Polyamines are small flexible organic polycations found in almost all cells. They likely existed in the last universal common ancestor of all extant life, and yet relatively little is understood about their biological function, especially in bacteria and archaea. Unlike eukaryotes, where the predominant polyamine is spermine, bacteria may contain instead an alternative polyamine, sym-homospermidine. We demonstrate that homospermidine synthase (HSS) has evolved vertically, primarily in the α-Proteobacteria, but enzymatically active, diverse HSS orthologues have spread by horizontal gene transfer to other bacteria, bacteriophage, archaea, eukaryotes, and viruses. By expressing diverse HSS orthologues in *Escherichia coli*, we demonstrate in vivo the production of co-products diaminopropane and N₁-aminobutyldcadaverine, in addition to sym-homospermidine. We show that sym-homospermidine is required for normal growth of the α-proteobacterium *Rhizobium leguminosarum*. However, sym-homospermidine can be replaced, for growth restoration, by the structural analogue spermidine and sym-norspermidine, suggesting that the symmetrical or unsymmetrical form and carbon backbone length are not critical for polyamine function in growth. We found that the HSS enzyme evolved from the alternative spermidine biosynthetic pathway enzyme carboxyspermidine dehydrogenase. The structure of HSS is related to lysine metabolic enzymes, and HSS and carboxyspermidine dehydrogenase evolved from the aspartate family of pathways. Finally, we show that other bacterial phyla such as Cyanobacteria and some α-Proteobacteria synthesize sym-homospermidine by an HSS-independent pathway, very probably based on deoxyhypusine synthase orthologues, similar to the alternative homospermidine synthase found in some plants. Thus, bacteria can contain alternative biosynthetic pathways for both spermidine and sym-norspermidine and distinct alternative pathways for sym-homospermidine.

Polyamines are primordial, small flexible organic polycations found in almost all cells of bacteria, archaea, and eukaryotes. In bacteria and archaea, the key polyamines (see Fig. 1A) are the triamines spermidine, sym-norspermidine, and sym-homospermidine (referred to herein as norspermidine and homospermidine), and occasionally more than one triamine can be found in the same cell. In eukaryotes, which contain spermidine (and in some plants, yeasts, and animals, the tetraamine spermine), polyamines are required for growth, cell proliferation, and normal cellular physiology. Polyamine biosynthesis is essential in the fungi *Saccharomyces cerevisiae* (2), *Schizosaccharomyces pombe* (3), *Aspergillus nidulans* (4), and *Usitilago maydis* (5), the kinetoplastid parasites *Trypanosoma brucei* (6) and *Leishmania donovani* (7), and the diplomonad parasite *Giardia lamblia* (8). In mouse, polyamines are essential for early embryo development (9, 10), and they are also essential for seed development in the flowering plant *Arabidopsis thaliana* (11).

The universal distribution of polyamines suggests that they were present in the last universal common ancestor. During the time since the last universal common ancestor (and perhaps before), polyamines have acquired multiple diverse roles in cellular physiology. Although polyamines have likely been present throughout the evolution of life, the biosynthetic strategy for their production has fractured into multiple parallel and intersecting pathways composed of diverse modules for diamine and triamine synthesis. Occasionally, tetraamine and longer linear chain and branched polyamines are found (12). In eukaryotes, the triamine spermidine is synthesized from the precursor diamine putrescine by the addition of an aminopropyl group donated by decarboxylated S-adenosylmethionine. The enzymes involved in eukaryotic spermidine biosynthesis, S-adenosylmethionine decarboxylase and spermidine synthase, are also present in many bacteria and archaea and have been extensively characterized (13–19). The decarboxylated S-adenosylmethionine pathway is also involved in norspermidine and norspermine biosynthesis in thermophilic Crenarchaeota and Clostridia (20, 21) and probably in other bacterial species. An entirely independent pathway for spermidine and norspermidine biosynthesis is present in some bacteria, based on aspartate β-semialdehyde as a carbamoylpropyl group donor (22–24). This pathway involves a carbamoylamine intermediate produced from diaminopropane or putrescine by carbamoyl(nor)spermidine.
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dehydrogenase (CANSDH), which is then decarboxylated by a carboxy(nor)spermidine decarboxylase (CANSDC) to produce norspermidine or spermidine (24). The CANSDH/CANSDC pathway is found in all subdivisions of the Proteobacteria, in some of the Firmicutes, and in Termite Group 1 (24).

