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A New Heat Shock Gene, *agsA*, Which Encodes a Small Chaperone Involved in Suppressing Protein Aggregation in *Salmonella enterica* Serovar Typhimurium

Toshifumi Tomoyasu,[1]* Akiko Takaya,[1] Tomomi Sasaki,[1] Takahiro Nagase,[2] Reiko Kikuno,[2] Mizue Morioka,[3] and Tomoko Yamamoto[1]

Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Inage-ku, Chiba 263-8522,[1] Kazusa DNA Research Institute, Kisarazu, Chiba 292-0812,[2] and Department of Biological Science, Graduate School of Science, The University of Tokyo, Tokyo 113-0033,[3] Japan

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We discovered a novel small heat shock protein (sHsp) named AgsA (aggregation-suppressing protein) in the thermally aggregated fraction from a *Salmonella enterica* serovar Typhimurium *dnaK*-null strain. The −10 and −35 regions upstream of the transcriptional start site of the *agsA* gene are characteristic of σ^{32}- and σ^{72}-dependent promoters. AgsA was strongly induced by high temperatures. The similarity between AgsA and the other two sHsps of *Salmonella* serovar Typhimurium, IbpA and IbpB, is rather low (around 30% amino acid sequence identity). Phylogenetic analysis suggested that AgsA arose from an ancient gene duplication or amplification at an early evolutionary stage of gram-negative bacteria. Here we show that overproduction of AgsA partially complements the Δ*dnaK52* thermosensitive phenotype and reduces the amount of heat-aggregated proteins in both Δ*dnaK52* and Δ*rpoH* mutants of *Escherichia coli*. These data suggest that AgsA is an effective chaperone capable of preventing aggregation of nonnative proteins and maintaining them in a state competent for refolding in *Salmonella* serovar Typhimurium at high temperatures.

The heat shock response is induced upon exposure of cells to a large variety of environmental stresses including heat shock and by pathophysiological and metabolic states (32, 33, 36). It consists of the compartment-specific induction of heat shock genes, which in gram-negative bacteria are under the common transcriptional control of σ^{32}, the transcriptional activator of the heat shock regulon (3, 16, 35, 51). The heat shock proteins (Hsps) include the major cytosolic chaperones such as ClpB, the DnaK chaperone system, GroEL/ES, HtpG, IbpA, IbpB, and a number of proteases (4, 13, 16, 35, 42, 51). The ensemble of molecular chaperones and proteases constitutes a cellular system for de novo folding and quality control of proteins that relies on the ability of chaperones and proteases to refold or degrade misfolded proteins (4, 17). Hsp104 (ClpB) and the Hsp70 (DnaK) bichaperone system are known to disaggregate and refold the denatured proteins efficiently (14, 15, 31, 46).

Members of the small heat shock protein (sHsp) family are found in most organisms. The sHsps are characterized by (i) a molecular mass typically between 12 and 42 kDa, (ii) a conserved central domain generally referred to as the α-crystallin domain, (iii) the formation of large oligomeric complexes, and (iv) an ATP-independent chaperone activity (6, 20, 30, 47). A number of in vitro studies suggest that sHsps bind nonnative proteins, prevent their aggregation, and maintain them in a state competent for refolding by the ATP-dependent chaperones, the ClpB-DnaK bichaperone system, and GroEL/GroES (11, 20, 28, 47).

However, only a few reports on their intracellular functions have been published (1, 45). The *Legionella pneumophila* sHsp "*gspA*" mutant was shown to be more susceptible to in vitro stress stimuli (oxidative, heat, acid, and osmotic) (1). Kuczýnska-Wísnik et al. have shown that *Escherichia coli* IbpA and IbpB are able to prevent the aggregation of endogenous proteins induced by extreme heat shock (24). It has also been reported that the overproduction of sHsps makes eukaryotic and prokaryotic cells more resistant to stresses such as heat, hydrogen peroxide, and superoxide anion (21, 23, 27). However, the growth rates and survival of sHsp mutant strains from *Saccharomyces cerevisiae* and *E. coli* were not affected under a variety of stress conditions (21, 37).

So far, most chaperone studies for bacteria have been performed using the model strain *E. coli*. *Salmonella enterica* serovar Typhimurium is a facultatively intracellular pathogen that causes gastroenteritis in humans and systemic diseases similar to typhoid in mice. It is known that large numbers of general and specific chaperones as well as specific virulence factors are required to successfully colonize the host organism and to avoid clearance by the immune system. In this study, we discovered a novel sHsp named AgsA (aggregation-suppressing protein) in the fraction of thermally aggregated proteins from *Salmonella* serovar Typhimurium. Our data indicate that AgsA suppresses aggregation of nonnative proteins and denatured proteins and that it reduces the amount of heat-aggregated proteins in cells.

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Phone: 81-43-290-2930. Fax: 81-43-290-2929. E-mail: tomoyasu@p.chiba-u.ac.jp.

MATERIALS AND METHODS

Strains and culture conditions. Bacterial strains are listed in Table 1. Luria broth (L broth) and SS medium were used with ampicillin (AMP; 50 μg/ml), chloramphenicol (CHL; 20 μg/ml), kanamycin (KAN; 20 μg/ml), and nalidixic acid (NAL; 25 μg/ml). To construct the strain carrying the *agsA ibpAB* triple

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics[a]	Reference or source
E. coli		
DH5α Z1	F$^-$ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ*(lacZYA-argF)*U169 *deoR* [ϕ80d*lac* Δ(*lacZ*)M15] *tetR lacI*^{q} Spec^r	29
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2 Tc::Mu(Km^r) λ*pir*	41
S17-1λ*pir*	RP4-2 Tet::Mu-Kan::Tn*7* λ*pir*	41
BB1553	Same as MC4100 except for Δ*dnaK52*::Cm	2
CS5257	BB1553 pBB528 (*lacI*^{q}) pTKY605 (*agsA*)	This study
BB7224	MC4100 Δ*rpoH*::Km *suhX401 araD*$^+$	46
CS5262	BB7224 pDMI, 1(*lacI*^{q}) pTKY605 (*agsA*)	This study
Salmonella serovar Typhimurium		
χ3306	SR-11 *gyrA1816*; virulent	Provided by R. Curtiss III
CS2042	Same as χ3306 except for Δ*ibpAB*::Cm	This study
CS2458	Same as χ3306 except for Δ*agsA*::Km	This study
CS2021	Same as χ3306 except for Δ*dnaK*::Cm	This study
CS2565	Same as χ3306 except for Δ*agsA*::Km *ibpAB*::Cm	This study

[a] Cm, CHL resistance; Km, KAN resistance.

mutation, bacteriophage P22 was propagated on CS2458 (Δ*agsA*::Km) and the resultant lysate was used for infection of CS2042 (Δ*ibpAB*::Cm). The transductants were selected for KAN resistance.

