Evolution and Multiplicity of Arginine Decarboxylases in Polyamine Biosynthesis and Essential Role in Bacillus subtilis Biofilm Formation

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Arginine decarboxylases (ADCs; EC 4.1.1.19) from four different protein fold families are important for polyamine biosynthesis in bacteria, archaea, and plants. Biosynthetic alanine racemase fold (AR-fold) ADC is widespread in bacteria and plants. We report the discovery and characterization of an ancestral form of the AR-fold ADC in the bacterial Chloroflexi and Bacteroidetes phyla. The ancestral AR-fold ADC lacks a large insertion found in Escherichia coli and plant AR-fold ADC and is more similar to the lysine biosynthetic enzyme meso-diaminopimelate decarboxylase, from which it has evolved. An E. coli acid-inducible ADC belonging to the aspartate aminotransferase fold (AAT-fold) is involved in acid resistance but not polyamine biosynthesis. We report here that the acid-inducible AAT-fold ADC has evolved from a shorter, ancestral biosynthetic AAT-fold ADC by fusion of a response regulator receiver domain protein to the N terminus. Ancestral biosynthetic AAT-fold ADC appears to be limited to firmicute bacteria. The phylogenetic distribution of different forms of ADC distinguishes bacteria from archaea, euryarchaeota from crenarchaeota, double-membraned from single-membraned bacteria, and firmicutes from actinobacteria. Our findings extend to eight the different enzyme forms carrying out the activity described by EC 4.1.1.19. ADC gene clustering reveals that polyamine biosynthesis employs diverse and exchangeable synthetic modules. We show that in Bacillus subtilis, ADC and polyamines are essential for biofilm formation, and this appears to be an ancient, evolutionarily conserved function of polyamines in bacteria. Also of relevance to human health, we found that arginine decarboxylation is the dominant pathway for polyamine biosynthesis in human gut microbiota.

The evolution of central metabolic pathways was one of the key biochemical developments in the early stages of primordial life (1). Metabolic pathways evolve through mechanisms such as gene duplication followed by neofunctionalization of one of the copies to produce a new enzyme with an altered substrate preference (2–4). Other mechanisms involved in the evolution of metabolic pathways include gene fusions, which can be fusion of two paralogous genes to achieve gene elongation. An example is the formation of eukaryotic S-adenosylmethionine decarboxylase (AdoMetDC),5 a polyamine biosynthetic enzyme, by fusion of two prokaryotic AdoMetDC genes (5). Gene fusions may bring together different protein functional modules such as regulatory and enzymatic components and are often generated independently in different lineages by fusion of open reading frames within operons (6). New steps in metabolic pathways can be recruited from preexisting pathways in the same cell (the “patchwork” model of pathway evolution) (7) or from other organisms by horizontal or endosymbiotic gene transfer. Convergent evolution of the same enzymatic activity in different organisms by completely different, evolutionarily unrelated proteins (recently described as non-homologous isofunctional enzymes) (8) is a result of independent biochemical invention (9).

The biosynthesis of polyamines is an excellent model for studying processes of metabolic pathway evolution. Polyamines are small organic polycations, usually linear di-, tri-, and tetra-amines, that are found in almost all cells. Synthesis of polyamines is achieved through biosynthetic modules: a diamine is produced directly or indirectly from an amino acid, and a triamine is produced from the diamine by the addition of an aminopropyl or aminobutyl group. Tetra-amines are produced from triamines by aminopropyl or more rarely by aminobutyl group addition. The modular nature of polyamine biosynthesis in bacteria, archaea, and plants is an excellent model for studying processes of metabolic pathway evolution. Polyamines are small organic polycations, usually linear di-, tri-, and tetra-amines, that are found in almost all cells. Synthesis of polyamines is achieved through biosynthetic modules: a diamine is produced directly or indirectly from an amino acid, and a triamine is produced from the diamine by the addition of an aminopropyl or aminobutyl group. Tetra-amines are produced from triamines by aminopropyl or more rarely by aminobutyl group addition. The modular nature of polyamine biosynthesis...
biosynthesis facilitates horizontal and endosymbiotic transfer (10).