In addition to spermidine and norspermidine, the other prevalent triamine is homospermidine. The existence of organisms that use only the unsymmetrical spermidine or only the symmetrical homospermidine as their sole triamine has profound implications for the molecular mechanisms of action of polyamines. Spermidine binds to RNA and influences ribosome function and mRNA translation (25) and also binds to DNA, thereby influencing DNA structure and compaction (26). It has been suggested that the manner of binding of polyamines to nucleic acids is specific; however, if cells cope with only spermidine or only homospermidine, the exact nature of the polyamine structure, and by inference, mode of binding, would not appear to be critical for function. Although homospermidine is generally considered to be an unusual polyamine, it is found in nitrogen-fixing Cyanobacteria (27), in the Chloroflexi and Siphonobacteria of the Bacteroidetes (30, 31), in the Chlorobi (28, 29), in the Flavobacteriaceae, Flexibacteriaceae, and Planctomycetes (29). Within the Prochloroales and Sphingobacteriaceae of the Bacteroidetes (30, 31), in the Thermus phylum (32), and in the Gemmatimonadetes, Acidobacteria, and Planctomycetes (29). Within the Proteobacteria, homospermidine is found in 18 out of 35 analyzed genera of α-Proteobacteria; it is widespread in the δ-Proteobacteria, absent in the ε-Proteobacteria, and prominent in the pathogenic γ-proteobacterial genus Legionella (33). However, homospermidine appears to be absent from the Aquificae, Thermotogae, Firmicutes, and Actinobacteria (29, 32). Within the archaea, homospermidine is found in methanogenic archaea (34). Among single-celled eukaryotes, homospermidine has also been found in the slime mold Physarum (35). Certain plant lineages produce homospermidine for pyrroline-5-carboxylate dehydrogenase (CANSDH), which is then decarboxylated by a carboxy(nor)spermidine decarboxylase (CANSDC) to produce norspermidine or spermidine (24). The CANSDH/CANSDC pathway is found in all subdivisions of the Proteobacteria, in some of the Firmicutes, and in Termite Group 1 (24).

Both spermidine and norspermidine can be synthesized by alternative pathways, the S-adenosylmethionine decarboxylase/spermidine synthase pathway and the CANSDH/CANSDC pathway. Our goals were to determine the prevalence and investigate the physiological role of the bacterial homospermidine biosynthetic pathway, to determine whether there is evidence for alternative homospermidine biosynthetic pathways, and to investigate the evolutionary origin of homospermidine biosynthesis.

**EXPERIMENTAL PROCEDURES**

Reagents—Samples of homospermidine were kind gifts from Deborah Kramer (Roswell Park Cancer Institute, Buffalo, NY), Akira Shirahata (Josai University, Saitama, Japan), and Shiv Kumar Sharma and Patrick Woster (Wayne State University, Detroit, MI). All other polyamines were obtained from Sigma-Aldrich.

Bacterial Growth and Polyamine Extraction—Bradyrhizobium japonicum N2P5549 and Rhizobium leguminosarum bv. viciae 3841 were obtained from Anne Edwards and Alan Downie (John Innes Centre, Norwich, UK). B. japonicum was grown with constant agitation at 28 °C in TY liquid medium. Cells were harvested in 1-ml aliquots after 9 days of growth and washed with 20 mM Tris-Cl, pH 6.8, and pellets were stored at −80 °C. Polyamines were extracted from cell pellets using 0.5 volume of 5% trichloroacetic acid and incubated at 4 °C for 16 h with occasional vortexing. Centrifugation (18,000 × g, 10 min) provided a clear supernatant containing the polyamines. R. leguminosarum was grown in AMS minimal medium (43) at 28 °C with constant shaking. The ornithine decarboxylase (ODC) inhibitor α-difluoromethylornithine was a kind gift of Patrick Woster, Wayne State University, and was filter-sterilized before being added to AMS minimal medium. The cells were harvested in 1-ml aliquots and washed with 0.5 volume of 20 mM Tris-Cl, pH 6.8, and cell pellets were stored at −80 °C. Polyamines were extracted from cell pellets by resuspending pellets in 200 µl of MOPS lysis buffer (100 mM MOPS, 50 mM NaCl, 20 mM MgCl2) and then subjecting to a freeze-thaw cycle three times before the addition of 60 µl of 40% trichloroacetic acid. The suspension was left on ice for 5 min and then centrifuged (18,000 × g, 10 min) to remove cell debris. The clear supernatant was analyzed for polyamine content. Anabaena variabilis ATCC 29413 and Caulobacter crescentus CB15 were obtained from the American Type Culture Collection. A. variabilis was grown in ATCC liquid medium 616 (BG-11) at 25 °C under lights (16-h light/8-h dark) with constant shaking for 2
weeks. C. crescentus was grown in M2 minimal salts medium (44) with 0.2% glucose with constant agitation at 30 °C for 36 h. For both A. variabilis and C. crescentus, 2-ml aliquots were taken, and cells were harvested by centrifugation. Cell pellets were washed twice with 0.5 volume of phosphate-buffered saline, pH 7.4, and stored at −80 °C. Polyamines were extracted using the MOPS lysis buffer method as described for R. leguminosarum. Extraction of polyamines from E. coli cells was performed using the same approach used for R. leguminosarum.

Polyamine Analysis by High Performance Liquid Chromatography—HPLC analysis of polyamines was performed using precolumn derivatization of the amine groups using the AccQ-Flour™ reagent kit (Waters Corp., Milford, MA) following the manufacturer’s instructions. In each case, 2.5 µl of cell extract was included in the derivatization reaction, along with 0.5 µM of an internal standard, diaminononane (diaminoheptane for B. japonicum and R. leguminosarum). Reactions were performed in Accu-Fluor borate buffer in a total volume of 100 µl and heated to 55 °C for 10 min. Derivatized polyamine samples were analyzed by HPLC using a reverse-phase C18 column (Phenomenex, Luna 5 µm) on a Dionex Summit HPLC System. Polyamine separation was performed using 10 µl of derivatized sample. The system was operated at 33 °C and eluted with a 50–2000 with a scan range of m/z 50–2000 with a scan rate of 1000 D/s and a step size of 0.08 Da.