Construction of Δ*ibpAB*, Δ*agsA*, and Δ*dnaK* mutants. To construct the Δ*ibpAB*::Cm mutant CS2042, the DNA fragment between nucleotides (nt) −55 and 1089 in the *ibpAB* operon coding region with promoters was initially amplified by PCR and cloned into pT7Blue-2 (Novagen). The resultant plasmid, pTKY577, was cleaved at the *Bst*XI and *Bgl*II sites (nt 226 and 831) in the cloned fragment, and the overhanging ends were filled in and ligated to the CHL resistance cassette, which was generated from *Bam*HI-digested pNK2884 (50) and then filled. The resultant plasmid, pTKY580, was cleaved at the *Mlu*I and *Sal*I sites in the vector. The generated *ibpAB*::Cm fragment was ligated to the *Mlu*I and *Sal*I sites of pTKY513, which is a previously constructed transferable suicide vector (43). The resultant mutator plasmid, pTKY583, was introduced into strain SM10(λ*pir*), which provides the π protein required for the replication of the suicide vector by transformation. The chromosomal *ibpAB* operon was replaced by the *ibpAB*::Cm construct by conjugative crosses as previously described (49). The mutant was selected for resistance to CHL and NAL. A double-crossover event resulting in the Δ*ibpAB*::Cm mutant was assessed by its sensitivity to AMP. Disruption of the *ibpAB* operon was checked by immunoblotting of the heat-aggregated proteins with an anti-*E. coli* IbpB serum (data not shown).

The *agsA* mutant CS2458 was constructed in the same way as the Δ*ibpAB*::Cm mutant. Synthetic oligonucleotides (5′-TTTCGTTAACCACTTAGAATTC) and (5′-GGCCAAGCTTATGATTTGTGTTCAATCGCC), which have an *Eco*RI or *Hin*dIII recognition sequence, were used as PCR primers. The amplified fragment containing the *agsA* coding region and its promoters was cloned into pHSG398 (44). The resultant plasmid was cleaved at the *Aha*II site (nt 256) in the cloned fragment and ligated to the KAN resistance cassette, which was generated from *Pst*I-digested pUC4K (48) and then blunted. The resultant plasmid, pTKY584, was cleaved at the *Eco*RI and *Fsp*I sites in the vector, and the overhanging *Fsp*I end was filled in. The generated *agsA* fragment was ligated to the *Eco*RI and filled-in *Sal*I sites of pTKY513. The resultant mutator plasmid, pTKY585, was introduced into strain S17-1(λ*pir*). The chromosomal *agsA* gene was replaced by the *agsA*::Km construct by conjugative crosses. The mutant was selected for resistance to KAN and NAL. A double-crossover event resulting in the Δ*agsA*::Km mutant was assessed by its sensitivity to AMP. Disruption of the *agsA* gene was checked by PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the heat-aggregated proteins (data not shown).

To construct the *dnaK* mutant CS2021, the DNA fragment between nt 53 and 1000 in the *dnaK* coding region was initially amplified by PCR and cloned into pT7Blue-2. The resultant plasmid, pTKY505, was cleaved at the *Eco*47III site (nt 525) in the cloned fragment, filled in, and ligated to the CHL resistance cassette, which was generated from *Bam*HI-digested pNK2884 and then filled in. The resultant plasmid, pTKY508, was cleaved at the *Sal*I and *Sma*I sites in the vector. The generated Δ*dnaK*::Cm fragment was ligated to the *Sal*I and *Sma*I sites of pCVD442 (10). The resultant mutator plasmid, pTKY507, was introduced into strain SM10(λ*pir*). The chromosomal *dnaK* gene was replaced by the *dnaK*::Cm construct by conjugative crosses. The mutant was selected for resistance to CHL and NAL. A double-crossover event resulting in the Δ*dnaK*::Cm mutant was assessed by its sensitivity to AMP. Disruption of the *dnaK* gene was checked for a thermosensitive phenotype and by immunoblotting of the total proteins with an anti-DnaK serum (data not shown).

Construction of AgsA-overproducing plasmid. To construct the AgsA-overproducing plasmid, the DNA fragment in the ribosome binding site and the *agsA* coding region was initially amplified by PCR. Synthetic oligonucleotides (5′-GGCCGAATTCAGGAGGTTAATGATGGCATCT) and (5′-GGCCAAGCTTATGATTTGTGTTCAATCGCC), which have an *Eco*RI or *Hin*dIII recognition sequence, were used as primers in a PCR. The amplified fragment was digested with *Eco*RI and *Hin*dIII and then cloned into pUHE21-2fdΔ12 (12). The resultant plasmid, pTKY605, allows the isopropyl-β-D-thiogalactopyranoside (IPTG)-controlled induction of AgsA.

Databases and protein sequence analysis. The database of nucleotide sequences of completed and unfinished eukaryotic and prokaryotic genomes at the National Center for Biotechnology Information (NCBI; National Institutes of Health) was used. The nonredundant database was searched with the gapped BLAST program and PSI-BLAST as described on the website (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned by homology searching of the maximum matching program with GENETYX-MAC 7.3. Multiple alignments were constructed with the CLUSTALW program. For the construction of a phylogenic tree, large inserts and ambiguously aligned regions were removed from the multiple alignments, and then commonly conserved domains were chosen (97 amino acid [aa] residues). Phylogenetic trees were constructed by using the PHYLIP package.

Isolation of aggregated proteins and gel electrophoresis. Isolation of aggregated proteins was performed as described previously (46). Quantification of the amount of aggregated proteins was performed using the Bradford assay reagent (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard. Gel electrophoresis was carried out according to the method of Laemmli (26) by using SDS–15% polyacrylamide gels and by staining with Coomassie brilliant blue.

Identification of protein. To determine the N-terminal protein sequence, proteins were separated by two-dimensional gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore). The proteins were stained with Coomassie brilliant blue, cut out, and analyzed with a Shimazu PPSD-21 protein sequencer.