By far, in all three domains of life, the most common diamine is putrescine (1,4-diaminobutane), which can be used to form spermidine (by aminopropyl addition) or sym-homospermidine (by aminobutyl addition). Almost all eukaryotes synthesize putrescine directly from ornithine by the action of ornithine decarboxylase (ODC). However, plants possess an additional, indirect pathway for putrescine biosynthesis from arginine, through the action of arginine decarboxylase (ADC) (11). It is unclear which bacteria use the ADC route to synthesize putrescine, and the relative importance of the ODC and ADC routes in bacterial polyamine biosynthesis is also unclear. The product of ADC, arginine, may be converted directly to putrescine by arginine ureohydrolase (AUH) (12) or indirectly via N-carbamoylputrescine by arginine deiminase/iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (NCPAH) (13).

There are currently four known protein fold types containing an ADC. The alanine racemase fold (AR-fold) includes solved structures of the eukaryotic ODC from human and Trypanosoma brucei (14, 15); bifunctional lysine/ornithine decarboxylase (LODC) from Vibrio vulnificus (16); meso-diaminopimelate decarboxylase (DAPDC) from Methanocaldococcoides jannaschii, Mycobacterium tuberculosis, and Helicobacter pylori (17–19); and carboxy(nor)spermidine decarboxylase (CNSDC) and ADC (20). The AR-fold ADC is substantially longer than the other members of the AR-fold basic amino acid decarboxylases (16), due to a large insertion relative to other members of the family. Acid-inducible ADC of Escherichia coli, which is not involved in polyamine biosynthesis, belongs to the aspartate aminotransferase fold (AAT-fold), which also includes the E. coli and Lactobacillus 30a biosynthetic ADC and also bacterial acid-inducible lysine decarboxylases. A pyruvoyl-dependent ADC (pylADC) is present in M. jannaschii and most of the euryarchaeaota (21), whereas Sulfolobus solfataricus and most of the crenarchaeaota possess an ADC that has recently evolved from the pyruvoyl-dependent AdoMetDC (22). An acid-dependent pylADC, probably not involved in polyamine biosynthesis, is present in Chlamyphila species (23). In addition, large algal viruses, such as Paramecium bursaria chlorella virus-1, possess an AR-fold ADC that highly resembles and has recently evolved from an AR-fold ODC (24, 25). Whatever the form of ADC enzyme encoded, in bacteria, ADC is encoded by the speA gene.

Although polyamines are primordial constituents of life, it has been difficult to elucidate their physiological roles. In eukaryotes and archaea, spermidine is required for hypusine formation, which is essential for cell growth in eukaryotes and archaea (26, 27). Furthermore, in archaea, it was shown recently that arginine is required for an essential modification of a cytidine nucleoside in a RNA nucleoside (to produce the modified nucleoside argamidine) required for decoding the AUA triplet (28, 29). A number of studies have concluded that the main physiological role of spermidine is in hypusine formation for elf5a function, both in the yeast Saccharomyces cerevisiae (30, 31) and in mammalian cells (32, 33). Nevertheless, there are also studies demonstrating that polyamines affect cellular function independently of hypusine formation (34, 35). The essential requirements for spermidine and agmatine in hypusine and agmatidine formation, respectively, obscure other core physiological functions of polyamines in eukaryotes and archaea. Thus, bacteria, which lack both hypusine and agmatidine modifications, should present a more transparent model for determining physiological functions of polyamines. However, aerobic growth of E. coli is barely affected by complete poliamine depletion (36). In Yersinia pestis and Vibrio cholerae, depletion of either all polyamines or of triamines, respectively, resulted in a slight reduction (40%) of aerobic growth of planktonic cells (37, 38). In contrast, poliamine depletion abolished aerobic growth of Pseudomonas aeruginosa PAO1 cells (13) and significantly inhibited growth of Rhizobium leguminosarum (10). Although polyamine depletion had relatively little effect on growth of planktonic Y. pestis and V. cholerae cells, poliamine depletion in both species abolished biofilm production (37, 38). Biofilm formation is thus a key physiological process where polyamines are required independently of hypusine or agmatidine formation, at least in two species of γ-Proteobacteria.