Cloning of B. japonicum hss—The hss ORF was amplified from genomic DNA by PCR using Takara Ex-Taq (BioWhittaker) and the following primers: forward, 5’-ATGGATCCATGACCCCCCGCTCG-3’; reverse, 5’-CTTCTTCAACGCACCAAGATATTTCCGGAA-3’, incorporating restriction sites BamHI and HindIII at the 5’ and 3’ end of the ORF, respectively. PCR product was ligated into the pGEM-T Easy vector (Promega). The DNA sequence of the amplified hss ORF was verified before being digested with BamHI and HindIII and ligated into the corresponding sites of the protein expression vector pET21a (Novagen) to produce an N-terminally T7-tagged HSS ORF.

Synthetic Genes—The following hss orthologue ORFs were synthesized de novo with codon usage optimized for expression in E. coli and S. cerevisiae (by Genscript Corp., Piscataway, NJ): Paramecium tetraurelia (GenBank™, XP_001452042), Ralstonia phage φRS1 (GenBank: YP_0019499984), Saweryia marylandensis (assembled from expressed sequence tags (ESTs) (GenBank: EC825861 (amino acids 1–237), EC824408 (amino acids 278–493), and EC825418 (amino acids 96–338), and Opitutus terraë PB90-1 (GenBank: YP_001819503), Global Ocean Sampling (GOS) marine metagenome (GenBank: ECW30472). In each case, BamHI and XhoI sites were incorporated at the 5’ and 3’ end, respectively. Synthetic genes were ligated as BamHI/XhoI fragments into the similarly digested pET21 expression vector (Novagen) to produce N-terminally T7-tagged ORFs. The P. tetraurelia ORF was cloned into pET21a; the other four were cloned into pET21b. All constructs were verified by sequencing prior to protein expression studies.

Expression of HSS Orthologues in E. coli—The pET21 constructs containing the six hss orthologues and the empty vector control were transformed into E. coli BL21(DE3) cells (Novagen). TriPLICATE cultures for each construct were grown in 10 ml of LB broth containing 100 µg/ml ampicillin. Protein expression was induced using the Novagen Overnight Express™ Autoinduction System 1 and grown at 25 °C for 24 h. Cells were harvested in 2-ml aliquots by centrifugation and washed twice in 0.5 volume of phosphate-buffered saline, pH 7.4, and cell pellets were stored at −80 °C.

Enzymatic Assay of B. japonicum HSS and R. leguminosarum ODC—The HSS assay was performed at 28 °C in a volume of 100 µl containing 1 µg of recombinant HSS protein, 0.5 mM NAD⁺, 5.0 mM putrescine, 70 mM KCl, 1 mM dithiothreitol, 50 mM Tris-Cl, pH 8.4. After 4 min, the reaction was stopped with 5% trichloroacetic acid. Assay conditions were altered as appropriate for kinetic analysis. Ornithine decarboxylase activity was assayed as described previously (45).
RESULTS AND DISCUSSION

A gene encoding HSS was first identified in the \( \alpha \)-proteobacterium \( R. \) viridis (38), and the enzymatic activity of an orthologue was confirmed for the \( P. \) bursaria Chlorella virus HSS (39). Kinetic behavior of the corresponding purified recombinant enzymes was not reported, and the only kinetic analysis of HSS reported has been for HSS activity (42, 46) from purified extracts of the \( \gamma \)-proteobacterium Acinetobacter tartarogenes (46) and \( R. \) viridis (42). As a first step in an evolutionary analysis of HSS, we cloned the \( hss \) orthologue of the \( \alpha \)-proteobacterium \( B. \) japonicum to compare the kinetic behavior of the recombinant enzyme with that of HSS activity reported for purified extracts from \( A. \) tartarogenes and \( R. \) viridis. We chose \( B. \) japonicum because homospermidine formation has been detected in cell-free extracts from this species (47). Fig. 1B shows the polyamine profile of \( B. \) japonicum at stationary phase, and for comparison, another \( \alpha \)-proteobacterium \( R. \) leguminosarum (Fig. 1C), both grown in defined minimal medium (rich medium contains substantial amounts of putrescine and spermidine). Both species contain homospermidine as their only triamine, i.e. no spermidine or norspermidine, and each contains the homospermidine precursor putrescine. The \( hss \) orthologue of \( B. \) japonicum (GenBank: NP_774402) was cloned and expressed in \( E. \) coli. Purified recombinant N-terminally, T7-tagged protein was assayed with putrescine as substrate, and the product homospermidine was quantified by fluorescence HPLC. Table 1 shows the kinetic behavior of the recombinant \( B. \) japonicum HSS when compared with published HSS activity (42, 46) from purified extracts of \( A. \) tartarogenes and \( R. \) viridis. The overall catalytic efficiencies of the \( B. \) japonicum recombinant HSS and the endogenous \( A. \) tartarogenes HSS are \( \approx 5 \)-fold higher than the \( R. \) viridis enzyme. This is the result of a lower \( K_m \) in the case of the \( B. \) japonicum enzyme and a higher \( K_{cat} \) in the case of the \( A. \) tartarogenes enzyme.