Plating efficiency and spot test. Cells were grown in L medium for 3 h at 30°C to mid-logarithmic phase. Aliquot of cultures were diluted from 10^{-1} to 10^{-5} in L medium. Aliquots (100 μl) were plated, and 5-μl aliquots were spotted onto L agar plates containing 250 μM IPTG. Plates were incubated at 30 or 37°C for 24 h, and colony numbers were determined afterwards.

Survival ratio of *shsp* mutants. Cells were grown in L medium for 3 h at 30°C to mid-logarithmic phase. Aliquots (0.5 ml) of wild-type cultures and of each *shsp* mutant culture (0.5 ml) were mixed. An aliquot (100 μl) of the mixed culture was diluted in L medium without heat treatment. The rest of the culture (900 μl) was shifted to 70°C for 1 min and then diluted in L medium. The diluted cultures were plated onto an L agar plate, and total cell numbers were counted after incubation for 24 h at 30°C. Among them, the numbers of viable bacteria of strains carrying the Δ*agsA*::Km and Δ*ibpAB*::Cm mutations were determined by selecting KAN- and CHL-resistant bacteria, respectively. The survival ratios were calculated as the ratio of the number of surviving *shsp* mutant cells to the

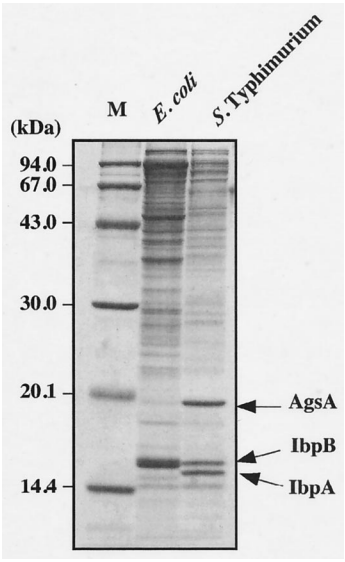

FIG. 1. Detection of aggregated proteins in *dnaK*-null mutants. *dnaK*-null mutant cells were grown to mid-exponential growth at 30°C and incubated further at 42°C for 1 h. The aggregated proteins were isolated and analyzed by SDS–15% PAGE as described in Materials and Methods. M, molecular weight marker; *E. coli*, BB1553 (Δ*dnaK52*); *Salmonella* serovar Typhimurium, CS2021 (*dnaK*::Cm).

number of surviving wild-type cells (*shsp*/WT) in the heat-treated sample divided by the ratio (*shsp*/WT) for the non-heat-treated sample.