Here we demonstrate that an ancestral form of the AR-fold ADC exists in species of the Chloroflexi (green non-sulfur bacteria) and Bacteriodetes phyla. The ancestral AR-fold ADC lacks the characteristic insertion of the longer AR-fold ADCs, which is found in most phyla of the double-membraned bacteria. We also show that the firmicute AAT-fold biosynthetic ADC is an ancestral form of the acid-inducible ADC found in E. coli and lacks the N-terminal wing domain necessary for decamer formation. We found that the N-terminal wing domain of the acid-inducible AAT-fold ADC is derived from a response regulator protein receiver (REC) domain that has fused to the N terminus of a biosynthetic AAT-fold ADC-like protein. The Bacillus subtilis biosynthetic AAT-fold ADC is shown to be essential for biofilm formation, establishing that polyamines are involved in biofilm formation in single-membranated as well as doubled-membranated bacteria. This indicates that a role in biofilm formation may be an ancient physiological function of polyamines in bacteria. We map the phylogenetic distribution of the different ADC enzymes and show that there is a clear division between double-membranated and single-membranated prokaryotes and between single-membranated bacteria and archaea. Finally, we show that the ADC route for putrescine biosynthesis is the dominant pathway for polyamine formation in the human gut microbiota.

EXPERIMENTAL PROCEDURES
Materials—All materials were of the highest grade available and were purchased from Sigma unless otherwise stated. L-[1-14C]Ornithine hydrochloride (57.1 mCi/mm mol), L-[U-14C]Arginine monohydrochloride (346 mCi/mm mol), and L-[U-14C]Lysine monohydrochloride (320 mCi/mm mol) were purchased from PerkinElmer Life Sciences.

Bacterial Strains and Growth Conditions—E. coli XL2 Blue and BL21 (DE3) pLysS were purchased from Stratagene and Novagen, respectively. B. subtilis 168 was a kind gift from Dr.
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Stephan Bornemann (John Innes Centre), and B. subtilis genomic DNA was prepared using standard procedures. The original B. subtilis 168 ΔspeA (BSP 7010) and ΔyaaO knock-out strains were a kind gift of Drs. Agnieszka Sekowska and Antoine Danchin (Institut Pasteur; the ΔyaaO gene knock-out strain was originally made by Dr. Asai Kei). Strains of B. subtilis were grown routinely in Luria-Bertani (39) medium (10 g/liter NaCl, 5 g/liter yeast extract, and 10 g/liter tryptone) at 37 °C, unless otherwise stated. Where appropriate, MSgg medium (5 mM potassium phosphate and 100 mM MOPS at pH 7.0 supplemented with 2 mM MgCl2, 700 μM CaCl2, 50 μM MnCl2, 50 μM FeCl3, 1 μM ZnCl2, 2 mM thiamine, 0.5% glycerol, 0.5% glutamate) (41) was used for analysis of biofilm formation. The B. subtilis strains used in this study for biofilm analysis were the strain 3610 (prototroph) obtained from the Bacillus Genetic Resource Center and strain NRS3088 (3610 ΔspeA (spc); created in this study). Phage transductions into the B. subtilis strain background NCIB3610 were conducted as described previously (40).

When required, antibiotics were used at the following concentrations: erythromycin (1 μg/ml) with lincomycin (25 μg/ml) and spectinomycin (100 μg/ml). When required, polyamines were added to the MSgg medium at a concentration of 0.5 mM where specified.

Biofilm Image Analysis—Analysis of biofilm formation was performed essentially as described (41, 42). Strains of B. subtilis to be tested were grown to mid-late exponential phase in liquid medium containing 100 μM amines were added to the MSgg medium at a concentration of 0.5 mM where specified.

Cloning and Gene Synthesis—The open reading frame (43) of B. subtilis speA (NP_389346) was amplified by PCR from genomic DNA. Putative speA ORFs from Clostridium difficile 630 (YP_001087362), Chloroflexus auranticus J-10-fl (YP_001634722), and Gramella forsetii KT0803 (YP_863630) were synthesized by Genscript (Piscataway, NJ) with codons optimized for expression in E. coli. The speA ORFs from B. subtilis and C. difficile were subcloned into the BamHI and Xhol sites of pET21a, and those from Chloroflexus aurantius and G. forsetii were subcloned into the Ndel and BamHI sites of pET15b.