Having validated the enzymatic activity of the \( B. \) japonicum HSS, we used the amino acid sequence of this protein to search available genomes for HSS orthologues using PBLAST. In addition, we searched ESTs by TBLASTN. A neighbor-joining phylogenetic tree of HSS orthologues is shown in Fig. 2 (the alignment on which the tree is based is shown in supplemental Fig. S1). The most conspicuous aspect of the phylogenetic distribution of HSS is that it is mainly represented in the \( \alpha \)-Proteobacteria. Representative \( \alpha \)-proteobacterial HSS orthologues are depicted in Fig. 2, but in total, there are \( hss \) genes in 69 sequenced \( \alpha \)-proteobacterial genomes.

Phylogenetically Distant HSS Orthologues Synthesize Homospermidine in \( E. \) coli—As little as 26% sequence identity is shared by distant HSS orthologues, so it is important that credible biochemical proof is provided of their corresponding HSS
enzymatic activity. Six of the HSS orthologues represented in Fig. 2 were expressed in E. coli, a species that does not synthesize homospermidine. The hss genes chosen were from the /H9251-Proteobacterium B. japonicum, a nitrogen-fixing symbiont of soybean (48); O. terrae (49), an obligate bacterial anaerobe from rice paddy fields and belonging to the diverse Verrucomicrobia phylum; the GOS marine metagenome; Ralstonia phage RSL1; the eukaryotic amitochondriate heterolobosean amoeba S. marylandensis (50); and the eukaryotic, single-celled, ciliate P. tetraurelia (51). Except for the B. japonicum hss orthologue, which was cloned by PCR from genomic DNA, all other HSS genes were synthesized with E. coli-optimized codons. Gene synthesis was essential in the case of the P. tetraurelia hss gene because ciliates use a different genetic code, and in the case of the GOS marine metagenome sequence, because its genomic provenance is unknown. The host E. coli cells without the recombinant HSS accumulate putrescine, cadaverine, and spermidine (Fig. 3, black peaks), and expression of each of the six hss orthologues in E. coli resulted in accumulation of homospermidine in the host cells (Fig. 3, peak f). Some of the HSS orthologues also produced an accumulation of diamino propane (Fig. 3, peak a: B. japonicum, Ralstonia phage RSL1, and O. terrae), and all except P. tetraurelia produced an additional peak of unknown identity (Fig. 3, peak g).

The unknown peak was analyzed by LC-MS/MS (supplemental Fig. S2) and was determined to be N1-aminobutylicadaverine; identity of the homospermidine peak (Fig. 3, peak f) was also confirmed by LC-MS/MS (supplemental Fig. S2). This is the first demonstration of the heterogeneous biosynthesis of N1-aminobutylicadaverine in vivo. It is also the first demonstration in vivo of the production of diamino propane from spermidine by HSS (see below).

Cells of B. japonicum do not synthesize cadaverine, so the HSS co-product N1-aminobutylicadaverine is not detected those cells (Fig. 1B). The production of the HSS co-product diamino propane and N1-aminobutylicadaverine was previously shown in vitro with purified cellular extracts of R. viridis (42). Böttcher et al. (42) proposed a two-site mechanism for catalysis by bacterial HSS. The substrate putrescine (or spermidine) binds “site A,” where it is then oxidatively deaminated by an NAD+-dependent mechanism,

| TABLE 1 |
| --- |
| **Comparison of kinetic parameters of homospermidine synthase activities** |
| For the B. japonicum recombinant HSS enzyme, homospermidine product formation was monitored by HPLC using both benzoylation and detection by UV and AccQ-Fluor reagent and detection by fluorescence. A minimum of three assays was performed for each enzyme preparation. |
|   | V_max | k_cat | K_m | K_cat/K_m | Optimum pH |
| --- | --- | --- | --- | --- | --- |
| B. japonicum recombinant HSS | 1.4 ± 0.13 | 1.18 ± 0.12 | 0.05 ± 0.02 | 23,600 ± 12,000 | 8.4 |
| R. viridis HSS | 1.32 | 1.2 | 0.26 | 4,600 | 8.8 |
| A. tartarogenes HSS | 8.8 | 8 | 0.28 | 28,600 | 8.7 |

**FIGURE 2.** Neighbor-joining tree of HSS orthologues. The unrooted tree was built using PAUP* (as described in Lee et al. (45)) with percentage values from 1000 bootstrap replicates indicated; values less than 50% are not shown. The alignment on which the tree is based is presented in supplemental Fig. S1. Only representative /H9251-Proteobacteria are shown (a more complete representation is shown in supplemental Fig. S2). Orthologues with four red asterisks were characterized in this study, and those with one asterisk were characterized previously.
and ammonia is released with putrescine or dianaminopropionate as substrates, respectively. The resulting semialdehyde reacts with a second amine (putrescine or cadaverine) bound in “site B” to produce an imine intermediate that is reduced by NADH to release either homospermidine or N₁-aminobutylcadaverine. Thus, because the E. coli expression host used in our studies contains putrescine, cadaverine, and spermidine, expression of the various HSS enzymes in E. coli produced diaminopropionate or ammonia as a co-product when spermidine or putrescine was a substrate in site A and homospermidine or N₁-aminobutylcadaverine when putrescine or cadaverine was the co-substrate in site B.