RESULTS

Identification of a new sHsp, AgsA. Our aim was to analyze the physiological role of sHsps in *Salmonella* serovar Typhimurium. Since sHsps have been known to coaggregate with heat-denatured proteins in an *E. coli* Δ*dnaK52* strain (31, 46), we isolated and analyzed heat-aggregated proteins in a *dnaK*-null mutant of *Salmonella* serovar Typhimurium by SDS-PAGE (Fig. 1). We used a previously developed protocol for cell lysis and aggregate purification, which greatly increased the sensitivity of detection of aggregated protein species and minimized contamination of membrane proteins (46). We found that three major proteins (14.5, 15.0, and 18.0 kDa) were accumulated in the aggregated fraction (Fig. 1). It was generally believed that most of the members of the *Enterobacteriaceae* have two sHsps, IbpA and IbpB. The 14.5- and 15.0-kDa proteins were identified by immunoblotting using a specific antiserum against *E. coli* IbpB (31) as the IbpA and IbpB homologues of *Salmonella* serovar Typhimurium, respectively (data not shown). The 18.0-kDa protein was identified to the product of an uncharacterized open reading frame (ORF) of

```
1
TGA AGG GAC AGC GGA AAT GGC AAT GAA CGG GAT GAG ACT TTC GTT AAC CAC TTA AAT TCG
61                                  -35 σ32                 -10 σ32
TGA CTA AAA AAA GGC TTT TAT AGA CTT GAA AAT GTT TTT CCC GTC CCT ATG TAG TCA GTG
121                 -35 σ70                 -10 σ70                 +1
GAC AAG CAA TGC TTG CCT TGA TGT TGA ACT TTT GAA TAG TGA TTC AGG AGG TTA ATG ATG
                                                    SD              M   M
181
GCA CTC AGA ACC TTG TCA GCA CTT CCC GTG TTT GCT GAT TCT CTT TTC TCT GAC CGT TTC
A   L   R   T   L   S   A   L   P   V   F   A   D   S   L   F   S   D   R   F
241
AAC CGT ATT GAT AGA CTT TTC AGT CAA TTA ACA GGA GAT ACG CCA GTC GCT GCG ACG CCA
N   R   I   D   R   L   F   S   Q   L   T   G   D   T   P   V   A   A   T   P
301
GCT TAC GAT CTG CAA AAG CGC GAT GCG AAT AAC TAT CTG CTT ACC GTG AGC GTT CCT GGC
A   Y   D   L   Q   K   R   D   A   N   N   Y   L   L   T   V   S   V   P   G
361
TGG AAA GAG GAA GAG CTT GAA ATT GAA ACG GTT GGC GGC AAC CTG AAT ATT ACG GGT AAA
W   K   E   E   E   L   E   I   E   T   V   G   G   N   L   N   I   T   G   K
421
CAC ACT GAA GAG ACG GTA GAG GAT CAG ACG CAC TGG ATT TAT CGT GGT ATT CGT AAG GCG
H   T   E   E   T   V   E   D   Q   T   H   W   I   Y   R   G   I   R   K   A
481
GAT TTC CAG TTG AGT TTT TCT TTG CCT GAA CAT GCT AAG GTG AAT AAT GCG AAA CTG GAA
D   F   Q   L   S   F   S   L   P   E   H   A   K   V   N   N   A   K   L   E
541
CAG GGC CTC TTG TTG GTC GAG ATT TAC CAG GAG ATC CCT GAA AGC GAG AAA CCG AAA AAA
Q   G   L   L   L   V   E   I   Y   Q   E   I   P   E   S   E   K   P   K   K
601
ATT GCC ATA GAA AGC AAA CCA AAG GCG ATT GAA CAC AAA TCA TAA TCG CGT TAA TGG GAG
I   A   I   E   S   K   P   K   A   I   E   H   K   S   *
661
CGT TTA GAC GGG CCT TCA TTG TCT GTA GCG AAA AGT ATC CCG TCA TGC TTG TAG GCA CAG
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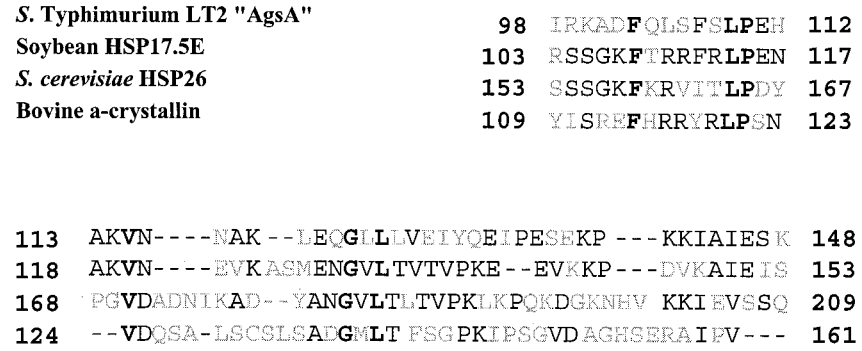
FIG. 2. Nucleotide sequence of the *Salmonella* serovar Typhimurium *agsA* gene. The nucleotide sequence of the *agsA* gene was taken from the completed *Salmonella* serovar Typhimurium LT2 genomic sequence at the National Center for Biotechnology Information (National Institutes of Health) by BLAST search. Position +1 marks the start of the AgsA ORF, which is translated. Bold letters show the N-terminal amino acid sequence that was determined by N-terminal sequencing. The underlined region represents the sequence of the ribosome binding site (SD). The putative promoter regions (−35 to −10) are boxed. σ^{32}, putative σ^{32} promoter; σ^{70}, putative σ^{70} promoter.

A



B

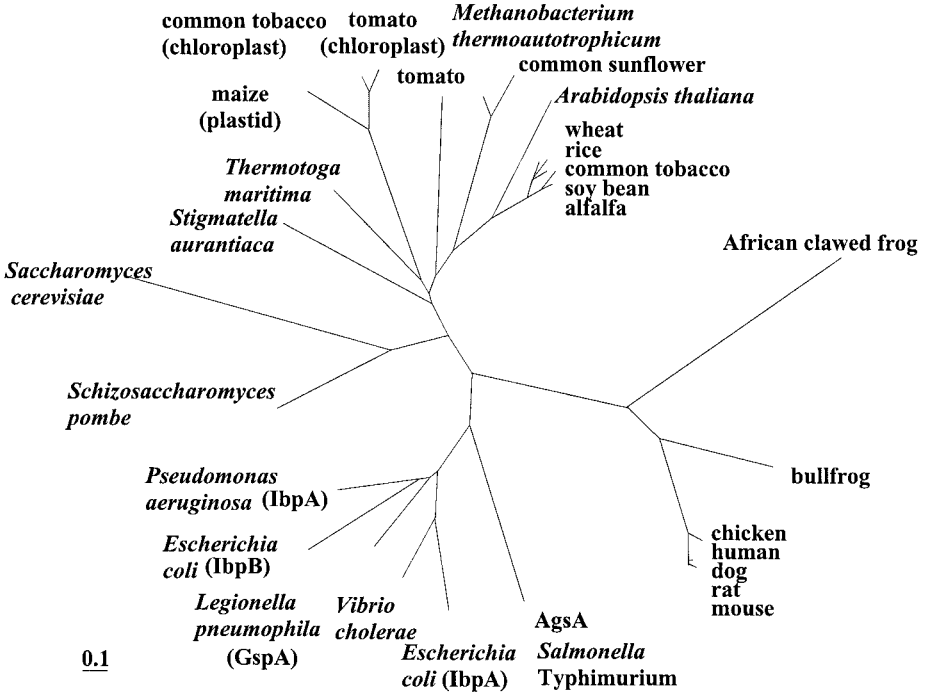


FIG. 3. (A) Alignment of the most highly conserved carboxy-terminal amino acid sequence of AgsA and various sHsps. Positions where four proteins have identical amino acids are boldfaced. Two or more identical amino acids in one position are indicated by plain text. Gaps are indicated by dashes. (B) Phylogenetic tree of the sHsps. Phylogenetic trees were constructed by using the PHYLIP package as described in Materials and Methods. Proteins (with accession numbers in parentheses) are as follows: mouse, Hsp27 (A53423); rat, Hsp27 (JN0924); dog, Hsp27 (JC4244); human, Hsp27 (HHHU27); chicken, Hsp25 (A39644); bullfrog, αB-crystallin (S54824); African clawed frog, Hsp30C (JN0274); alfalfa, Hsp18 (S16248); soybean, Hsp17.5 (T07629); common tobacco, Hsp18p (T03958); rice, Hsp17 (T04171); wheat, Hsp17 (HHWT17); *Arabidopsis thaliana*, Hsp22 (S71188); common sunflower, Hsp17.9 (S46310); *Methanobacterium themoautotrophicum*, Hsp17 (F69214); tomato, Hap17.6 (T07602); tomato (chloroplast), Hsp21 (T06324); common tobacco (chloroplast), Hsp26a (T02018); maize (plastid), Hsp26 (T03379); *Thermotoga maritima*, Hsp17 (T46658); *Stigmatella aurantaca*, Hsp SP21 (A49942); *Saccharomyces cerevisiae*, Hsp26 (P15992); *Schizosaccharomyces pombe*, Hsp16 (T40376); *Pseudomonas aeruginosa* IbpA (D83256); *Escherichia coli* IbpB (G65170); *Legionella pneumophila*, GspA (S49042); *Vibrio cholerae*, Hsp16 (D82373); *Escherichia coli* IbpA (A45245).