Protein Expression and Purification—For expression of putative decarboxylases, E. coli BL21 was transformed with protein expression plasmids and grown to an A600 nm of 0.3 in LB liquid medium containing 100 μg/ml ampicillin at 37 °C. Protein expression was induced with 0.4 mM isopropyl β-D-galactopyranoside, and cultures were incubated for a further 3 h at 37 °C. T7-tagged proteins were purified using the T7-Tag affinity purification kit (Novagen) according to the manufacturer’s instructions. For purification of His-tagged proteins, cells were resuspended in 20 mM sodium phosphate (pH 8.0) containing 500 mM NaCl, 20 mM imidazole, and 0.02% (v/v) Brij35 before being broken by sonication. The cell lysate was cleared by centrifugation and applied to a HiTrapTM chelating HP column (GE Healthcare) that had been charged with Ni2+ and equilibrated with the above buffer. The column was washed with the above buffer, and bound protein was eluted using a 0.02–1 M imidazole gradient. Purified proteins were concentrated using Amicon Ultra-4 centrifugal filter units, buffer-exchanged with 20 mM Tris-HCl (pH 7.5) containing 20% (v/v) glycerol and 2 mM dithiothreitol and stored at −80 °C.

Polyamine Analysis—For HPLC analysis, polyamines were labeled using the AccQ-FlorTM reagent kit (Waters Corp., Milford, MA) according to the manufacturer’s instructions. Labeling reactions contained 5 μl of stopped enzyme assay and 1.25 mM 1,7-diaminoheptane as an internal standard and were heated to 55 °C for 10 min. Derivatized polyamine samples were analyzed by HPLC using a reverse phase C18 column (Luna 5 μ, Phenomenex) on a Dionex Summit HPLC System. Polyamine separation was performed using 10 μl of derivatized sample. The system was operated at 33 °C and equilibrated with Eluent A (70 mM acetic acid, 25 mM triethylamine, pH 4.82) at 1.2 ml/min. Elution was performed using the following linear gradients of Eluent B (80% acetonitrile): 22% for 5 min, 39% for 12 min with 6% methanol, 33% for 30 s with 14% methanol, 10% for 6.5 min with 70% methanol, and finally 100% for 21 min. Polyamines were monitored by fluorescence (Dionex RF 2000 detector) with a 248-nm excitation filter and a 398-nm emission filter and identified by comparison of retention times with known standards that were derivatized and analyzed in parallel with the enzyme assays.

Enzyme Assays—Amino acid decarboxylase activity was measured using a stopped 14CO2 release assay (44). Unless otherwise stated, assays were buffered in 50 mM HEPES (pH 7.5) containing 50 mM NaCl, 2 mM DTT, and 0.1 mM pyridoxal 5’-phosphate (PLP) and contained 3.7 kBq L-[1-14C]ornithine hydrochloride, L-[U-14C]arginine monohydrochloride, or L-[U-14C]lysine monohydrochloride, 0.04–10 mM unlabeled substrate, and 0.1–5 μg of purified protein. Reactions were incubated at 30–80 °C for 10 min before being stopped by the addition of 5% (v/v) trichloroacetic acid, and 14CO2 release was quantified by liquid scintillation counting. For determining the pH optima of enzymes, reactions were performed in the following series of buffers: 50 mM sodium acetate (pH 3.5, 4.5, or 5.5), 50 mM MES (pH 6.5), 50 mM HEPES (pH 7.5), and 50 mM CHES (pH 8.5 or 9.5).

Molecular Modeling—Enzyme structures were presented using the molecular graphics program PyMOL (45).

Bioinformatics Analysis—Sequence alignment and neighbor joining tree building were performed as described previously (16).

RESULTS

An Ancestral Form of the Alanine Racemase Fold of Biosynthetic Arginine Decarboxylase—The biosynthetic AR-fold ADC has an interdomain insertion of between 90 and 105 amino acids compared with other enzymes of the same structural class (16). This insertion is located between the βα-barrel N-terminal domain and the C-terminal β-barrel domain, relative to the known structures of DAPDC, ODC,
AR-fold ADC proteins are usually at least 100 amino acids bigger than other members of the family. The position of the interdomain insertion in the crystal structure of the *V. vulnificus* ADC monomer (20) is shown in Fig. 1A. A four-helix bundle insertion, which is absent in the ancestral form, is depicted at the bottom of the structure in tan, and the rest of the enzyme (which resembles ODC and DAPDC) is shown in dark blue for α-helices and light blue for β-strands. The pyridoxal 5′-phosphate co-factor and agmatine are shown by colored spheres. A is based on the structure determined by Deng et al. (20).

Each monomer is depicted in a different color with the N-terminal wing domains at the center of the doughnut and rendered a lighter colored version of the remainder of the corresponding monomer, the remainder being equivalent to the short biosynthetic AAT-fold ADCs. B is based on the structure determined by Andrell et al. (51).