Vertical and Horizontal Transfer of HSS—It is only in the α-Proteobacteria that there seems to be extensive vertical inheritance of hss. A more complete representation of the α-proteobacterial HSS orthologues is shown in the neighbor-joining tree depicted in supplemental Fig. S3. The HSS orthologues from the Rhodospirillales order are ancestral to the Rhodobacterales and Rhizobiales orders in this tree, which is the evolutionary relationship of those orders within the α-Proteobacteria (52, 53). There are no HSS orthologues in the most basal α-proteobacterial order, the Rickettsiales, which are mainly represented by species with reduced genomes. There are also no HSS orthologues in the Sphingomonadales and Caulobacteriales orders, which diverged after the hss-containing Rhodospirillales (52, 53), suggesting hss gene loss in the ancestor of those orders.

Outside of the α-Proteobacteria, the distribution of hss orthologues in bacteria is sporadic and limited. There are a few β- and γ-proteobacterial species containing hss orthologues and one species each of the Verrucomicrobia, O. terrae (Figs. 2 and 3), and of the Chlorobi, Pelodictyon phaeoclatratiforme. Key bacterial pathogens possess hss orthologues including Legionella pneumophila, which was shown previously to contain homospermidine (33). Other pathogens with an hss orthologue include Brucella species (causative agent of brucellosis), Pseudomonas aeruginosa, and the plant pathogen Pseudomonas syringae.

Four closely related methanomicrobical species of the archaea contain hss, coincident with the known presence of homospermidine in methanogens (34) and high rates of horizontal gene transfer (54). An hss orthologue (Figs. 2 and 3) is present in the 240-kbp linear double-stranded DNA genome of the Myovirus-type phage φRSL1 that infects the soil-borne β-proteobacterial plant pathogen Ralstonia solanacearum. There is no hss orthologue in the genome of R. solanacearum, and the phage protein exhibits only 37% identity with the HSS of Ralstonia eutropha. The recently completed φRSL1 genome also contains an orthologue of glutathionylspermidine synthetase (55). This is not the first case of a polyamine biosynthetic gene being found in a phage genome. Orthologues of the gene encoding S-adenosylmethionine decarboxylase are found in phage of cyanobacteria and Thermus thermophilus (56). Also shown in Figs. 2 and 3 is an HSS orthologue (GenBank:
ECW30472) from the GOS marine metagenome (57), which possesses 69% identity with the closest match from sequenced genomes: an HSS from the verrucomicrobium O. terrae. All of the putatively full-length HSS orthologous sequences in the GOS marine metagenome exhibit at least 50% identity to this sequence. It is notable that the sequenced genomes of Pelagibacter ubique, Prochlorococcus species, and Synechococcus species do not contain hss orthologues, and ~50% of the GOS marine metagenome sequence that can be recruited to known genomes belongs to these three groups (57).

Orthologues of hss are present in each of six chlorovirus genomes. Chloroviruses are large double-stranded DNA viruses (e.g. P. bursaria Chlorella virus-1 (PCBV-1) has a 331-kbp genome) that infect single-celled eukaryotes such as the green alga Chlorella, an endosymbiont of the ciliate P. bursaria. The presence of hss in the chloroviral genomes is intriguing because these genomes also contain a biosynthetic pathway consisting of an unusual arginine decarboxylase (58), an agmatine deiminase and N-carbamoylputrescine amidohydrolase (59), that together produce putrescine from arginine. An HSS orthologue from PCBV-1 was shown previously to be functional, producing homospermidine from two molecules of putrescine (39). The evolutionary origin of the chloroviral hss is uncertain. The algal host Chlorella seems an unlikely source because no plant or algal species is known to possess an hss orthologue including the draft genome sequences of Chlorella sp. NC64A and Chlorella vulgaris C-169 (JGI Genome Portal). Chloroviral genomes are from viruses that infect the Chlorella endosymbiont of the bacteriovorus single-celled eukaryote P. bursaria; the genome of P. tetraurelia contains four paralogous copies of hss (one of these paralogues is shown in Figs. 2 and 3). If the Paramecium cell was the source of the hss gene in the chlorovirus genome, the genetic code of the gene would have to be reprogrammed. Ciliates such as Paramecium use a different genetic code (60) where the TAA and TAG stop codons encode glutamine, whereas chloroviruses use the normal genetic code.

Multiple ESTs with similarity to HSS were detected among sequences from the amitochondriate bacteriovorus heterolobosean amoeba S. marylandensis. An intact ORF for the S. marylandensis HSS orthologue was assembled from overlapping ESTs and is indicated in Figs. 2 and 3. This is another probable case of hss acquisition by horizontal gene transfer from a bacterium to a bacteriovorus eukaryote. We were able to assemble most of an HSS orthologue from the bacteriovorus slime mold Physarum polycephalum (supplemental Fig. S4). Homospermidine was detected previously in axenically grown cultures of P. polycephalum (35), providing persuasive evidence that the P. polycephalum hss orthologue is functional. The closest matches to the P. polycephalum hss orthologue are shown in Fig. S4. Studies of homospermidine synthase activity of recombinant hss orthologues from other organisms (supplemental Fig. S4) exhibit 71% identity with the α-proteobacterium Azorhizobium caulinodans and slightly less identity with other members of the Rhizobiales order. Such a high degree of sequence identity suggests a recent acquisition of the hss gene by M. ovata supported by the fact that the completed genome
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sequence is not a bacterial contaminant. Recently a bacterial sequence missing, suggesting aberrant mRNA processing. Taken together, this evidence suggests that the M. ovata hss orthologue. The 72-bp 5′-untranslated region and 106-bp 3′-untranslated region of the M. ovata hss mRNA have no sequence similarity to any bacterial sequence. In addition, three ESTs have portions of the HSS sequence missing, suggesting aberrant mRNA processing. Taken together, this evidence suggests that the M. ovata hss sequence is not a bacterial contaminant. Recently a bacterial spermidine in E. coli (65) and Bacillus subtilis (66). The presence of N³-acetylhomospermidine has been detected in *Vibrio parahaemolyticus* (67) but so far there have been no reports on the presence of acetylhomospermidine. There are orthologues of glutathionylspermidine synthetase (68) adjacent to HSS in the β-Proteobacteria *Cupriavidus taiwanensis* and R. eutropha (Fig. 4), suggesting that the encoded proteins may glutathionylate homospermidine. Only glutathionyl-
spermidine has been detected in bacteria, and its function is elusive (69).