the *Salmonella* serovar Typhimurium genome by determination of its N-terminal amino acid sequence as shown in Fig. 2. The translated amino acid sequence of this protein shows weak but significant similarity to the sHsps from the eukaryotes and to the structurally related α-crystallin lens proteins (Fig. 3A). Thus, the 18.0-kDa protein is a member of the sHsp protein family. We named this protein AgsA (aggregation-suppressing protein) on the basis of its function as described below.

Analysis of the area upstream of *agsA* revealed that the transcription is possibly regulated by two promoters (Fig. 2). One is similar to the consensus sequence of a promoter that is recognized by the σ^{70} housekeeping transcription factor, while

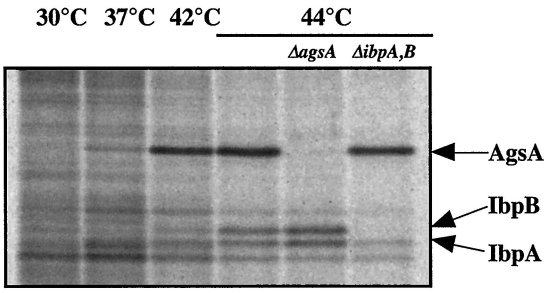


FIG. 4. Heat induction of AgsA. Cells of strains χ3306 (wild type), CS2042 (Δ*ibpAB*), and CS2458 (Δ*agsA*) were grown for 4 h at 30°C in M9 glucose minimum medium. Aliquots of the cultures were pulse-labeled for 2 min with an L-U-^{14}C-labeled amino acid mixture (Amersham Pharmacia Biotech) at 15 min after the shift from 30°C to the indicated temperature. Equal amounts of total proteins were analyzed by SDS-PAGE.

another promoter seems to be recognized by the σ^{32} heat shock transcription factor (8). In contrast to *ibpA* and *ibpB*, which form an operon together (7), it is likely that the *agsA* gene is transcribed in a monocistronic mRNA that could be translated to a polypeptide of 156 aa (including the N-terminal Met residue) with a molecular mass of 17.7 kDa. The result of N-terminal sequencing and determination of the predicted amino acid sequence suggested that AgsA contains neither Met nor Cys. This was confirmed by the result in which pulse-labeling of proteins with [^{35}S]Met-Cys failed to detect the stimulated synthesis of an sHsp corresponding to the molecular weight of AgsA by heat shock (data not shown). On the other hand, pulse-labeling of proteins with a ^{14}C-labeled amino acid mixture revealed a single sHsp corresponding to AgsA at high temperature. We observed a weak induction of AgsA at 37°C and strong induction at 42 and 44°C, stronger than that of IbpAB (Fig. 4). We also confirmed that there was no induction of AgsA in a Δ*agsA* strain, even at a high temperature (44°C) (Fig. 4).

Homology alignment and phylogenetic analysis. It is known that the carboxy-terminal domain called the α-crystallin domain displays the highest degree of conservation within the sHsp family (9). Homology alignment with eukaryotic sHsps revealed that AgsA has the highest similarity to soybean sHsps over the carboxy-terminal region of the protein (42% identical) (Fig. 3A). A BLAST search by comparison with the prokaryote genome database at the NCBI uncovered an ORF encoding a *Klebsiella pneumoniae* homologous protein with high similarity throughout the entire region (70% amino acid sequence identity). Another gene coding for a homologous protein (42% amino acid sequence identity) was found in the genome of *Buchnera* sp. APS, which is an endocellular bacterial symbiont harbored by pea aphids. The similarities between AgsA and IbpA or IbpB of *Salmonella* serovar Typhimurium are rather low (sequence identity to AgsA, 32% for IbpA [E values, $8e^{-17}$] and 31% for IbpB [E values, $5e^{-17}$]). In contrast to other members of the *Enterobacteriaceae*, including *E. coli*, that encode IbpA and IbpB homologues but not AgsA in their genomes, *K. pneumoniae* has three different sHsp homologues corresponding to AgsA, IbpA, and IbpB. Interestingly, *Buchnera* sp. APS has only one sHsp, which shows the highest homology to AgsA. A phylogenetic analysis suggested that AgsA arose from an ancient gene duplication or amplification at an early evolutionary stage of gram-negative bacteria that was followed by sequence divergence (Fig. 3B).