**FIGURE 1.** Structures of pyridoxal 5′-phosphate-dependent arginine decarboxylases. A, structure of the *V. vulnificus* AR-fold ADC monomer (Protein Data Bank entry 3N2O). The four-helix bundle insertion, which is absent in the ancestral form, is depicted at the bottom of the structure in tan, and the rest of the enzyme (which resembles ODC and DAPDC) is shown in dark blue for α-helices and light blue for β-strands. The pyridoxal 5′-phosphate co-factor and agmatine are shown by colored spheres. A is based on the structure determined by Deng et al. (20). B, structure of one pentamer layer of the decameric AAT-fold acid-inducible ADC of *E. coli* (Protein Data Bank entry 2VYC). Each monomer is depicted in a different color with the N-terminal wing domains at the center of the doughnut and rendered a lighter colored version of the remainder of the corresponding monomer, the remainder being equivalent to the short biosynthetic AAT-fold ADCs. B is based on the structure determined by Andrell et al. (51).

**FIGURE 2.** Neighbor joining tree of short and long form AR-fold ADC, bifunctional L/ODC, ODC, and DAPDC orthologues. Sequences were aligned using ClustalW. The tree was constructed using PAUP*. The numbers represent percentage support derived from 1000 bootstraps. The alignment used to construct the neighbor joining tree is displayed in supplemental Fig. S1.

**FIGURE 3.** Alignment of the same sequences around the interdomain insertion region of the long ADCs is shown in supplemental Fig. S1.
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**TABLE 1**

Kinetic constants for *C. auranticus* and *G. forsetii* ADCs

| Enzyme         | pH Temperature | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
|----------------|---------------|--------------|----------|-----------------|
| *C. auranticus*| 6.5 70        | 11 ± 0.4    | 0.59 ± 0.07 | 1800 ± 2000     |
| *G. forsetii*  | 8.5 30        | 0.48 ± 0.02 | 2.2 ± 0.02 | 220 ± 20        |

100,000 (16), indicating that the *V. vulnificus* ADC is ~5.5-fold more efficient than the *C. auranticus* ADC. Assay of the *G. forsetii* ADC in the presence of a 5 mM concentration of the ADC product agmatine revealed that the reaction product inhibited activity by ~30%. To determine the affinity of the product/inhibitor for the two ADC enzymes, kinetic constants were calculated at various concentrations of agmatine, giving \( K_i \) values of 0.92 ± 0.2 and 1.9 ± 0.4 mM for *C. auranticus* and *G. forsetii* ADCs, respectively. Differences in \( K_i \) values for agmatine were consistent with the substrate affinities of the two enzymes.

The short form AR-fold ADC found in the Chloroflexi phylum and Bacteroidetes phyla is probably the ancestral form of the AR-fold ADC enzyme. It is likely that the 90–105-amino acid insertion in the long form ADC is a polarized transition (i.e. it is much less probable that the insertion was removed without trace to produce a short form ADC than that a preexisting short form ADC acquired an insertion). Within the Chloroflexi, agmatine is converted to putrescine by AIH/NCPA. Within the Bacteroidetes, Sphingobacteria contain only AIH/NCPA orthologues, whereas flavobacteria possess only AUH orthologues. There is one species of Sphingobacteria encoding the long form ADC (*Rhodothermus marinus* DSM 4252; Figs. 2 and 3). An aberrant form of AR-fold ADC is encoded in *Francisella* species of the \( \gamma \)-Proteobacteria; it has an insertion of only 20 amino acids relative to the short
ADCs but is more similar to the long form ADC. It is found in a gene cluster containing an AIH gene disrupted by a frameshift (Fig. 4), with the implication that the ADC may not function for putrescine biosynthesis in these species. Gene clusters containing long and short form AR-fold ORFs together with other polyamine metabolic genes are common (Fig. 4).