Four species of bacteria contain paralogous pairs of hss genes. The two α-Proteobacteria Methylobacterium extorquens DM4 and Methylobacterium chloromethanicum CM4 contain HSS paralogues with 97 and 96% identity of the encoded proteins, respectively, and in the case of M. chloromethanicum CM4, one of the paralogues is located on a plasmid. The verrucomicrobium O. terrae and the β-proteobacterium C. taiwanensis contain HSS paralogous pairs with only 48 and 45% identity, respectively. Such a low level of identity between the paralogues of each HSS pair in O. terrae and C. taiwanensis obviously suggests different origins of each gene. However, the GC content of R. leguminosarum from cultures of R. eutropha H16, 0.03 nmol of CO₂/h respectively. Such a low level of identity between the paralogues 97 and 96% identity (Fig. 4). Both C. taiwanensis and R. eutropha are part of the Burkholderiaceae, whereas V. paradoxus is a member of the Comamonaceae. Together these data suggest that at least one of the C. taiwanensis hss paralogues is a species of the Burkholderiaceae, whereas V. paradoxus is a member of the Comamonaceae. Together these data suggest that at least one of the C. taiwanensis hss paralogues is part of a cluster/operon identical to that in the β-proteobacterium Variovorax paradoxus S110 (with the HSS protein sequences being 64% identical) and the other C. taiwanensis hss parologue is part of a cluster/operon similar to that found in the β-proteobacterium R. eutropha H16, with the HSS protein sequences being 88% identical (Fig. 4).

Homospermidine Is Required for Normal Growth in the α-Proteobacterium R. leguminosarum—An analysis of genomes containing hss orthologues reveals that in some bacterial species, the only route for putrescine biosynthesis is a eukaryotic-like alanine racemase fold ODC. The α-proteobacterium R. leguminosarum is such a species and, as shown in Fig. 1C, it contains only diaminopropane, putrescine, and homospermidine. In principle, inhibition of the ODC enzyme in R. leguminosarum should decrease putrescine content and, as a consequence, homospermidine levels. The specific, substrate-based suicide inhibitor of the alanine racemase fold ODC, α-difluoromethylornithine (α-DFMO) (70), was added to R. leguminosarum defined minimal growth medium. Cellular extracts from cultures of R. leguminosarum grown aerobically in the presence of 1.0 mM α-DFMO exhibited a specific ODC activity of 1.82 ± 0.03 nmol of CO₂/h⁻¹ mg⁻¹ of protein when compared with 4.29 ± 0.01 nmol of CO₂/h⁻¹ mg⁻¹ of protein for the control cultures, a more than 2-fold decrease in ODC activity caused by 1.0 mM α-DFMO. Growth of R. leguminosarum cells in increasing concentrations of α-DFMO resulted in correspondingly decreased growth rates (Fig. 5A), and concentrations of 5 mM or more of α-DFMO decreased growth by up to 4-fold. With concentrations of α-DFMO of more than 5 mM, there was a diminution of growth rate decrease with higher concentrations, so 5 mM α-DFMO was used for subsequent experiments. The polyamine content of R. leguminosarum cells grown aerobically with or without 5 mM α-DFMO is shown in Fig. 5B. There is a more than 10-fold decrease in putrescine and homospermidine content in the presence of the inhibitor after both 24 h and 48 h of growth. The 2–3-fold decrease in diamino- propane content may be due to changes in growth rate as diaminopropane concentration should not be directly affected by α-DFMO inhibition of ODC activity. To verify whether the decrease in growth rate caused by α-DFMO was due to polyamine depletion rather than an off-target effect, R. leguminosarum cells were grown aerobically in 5 mM α-DFMO-containing medium supplemented with 1 mM putrescine. As can be seen in Fig. 5C, putrescine restores growth of α-DFMO-treated cells almost completely, confirming that the effect of α-DFMO on cell growth is due to polyamine depletion. This is the first report to show that α-DFMO can inhibit bacterial growth, and the finding has interesting implications for the potential effect of α-DFMO on bacterial pathogens that contain only the eukaryotic-like alanine racemase fold ODC-encoding gene for putrescine biosynthesis. Such pathogens include the oral pathogen Treponema denticola; the agent of Q fever, Coxiella burnetii; Brucella species, which cause brucellosis, and Bartonella species, which cause diseases such as cat scratch fever. In principle, growth of each of these pathogens should be susceptible to inhibition by α-DFMO, and possibility there may be antibacterial potential for this anticancer (71), antitrypanosomal (72) drug that is clinically approved and used in humans. It should be
noted that α-DFMO is not active against the aspartate aminotransferase fold ODC found in *E. coli* (73).