Role of sHsps in bacterial thermotolerance. It is believed that sHsps constitute the system for protecting against irreversible aggregation of cellular proteins and assist in protein refolding by the ClpB-DnaK bichaperone system and GroESL after heat shock of *E. coli* (4, 11, 47). However, it has also been reported that no substantial differences were observed in the thermoresistance and tolerance phenotypes between *ibpAB* mutants and their parental strain (21). We examined the temperature sensitivity for growth (at 44°C), protein aggregation (at 44°C), and thermotolerance (at 52°C) phenotype of *Salmonella* serovar Typhimurium *agsA* and *ibpAB* mutant cells. The *shsp* mutant did not show any temperature-sensitive phenotype, accumulation of aggregated proteins, or defect in thermotolerance at the indicated temperatures (data not shown). It is well known that the ClpB-DnaK bichaperone system has a function crucial for the acquisition of heat resistance, thermotolerance, and protection of cellular proteins. It seems that the most defects of *agsA* and *ibpAB* mutations are sequestered by the function of the DnaK-ClpB bichaperone system. Therefore, we used the more intense condition to assess the role of the sHsps in the thermotolerance of *Salmonella* serovar Typhimurium. We determined the survival rates after transient exposure to a lethal temperature (70°C) (Fig. 5). When wild-type cells were shifted from 30 to 70°C, cell viability rapidly decreased, yielding about 0.01% viable cells after 60 s and no viable cells after 90 s of incubation. To avoid experimental error, we mixed the same numbers of wild-type and mutant cells and then exposed the cultures to 70°C for 1 min. The survival ratio was determined as described in Materials and Methods, and the relative ratio is shown in Fig. 5. In the *agsA* mutant strain, no decrease in viability was observed compared to the wild-type strain (relative survival rate, 113%).

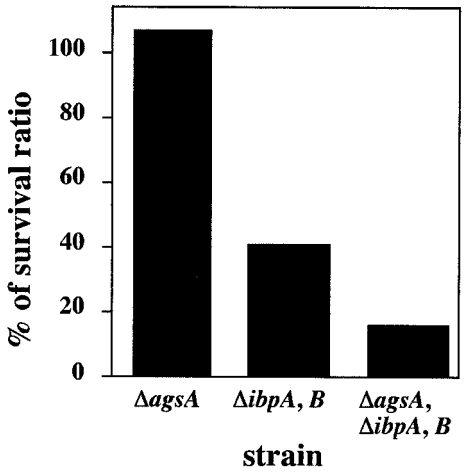


FIG. 5. sHsps are necessary for improvement of survival at 70°C. Cells of strain χ3306 (wild type) and mutants CS2458 (Δ*agsA*), CS2042 (Δ*ibpAB*), and CS2565 (Δ*agsA* and Δ*ibpAB*) were grown to mid-exponential growth at 30°C. The same numbers of wild-type and *shsp* mutant cells were mixed and then sifted at 70°C for 1 min. The numbers of viable *shsp* mutant cells were determined by using a drug resistance marker (Km for Δ*agsA*; Cm for Δ*ibpAB*). Survival ratios were calculated as described in Materials and Methods. Each value is the average from two different experiments.

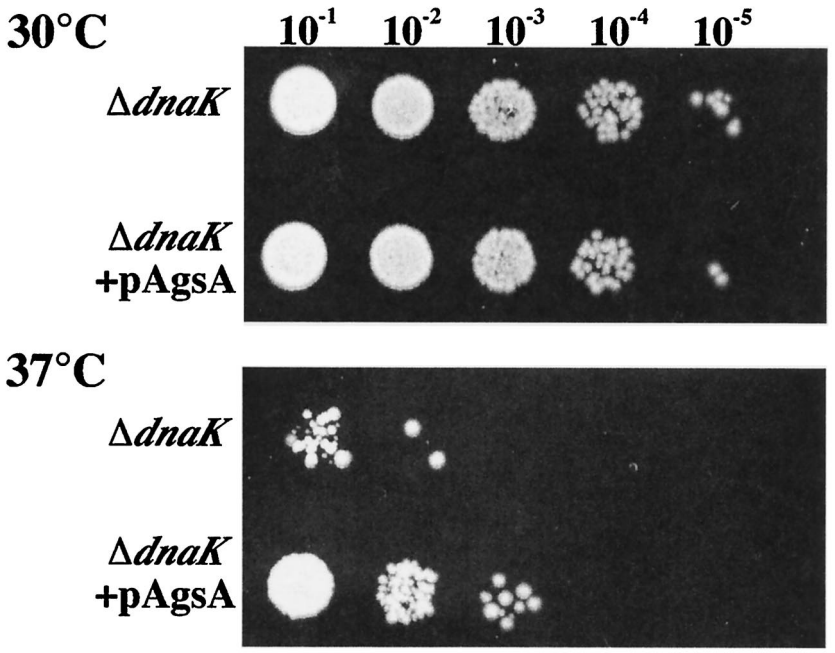


FIG. 6. AgsA partially suppresses the Δ*dnaK52* phenotype. Δ*dnaK52* (strain BB1553) and Δ*dnaK52* + pAgsA (strain CS5257) cells were grown in L medium at 30°C for 3 h. Various dilutions (10^{-1} to 10^{-5}) were spotted onto L agar plates supplemented with 250 μM IPTG and were incubated at 30 or 37°C for 24 h.

The *ibpAB* mutant showed a significantly lower viability (42%). The addition of the *agsA*::Km mutation to *ibpAB* mutants resulted in a further decrease in viability (15%). These results suggest that both the *agsA* and *ibpAB* genes play important roles in the survival of *Salmonella* serovar Typhimurium at lethal temperatures.

The heat sensitivity of a Δ*dnaK52* mutant is partially suppressed by the overproduction of AgsA. To examine the function of AgsA, we constructed the *agsA* recombinant plasmid pTKY605, which contains an IPTG-regulatable PA1lacO1 promoter. For this experiment, we chose an *E. coli* Δ*dnaK52* mutant (BB1553) which expresses DnaJ at low levels and carries a spontaneous suppressor mutation in *rpoH*, which partially inactivates σ^{32} (2). Cells of this strain grow with approximately wild type rates at 30°C but remain temperature sensitive for growth above 37°C. Since *E. coli* has no *agsA* gene on the chromosome, we decided to monitor the chaperone function of AgsA.

To assess the role of AgsA in the state of thermoresistance, we determined the plating efficiency at a sublethal temperature (37°C) for Δ*dnaK52* cells. When Δ*dnaK52* cells were incubated at 37°C, the growth of the cells was inhibited, and only about 0.02% of the cells could form colonies. Interestingly, the plating efficiency of a Δ*dnaK52* derivative carrying an AgsA-overproducing plasmid at 37°C was increased to 1.7%, suggesting that AgsA could partially complement the thermosensitive phenotype. This phenotype was also confirmed by using a spot test as shown in Fig. 6. These results suggest that AgsA partially compensates DnaK function and assists the growth of Δ*dnaK52* cells under sublethal conditions. We observed no significant differences in colony formation between induced and noninduced conditions (data not shown). This could be due to the accumulation of AgsA in cells carrying pTKY605 without an inducer, as indicated in Fig. 7A.