Ancestral Biosynthetic Arginine Decarboxylases of the Aspartate Aminotransferase Fold—The *E. coli* AAT-fold ADC is an acid-inducible enzyme with a monomer size of 756 amino acids. This ADC activity is not involved in polyamine biosynthesis but in an acid resistance system. Five AAT-fold basic amino acid decarboxylases are encoded in the *E. coli* genome: the acid-inducible ADC, acid-inducible and constitutive...
A polyamine biosynthetic ADC exhibiting sequence similarity to the E. coli AAT-fold LDC was identified previously in the firmicute bacterium B. subtilis (48). The biosynthetic AAT-fold ADC enzyme identity was established by disruption of the encoding speA gene and subsequent analysis of polyamine levels in the gene knock-out strain (48). No biochemical characterization of the enzyme was performed in that study (48); therefore, we characterized the kinetic behavior of the B. subtilis 168 biosynthetic ADC (NP_389346), cloned from genomic DNA and expressed from pET21a in E. coli and purified as an N-terminal T7-tagged fusion protein. The B. subtilis biosynthetic ADC was found to have an optimal pH of 7.7 and optimal temperature of >75 °C (supplemental Fig. S3), and at 37 °C, the $K_m$ of the substrate arginine was 0.63 mM and the $k_{cat}$ was 0.21 s$^{-1}$ (Table 2). Many of the firmicute bacteria contain two AAT-fold basic amino acid decarboxylase paralogues. The second paralogue in B. subtilis, encoded by the yaaO gene, was shown previously to play no detectable role in polyamine biosynthesis (48). There are two AAT-fold basic amino acid decarboxylase paralogues in the genome of C. difficile 630 (YP_001087362 and YP_001090072). The YP_001087362 open reading frame is clustered immediately adjacent to open reading frames orthologous to the polyamine biosynthetic enzymes AdoMetDC, spermidine synthase, and yaaO (Fig. 5). We therefore expressed YP_001087362 as an N-terminally T7-tagged protein in E. coli and purified the recombinant enzyme. The C. difficile YP_001087362 protein is a functional ADC (Table 2) but with relatively low efficiency compared with the B. subtilis enzyme ($K_m = 3.3$ mm, $k_{cat} = 0.018$ s$^{-1}$ at 60 °C). Another AAT-fold ADC was recently identified in the firmicute Selenomonas ruminantium (49). Its $K_m$ for arginine was even higher than the C. difficile ADC, at 5.6 mm, and the optimal temperature was 60 °C.

| Enzyme | pH | Temperature | Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-------|----|-------------|-----------|-----------|-------|---------------|
| B. subtilis | 7.5 | 37 | Arg | 0.21 | 0.63 | 340 |
| B. subtilis | 7.5 | 70 | Arg | 1.4 | 1.1 | 1300 |
| B. subtilis | 7.5 | 70 | Orn | 0.04 | 7.5 | 5.6 |
| C. difficile | 7.5 | 60 | Arg | 0.025 | 0.73 | 35 |

Biosynthetic AAT-fold ADCs from the Firmicutes are considerably shorter than the acid-inducible AAT-fold ADC from E. coli and the biosynthetic AAT-fold ADC from Lactobacillus 30a (S. ruminantium ADC, 485 aa; BAD80720; B. subtilis ADC, 490 aa (P21885); and C. difficile ADC, 491 aa (YP_001087362)). A crystal structure of the Lactobacillus 30a ODC revealed that the functional unit is a dimer, with the active sites formed across the interface between the two monomers. The enzyme assembles into a dodecamer composed of six dimers (50). Similarly, the acid-inducible ADC of E. coli is a decamer composed of five dimers (51). Monomer forms of both the E. coli acid-inducible ADC and the Lactoba-
cillus 30a biosynthetic ODC are composed of five structural domains: the wing, linker, PLP-binding region, the aspartate aminotransferase-like small domain, and the C terminus (50, 51) (supplemental Fig. S4). The wing domain is required for multimeric assembly (Fig. 1B) and is also required for acid induction of activity in the E. coli ADC (51). Lactobacillus 30a biosynthetic ODC is very similar in amino acid sequence to E. coli biosynthetic and acid-inducible ODCs, and probably the Lactobacillus 30a ODC was acquired by horizontal transfer because only the Lactobacilli within the Firmicutes possess the long form biosynthetic AAT-fold ODC. When the firmicute B. subtilis, C. difficile, and S. ruminantium biosynthetic ADCs are aligned with the Lactobacillus 30a ODC and acid-inducible ADC of E. coli, it is clear that the firmicute biosynthetic ADCs lack completely the wing domain of the E. coli and Lactobacillus 30a enzymes (supplemental Fig. S4). In addition, the biosynthetic AAT-fold ADCs have small deletions in each of the other four domains relative to the E. coli acid-inducible ADC and Lactobacillus 30a ODC enzymes (supplemental Fig. S4).