The diamine cadaverine was much less effective in restoring growth of cells grown in α-DFMO (Fig. 5C), although cadaverine was accumulated in cells (Fig. 5D). No aminobutylcadaverine was detected in these cells, probably because putrescine was so effectively depleted by α-DFMO (Fig. 5D). Surprisingly, spermidine was able to restore growth of the α-DFMO-treated cells almost as well as putrescine (Fig. 5C), which may have been due to the observed production of homospermidine from spermidine by the HSS enzyme (Fig. 5D). It has been shown *in vitro* that HSS is able to produce homospermidine, putrescine, and diaminopropane from spermidine (42). Norspermidine was less effective in this experiment than spermidine in restoring growth of α-DFMO-treated cells, and no other polyamines were produced from norspermidine. The effect of the triamines norspermidine, spermidine, and homospermidine on restoring growth of α-DFMO-treated *R. leguminosarum* cells was directly compared. Growth was again inhibited by 5 mM α-DFMO, and each triamine, including norspermidine, restored growth to a similar level (Fig. 5F) and was accumulated in cells to a similar level (Fig. 5F). As before, spermidine was converted by the HSS into homospermidine, putrescine, and diaminopropane, but homospermidine and norspermidine were not further metabolized (Fig. 5F). To directly compare the effects of exogenous spermidine and homospermidine on growth in *R. leguminosarum*, it will be necessary to have a gene deletion of *hss* in this species so that spermidine is not metabolized to homospermidine by HSS. The ability of norspermidine, spermidine, or homospermidine to restore growth of α-DFMO-treated cells indicates that the exact structure of the triamine is not critical for growth. In contrast to *R. leguminosarum*, complete depletion of polyamines in aerobically grown *E. coli* had little or no effect on growth (74).

**Evolution of Homospermidine Synthase**—A large number of related bacterial protein sequences exhibit a low level of sequence similarity to HSS and are annotated as saccharopine dehydrogenases. Closer inspection of these sequences reveals that they are orthologues of CANSDH, an enzyme involved in an alternative norspermidine/spermidine biosynthetic pathway in diverse bacteria (24). The sequence similarity is highest in the N-terminal half but extends throughout the proteins (supplemental Fig. S5). Although it is not immediately obvious why HSS should be related to CANSDH, the substrates, products, and proposed reaction mechanisms of the two enzymes are similar. In the case of CANSDH, L-aspartic-α-semialdehyde forms a Schiff base with either dianinopropane or putrescine, and in an NADPH-dependent step, carboxynorspermidine is formed with dianinopropane, or carboxyspermidine is formed with putrescine, with the release of a water molecule in both cases (75). The proposed intermediate imine structures for 1) HSS (42) and 2) CANSDH with putrescine (75) are: 1) H2N(CH2)4nCH(CH2)3NH2 and 2) H2N(CH2)4nCHCH2CH(NH2)COOH.

The similar substrates, products, reaction mechanisms, and sequence similarity indicate common descent for the genes encoding HSS and CANSDH. The carboxytriamine products produced by CANSDH require another enzyme, CANSDC, to produce the final product, spermidine or norspermidine (24). The more parsimonious evolutionary sequence of events would be that HSS evolved from CANSDH because evolution of CANSDH from HSS would require the simultaneous evolution of CANSDC.
A crystal structure of the _L. pneumophila_ HSS has been determined to 2.5 Å by the Northeast Structural Genomics Consortium (Protein Data Bank (PDB): 2ph5). The HSS is a dimer in the crystal structure with each monomer consisting of two domains (Fig. 6), an NAD\(^+\) binding Rossmann fold, and an inserted mixed β-sheet belonging to the glyceraldehyde 3-phosphate dehydrogenase-like C-terminal domain (GAPDH-like) superfamily. Four regions highly conserved between HSS and CANSDH are indicated on the HSS structure in Fig. 6, _upper panel_ (colored blue), and each of the conserved motifs interacts with the NAD\(^+\) cofactor, which is located in a deep cleft between the two domains (Fig. 6, _lower panel_). The nicotinamide ring of NAD\(^+\) is buried deep in the cleft and likely marks the active site (Fig. 6, _upper panel_). Although no structure is available for CANSDH, other structures closely resemble HSS (Fig. 7), including lysine 6-dehydrogenase (PDB: 22ZV) from the euryarchaeote _Pyrococcus horikoshii_ and saccharopine reductase (PDB: 1e5q) from the fungus _Magnaporthe grisea_. A minimal evolutionary core of these structures is represented by the more distantly related aspartate dehydrogenase fold (PDB: 2dc1), which includes both the NAD(P)-binding Rossmann-like domain and the more unique inserted GAPDH-like domain (Fig. 7A). The HSS (Fig. 7B), lysine 6-dehydrogenase (Fig. 7C), and saccharopine reductase (Fig. 7D) exhibit increasing elaborations of similar secondary structure elements relative to the aspartate dehydrogenase core. Within the unique inserted domain, each elaborated fold includes similar helical inserts, a β-hairpin that covers the face of the sheet, and an edge β-strand extended from the C terminus of the Rossmann-like domain. NAD\(^+\)/NADP is shown bound to the Rossmann domain in each structure. In the case of saccharopine reductase, the substrates are glutamate and 1,2-aminoiso-6-semialdehyde, which form a Schiff base intermediate that is reduced by NADPH to saccharopine (76). Lysine 6-dehydrogenase catalyzes the oxidative deamination of the ε-amino group of lysine in the presence of NAD\(^+\) to form 1,2-aminoiso-6-semialdehyde (77).

