). In contrast, Lon failed to degrade GST–SipA from cells that were never exposed to thymol even in reactions containing ATP (Figure 4G). Consistent with the results from experiments using live bacterial cells, both the SipA27–270 and SipA497–669 fragments of SipA endured SDS and β-mercaptoethanol at high temperature implied that the compound is covalently linked to its target molecules. Alternatively, one or more of its derivatives generated in bacterial cells may directly bind these proteins by covalent bonds. The two regions in SipA that independently generated in bacterial cells may directly bind these proteins by covalent bonds. The two regions in SipA that independently generated in bacterial cells may directly bind these proteins by covalent bonds.

The abundance of at least 35 proteins not directly related to bacterial virulence was affected in cells treated with thymol (Tables 4, 5). We examined whether such reduction was caused by a mechanism that requires the Lon protease by testing the heat shock protein IbpB (Tomoyasu et al., 2003) and the threonine/serine transporter TdcC (Kroger et al., 2012). Plasmids that direct the production of IbpB-Flag and TdcC-Flag were introduced into wild-type and the lon mutant, respectively. Thymol was added to the cultures for 4 h prior to the detection of the fusion proteins by immunoblotting with a Flag-specific antibody. The bacterial ICDH was probed and served as a loading control. The experiments were repeated at least three times each with independent biological samples.

The IC50 of thymol in its inhibition of SipA-lactamase translocation by T3SS-1 of S. typhimurium is about 0.05 mM, which is at least 20 times lower than its lowest MIC (150 μg/ml or 1 mM) against S. Typhimurium (Chauhan and Kang, 2014; Marchese et al., 2016). Our results demonstrate that 11 out of 46 proteins affected by thymol are associated with SPI-1, which indicates its considerable specificity against S. Typhimurium virulence. Instead of inhibiting the activity of the T3SS-1 machinery itself, thymol targets proteins associated with the SPI-1 for degradation, including effectors, chaperones, and the regulatory protein HilA, an OmpR/ToxR family transcriptional regulator that controls the expression of a large number of virulence genes (Bajaj et al., 1995; Thijs et al., 2007). It is worth noting that the protein levels of some known targets of HilA did not significantly decrease in thymol-treated cells in our proteomic analysis. Such discrepancies may arise from the reproducibility of the proteomic approaches or that the expression of these proteins is under the control of multiple layers of regulation, which is a common phenomenon in S. Typhimurium (Ellermeier and Slauch, 2007; Smith et al., 2016). Nevertheless, because mutants lacking one or more effectors such as SipA and SipB are partially defective in virulence (Hersh et al., 1999; Zhou et al., 1999), the protection seen in cell invasion clearly attributes to the additive effects of lowering the level of both effectors and the regulatory protein, which governs the expression of multiple effectors and other proteins involved in virulence (Bajaj et al., 1995; Ellermeier and Slauch, 2007; Thijs et al., 2007).

The fact that the putative complexes formed by thymol and fragments of SipA endured SDS and β-mercaptopoethanol at high temperature implied that the compound is covalently linked to its target molecules. Alternatively, one or more of its derivatives generated in bacterial cells may directly bind these proteins by covalent bonds. The two regions in SipA that independently interact with thymol do not share detectable homology, nor do the proteins that appeared to be targeted by this compound. The mechanism underlying the recognition of multiple proteins without similarity warrants further studies. It is possible that thymol is inserted into a transitional configuration shared by all of its targets during protein folding, thus preventing them from

**DISCUSSION**

Although it has been reported that thymol could reduce the virulent gene expression in S. Typhimurium (Giovagnoni et al., 2020; Qi et al., 2020), the underlying mechanism is still unclear. Our results reveal that natural monoterpene phenol thymol targets multiple S. Typhimurium virulence factors for Lon protease degradation, thus protecting the host against the bacterial infection.
assuming the proper final structures. This notion is consistent with the fact that thymol did not induce conformational changes in SipA if it was added after translation of the proteins had been complete (Figure 4F). The stable interactions between thymol and its target proteins may allow future work to determine the structures of the protein–drug complexes, which should provide not only the molecular details for the binding but also the information useful in the modification of this compound to increase its efficacy and specificity. Furthermore, the finding that thymol directs virulence factors for protease degradation opens a research avenue to develop anti-virulence strategies.

DATA AVAILABILITY STATEMENT

BALB/c mice were obtained from the Experimental Animal Center of Jilin University. All procedures involving animals were carried out in accordance with the Guidelines for the Use of Animals in Research, issued by Jilin University. The animal experiment protocols were ethically approved by the IACUC of Jilin University.

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AUTHOR CONTRIBUTIONS

LS and XD conceived the project; YZ, LS, and XD designed the experiments; YZ, YL, and JL performed the research; YL prepared the alkyne-thymol analog; YZ, YL, and LS wrote the article, and all authors made editorial input.

FUNDING

This work was supported by a grant from the National Natural Science Foundation of China (31620103918) and by research fund from the First Hospital of Jilin University.

ACKNOWLEDGMENTS

We thank Yufeng Yao (Shanghai Jiaotong University) for the HilA-specific antibody; Bradley Jones (the University of Iowa) and James Slauch (the University of Illinois) for bacterial strains; Jianhui Li (the Chinese Academy of Sciences) for technical assistance in circular dichroism spectroscopy analysis.
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