Suppression of protein aggregation by AgsA in Δ*dnaK52* and Δ*rpoH* mutants. The Hsp70 (DnaK) chaperone system is known to refold the denatured proteins efficiently, and the Δ*dnaK52* mutation causes a large accumulation of aggregation protein at nonpermissive temperatures (46). This phenotype of the Δ*dnaK52* mutant allowed us to analyze the chaperone function of AgsA. Thus, we examined the ability of AgsA protein to prevent protein aggregation in *E. coli* Δ*dnaK52* mutant cells at 42°C (Fig. 7A). Overproduction of AgsA did not perturb cell growth (data not shown). When AgsA was overproduced in Δ*dnaK52* cells by IPTG, a large amount of AgsA was produced (around 5% of total proteins) and copurified in the aggregated fraction (around 10% of aggregated proteins). Under nonoverproduced conditions, cells already had significant amounts of AgsA protein (around 0.5% of total proteins), which copurified in the aggregated fraction (around 1% of aggregated proteins). We then examined protein aggregation in Δ*dnaK52* cells with and without overproduction of AgpA. Without IPTG, about 10% of total proteins were aggregated in Δ*dnaK52* cells at 42°C (Fig. 7B). Interestingly, overproduction of AgsA decreased the amount of aggregated protein to 3% of total proteins at 42°C, suggesting that AgsA is able to prevent protein aggregation. Since the Δ*dnaK52* cells have only a small amount of aggregated proteins, less than 1% of total protein at 30°C, we failed to monitor the effect of AgsA overproduction reproducibly.

We also examined function of AgsA by overproduction in a Δ*rpoH* strain (BB7224). This mutant lacks σ^{32}, is therefore largely devoid of all major cytosolic chaperones except for GroEL/GroES, and has lower levels of proteases (25). The Δ*rpoH* strain carries an insertion element in the promoter region of the *groES groEL* operon, which drives constitutive expression of this operon and results in fourfold-increased

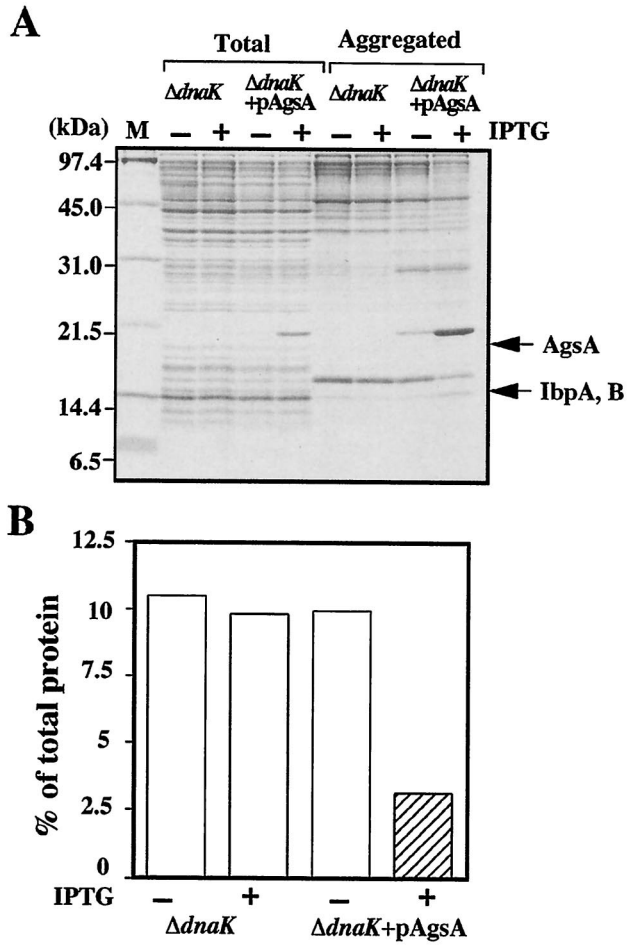


FIG. 7. AgsA prevents heat-induced aggregation of proteins in the Δ*dnaK52* mutant at 42°C. (A) Δ*dnaK52* (strain BB1553) and Δ*dnaK52* + pAgsA (strain CS5257) cells were grown in L medium that was either left unsupplemented (−) or supplemented with (+) 1 mM IPTG at 30°C for 2 h. Cells were cultured further at 42°C for 1 h. Aggregated proteins were isolated as described in Materials and Methods. Equal amounts of proteins (20 μg) were analyzed by SDS–15% PAGE. (B) The amount of aggregated protein was quantified by Bradford assay and calculated in relation to total protein content (set at 100%). Total, total cell lysate; Aggregated, aggregated proteins; M, molecular weight marker. Each value is the average from at least three different experiments.

GroEL/GroES levels compared with those in the wild type. This increase in these levels allows Δ*rpoH* cells to grow, albeit slowly, at temperatures up to 40°C (25). After growth at 30°C, the levels of heat shock proteins relative to those in the wild type were below 1% for DnaK, 20% for HtpG, 10% for IbpB, 20% for ClpB, and about 50% for Lon and HslVU (46). Lack of these chaperones and proteases results in a large accumulation of aggregation protein, that is, about 3 and 10% of total proteins were aggregated at 30 and 42°C, respectively (Fig. 8). It seems that this phenotype allows us to perform fine analysis of the AgsA chaperone function without effects of other chaperones and proteases. When AgsA was overproduced in Δ*rpoH* cells to the same extent as in Δ*dnaK52* cells (5% of total proteins) at 30 and 42°C, large amounts of AgsA were copurified in the aggregated fraction (corresponding to 10% of aggregated proteins) at both temperatures (Fig. 8). In the Δ*rpoH* cells overproducing AgsA, the amount of aggregated proteins decreased to 42% compared to that in noninduced cells at 42°C (Fig. 8), suggesting the ability of AgsA to suppress protein aggregation in Δ*rpoH* cells. Interestingly, overproduced AgsA also suppressed protein aggregation at a lower temperature, that is, the amount of aggregated protein decreased to 52% compared to that in noninduced cells at 30°C (Fig. 8). Taken together, our data clearly indicate that the AgsA is capable of protecting cellular proteins against aggregation.

DISCUSSION

In this study, we discovered a novel sHsp (AgsA) in the aggregated proteins accumulated in *Salmonella* serovar Typhimurium Δ*dnaK* cells expressed at high temperatures (Fig. 1). sHsps are classified into two classes, class A and B (34). Class A contains only bacterial proteins, and the similarity is not restricted to the α-crystallin domain but extends into the flanking amino- and carboxy-terminal regions. Class B proteins are much more divergent in length and sequence. They include prokaryotic as well as eukaryotic members from a wide variety of organisms. Homology alignment of AgsA with *Salmonella* serovar Typhimurium IbpA and IbpB shows weak similarity (32% sequence identity to AgsA for IbpA, and 31% for IbpB), though the homology extends into the flanking regions (data not shown). These findings suggest that AgsA belongs to class A, and it seems to have arisen from the same phylogenetic origin by duplication or amplification at an early evolutionary stage of *Enterobacteria* (Fig. 3B). Homologues of AgsA were found in the genomes of the *K. pneumoniae* and in a *Buchnera* sp. The mutualism between *Buchnera* and its host is so obligatory that neither organism in the association can reproduce independently. This symbiotic bacterium lost many genes, including essential genes, and depends completely on its host (40). The genome size of the *Buchnera* sp. is only one-seventh the size of the *E. coli* genome. Nevertheless, an AgsA homologue is still maintained in *Buchnera*. These results suggest that the AgsA homologue may have an unknown important function for the symbiosis of *Buchnera*.