The core evolutionary fold of HSS/CANSDH is found in other enzyme structures, most of which participate in metabolic pathways surrounding aspartate (supplemental Fig. S6). Minimal core fold representative aspartate dehydrogenase serves as the first step in _de novo_ NAD biosynthesis from aspartate (Fig. 8), whereas a similar GAPDH-like fold representative, homoserine dehydrogenase (PDB: 1ebf), leads to the biosynthesis of threonine or methionine from aspartate β-semialdehyde. The diaminopimelate pathway for lysine biosynthesis from aspartate includes two additional GAPDH-like enzymes, aspartate β-semialdehyde dehydrogenase (PDB: 1brm) and dihydrodipicolinate reductase (PDB: 1arz). Close sequence homologues of aspartate β-semialdehyde dehydrogenase fall within similar metabolic pathways (Fig. 8); one belongs to an alternative lysine biosynthetic pathway from aminoacidopate (N-acetyl-γ-aminoacipil-phosphate reductase), whereas the other participates in arginine biosynthesis (N-acetyl-γ-glutamylphosphate reductase (PDB: 1xyg)). Indeed, the four main genes belonging to the two pathways to lysine biosynthesis (diaminopimelate and α-aminoacidopate pathways) and the pathway to arginine biosynthesis are thought to have evolved from paralogous duplications followed by evolutionary divergence (78). Of note, the close HSS homologue, CANSDH, brings together a side product of arginine biosynthesis (putrescine) with the intermediate common to the lysine (diaminopimelate pathway), methionine, and threonine biosynthetic pathways (aspartate β-semialdehyde) to form spermidine. The remaining close homologues of HSS, lysine 6-dehydrogenase and saccharopine reductase, are involved in a reversible pathway of lysine degradation to aminoacidopate. Recurrence of the HSS-like enzymes in the aspartate and arginine family pathways is shown in Fig. 8. Structural relatedness of the HSS-like enzymes is depicted in a phylogenetic tree of structures in supplemental Fig. S6. It thus seems highly probable that HSS and carboxyspermidine dehydrogenase evolved from the aspartate family of amino acid bio-

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**FIGURE 8. Recurrence of the GAPDH-like fold in the aspartate, arginine, and polylamine pathways.** The metabolic routes for lysine (diaminopimelate and α-aminoacidopate pathway) and arginine biosynthesis are represented along with bifurcating pathways that stem from various metabolic intermediates (NAD biosynthesis from aspartate, threonine/methionine biosynthesis from aspartate β-semialdehyde, and polylamine biosynthesis from putrescine/aspartate β-semialdehyde). Enzymes with similar folds are indicated with similarly colored circles, and pathways with similar consecutive enzymes that are presumed to stem from a common ancestral pathway (78) are boxed in dashed lines. Genes encoding GAPDH-like fold enzymes are labeled in boxes (red and green) and are therefore not numbered. The abbreviations are: argC, N-acetyl-γ-glutamylphosphate reductase; AspDH, aspartate dehydrogenase; hom, homoserine dehydrogenase; dap8, dihydrodipicolinate reductase; lysY, N-acetyl-α-aminoacidopate semialdehyde dehydrogenase; LysDH, L-lysine dehydrogenase; dsh, saccharopine dehydrogenase.
Evolution of sym-Homospermidine Biosynthesis

An Alternative Homospermidine Biosynthetic Pathway—As discussed above, hss orthologues are absent from the Caudobacterales order of the α-Proteobacteria. We grew *C. crescentus* in a defined minimal growth medium and found that homospermidine was the only detectable polyamine in these cells (Fig. 9A). Similarly, we grew the photosynthetic filamentous cyanobacterium *A. variabilis* in defined minimal growth medium, and again the only detectable polyamine was homospermidine (Fig. 9B). Neither *C. crescentus* nor *A. variabilis* possess an hss orthologue. It has been amply demonstrated (and discussed above) that members of the Cyanobacteria, Chloroflexi, Chlorobi, δ-Proteobacteria, Bacteroidetes, Acidobacteria, and Planctomycetes contain homospermidine. None of these phyla contain an hss orthologue (except for *P. phaeoclasti-ratiforme* in the Chlorobi). The enzyme deoxyhypusynusine synthase (DHS) transfers an aminobutyl group from spermidine to a lysine residue in eukaryotic translation initiation factor eIF5A (79) to form the unique residue deoxyhypusine. DHS is also able, as a side reaction, to transfer an aminobutyl group to putrescine to form homospermidine (80). The plant homospermidine synthase, which is used solely for pyrrolizidine alkaloid biosynthesis, is unrelated to DHS and has evolved from DHS by convergent evolution of homospermidine biosynthesis. This is analogous to the duplication of the plant dhs gene and subsequent evolution of one parologue to retain only the innate homospermidine synthase activity.

Conclusion—The polyamine biosynthetic pathway exemplifies the importance of gene duplication and horizontal gene transfer in the generation of biosynthetic diversity. It also highlights how biosynthetic diversification can be buffered by product convergence, likely due to evolutionary selection for a constrained range of biosynthetic product structures.

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