We showed that overproduction of AgsA partially suppressed the Δ*dnaK52* thermosensitive phenotype at 37°C. However, AgsA overproduction could not rescue the lethality of the Δ*dnaK52* mutant at 42°C and did not result in a significant increase in viability at 50°C (data not shown). These data suggest that AgsA cannot assist in refolding for strongly damaged proteins but can protect the partially damaged proteins and assist their spontaneous refolding. However, AgsA could prevent aggregation of strongly damaged proteins at high temperatures (Fig. 7 and 8). Rajaraman et al. reported that human α-crystallin interacts with unfolded proteins to reactivate them at an early stage of denaturation and to protect them from aggregation at a later stage (38). The function of AgsA may be analogous to that of human α-crystallin. To know the function of sHsps in *Salmonella* serovar Typhimurium, we examined the effect of a lethal temperature (70°C) on the viability of the wild type and *agsA*, *ibpAB*, and *agsA ibpAB* triple mutants (Fig. 5). Compared to the wild-type strain, an *agsA*-null mutant exhibited no loss of viability at lethal temperatures. In contrast, an *ibpAB* mutant showed significantly lower viability at high temperatures. The *agsA ibpAB* triple mutant exhibited a stronger defect than the *ibpAB* mutant. These data show that the major contribution to thermotolerance comes

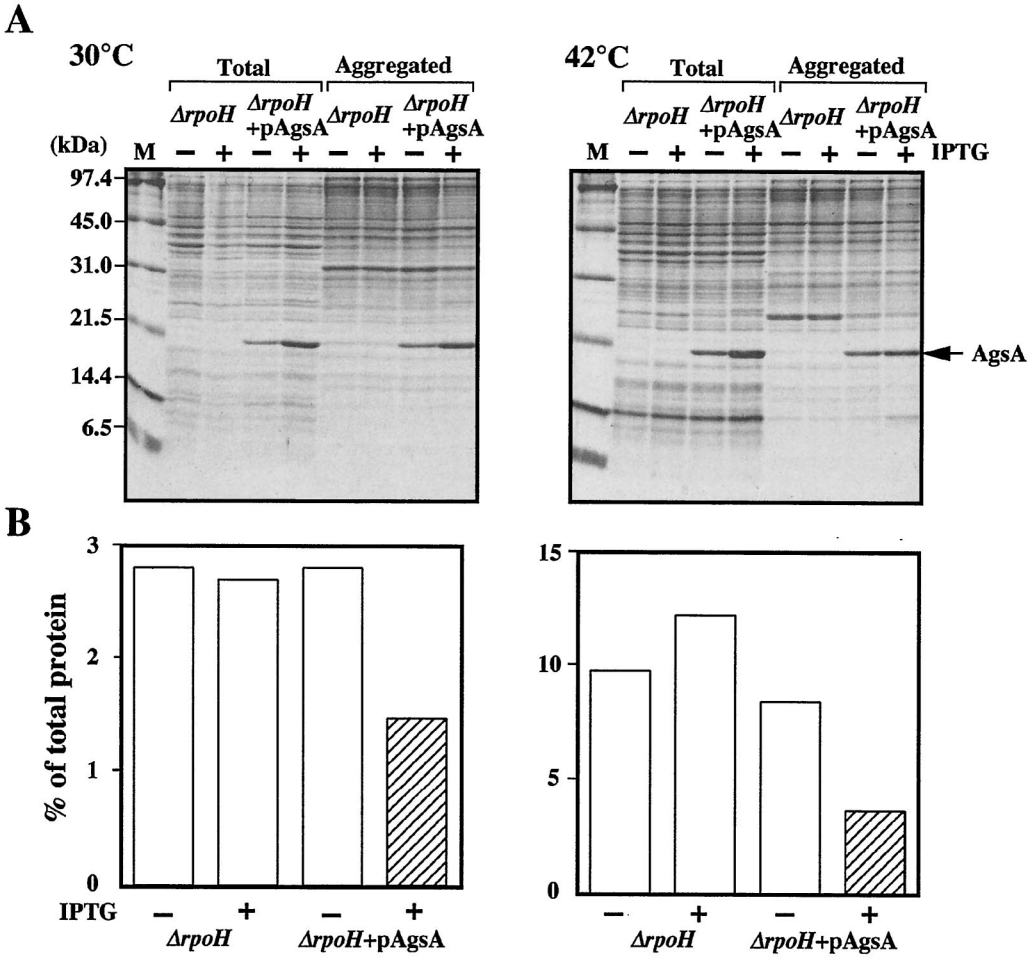


FIG. 8. AgsA prevents aggregation of proteins in a Δ*rpoH* mutant. (A) Δ*rpoH* (strain BB7224) and Δ*rpoH* + pAgsA (strain CS5262) cells were grown in L medium that was either left unsupplemented (−) or supplemented with (+) 1 mM IPTG at 30°C for 2 h. Cells were cultured further at 30 or 42°C for 1 h. Aggregated proteins were isolated as described in Materials and Methods. Equal amounts of proteins (20 μg) were analyzed by SDS–15% PAGE. (B) The amount of aggregated protein was quantified by Bradford assay and calculated in relation to total protein content (set at 100%). Each value is the average from at least three different experiments.

from IbpAB, and this advantage may account for the high conservation of IbpAB in *Enterobacteriaceae*.

It has been reported that the overproduction of *E. coli* IbpA and IbpB may possibly reduce the amount of aggregated proteins by long exposure (0.5 to 4 h) at an extremely high temperature, 50°C (24). It is also indicated that some sHsps form a stable multimer conformation at low temperatures and cannot associate with denatured proteins (19, 22, 39). Here we showed that overproduction of AgsA could reduce the amount of aggregated proteins even at a permissive temperature (30°C) in Δ*rpoH* cells. This data suggest that AgsA allows the forming of some active conformation even at low temperatures. Since *Salmonella* serovar Typhimurium synthesized only a small amount of AgsA at a low temperature (Fig. 4), it is impossible to discuss some important physiological function of AgsA in this condition at present. Δ*rpoH* cells have only small amounts of heat shock chaperones and proteases except for GroEL/GroES. It is well known that the protein aggregates can be suppressed by the DnaK chaperone system and the GroEL/GroES chaperone (but with lesser effects and to lesser extents than DnaK) and subsequently refolded into the native state. No other chaperones were reported to provide effective prevention of endogenous protein aggregation in the cells. We showed that AgsA overproduction could suppress protein aggregation in Δ*rpoH* cells. Since Δ*rpoH* and Δ*dnaK52* mutants have the GroEL/GroES chaperone in cells, we cannot exclude the possibility that AgsA may be functional only with GroEL/GroES. However, it is known that GroEL/GroES has limiting folding capacity, and their substrates are believed to be <60 kDa (5, 18). Our data clearly showed that AgsA could protect against aggregation of proteins larger than 60 kDa (Fig. 8). For aggregation prevention, AgsA seems to be functional without the help of another chaperone such as the DnaK-ClpB bichaperone system, GroEL/GroES, HtpG, IbpA, or IbpB.

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