A sequence comparison analysis using BLASTP of either the E. coli acid-inducible ADC or the Lactobacillus 30a ODC shows clearly that the wing domain of both proteins exhibits sequence homology to the signal receiver domains of response regulators, such as CheY (so-called REC domains). An alignment of the E. coli acid-inducible ADC wing domain with REC domains of diverse response regulator proteins is shown in supplemental Fig. S5. A number of aspartate residues are conserved between the ADC wing domain and the other REC domains and may be involved in metal binding. Comparison of the crystal structure of the acid-inducible ADC wing domain with other structures, using the Vector Alignment Search Tool at the National Center for Biological Information, shows an even greater similarity than sequence-based searches to the structures of many diverse REC domain-containing proteins. It was shown previously that the wing domain protrudes from the rest of the E. coli acid-inducible ADC protein structure (51). It is thus most likely that the acid-inducible ADC wing domain was acquired by fusion of a REC domain protein to the N terminus of a firmicute short form biosynthetic AAT-fold ADC. Because the wing domains are not found in other AAT-like proteins (51), it is very probable that the shorter biosynthetic ADCs found in the Firmicutes are ancestral to the longer acid-inducible ADC and the constitutive and inducible LDCs and ODCs found in E. coli. Potential orthologues of the firmicute biosynthetic AAT-fold ADCs are also encoded in Cyanobacteria, forming a distinct cyanobacterial clade. Genes for the corresponding enzymes from the filamentous cyanobacterium Anabaena variabilis ATCC 29413 (YP_322672; 488 aa) and Nostoc punctiforme PCC 73102 (YP_001864209; 504 aa) were synthesized with E. coli-optimized codons, expressed from pET21a in E. coli, and purified as T7-tagged proteins. Neither enzyme displayed any activity with arginine, ornithine, or lysine (results not shown), and they appear to be uninvolved in polyamine metabolism, similar to the AAT-fold ADC-like protein encoded by the yaaO gene of B. subtilis described above.
The Biosynthetic AAT-fold ADC of B. subtilis Is Essential for Biofilm Development — A gene disruption mutant of the B. subtilis 168 biosynthetic ADC was made previously by Sekowska et al. (48); however, the effect of the speA gene deletion on growth was not reported. We analyzed aerobic growth, in polyamine-free defined minimal medium, for both the biosynthetic ADC gene deletion (\(/H9004\)speA) strain made by Sekowska et al. (48) and a deletion strain of the related yaaO gene (\(/H9004\)yaaO). Deletion of either speA or yaaO had no discernible effect on aerobic growth of planktonic cells in liquid medium (Fig. 6, top), although polyamines were completely absent from the \(\Delta speA\) cells (Fig. 6, bottom). This experiment also confirmed that deletion of yaaO has no effect on polyamine levels in \(\Delta yaaO\) cells grown aerobically in polyamine-free medium. Polyamines are critical for biofilm formation in the \(/H9253\)-Proteobacteria Y. pestis and V. cholerae (37, 38, 52, 53). Therefore, the effect of disruption of \(\Delta speA\) on B. subtilis biofilm formation was examined. Phage transduction was used to replace the \(\Delta speA\) (ADC) gene of B. subtilis NCIB3610 with the \(/H9004\)speA region of B. subtilis 168 to facilitate biofilm analysis. The B. subtilis NCIB3610 strain forms complex and robust biofilms in comparison with the laboratory isolate 168 (41). Polyamine depletion in the \(/H9004\)speA gene knock-out strain resulted in abolition of biofilm formation using both complex
colony architecture and pellicle formation as two independent measures of biofilm formation capability (Fig. 7). The negative impact on biofilm formation was fully reversed by the addition of 0.5 mM agmatine or spermidine to the growth medium (Fig. 8). The addition of putrescine to the growth medium did not restore biofilm formation, which may be due to lack of uptake. Although biofilm formation in the B. subtilis/H9004 speA NCIB3610 background was abolished (Fig. 7A), aerobic growth of planktonic cells of the same/H9004 speA NCIB3610 strain in polyamine-free liquid medium was unaffected (Fig. 7B).

The Phylogenetic Distribution of Polyamine Biosynthetic ADCs—In addition to the AR-fold and AAT-fold pyridoxal 5'-phosphate-dependent ADCs, there are two types of pyruvoyl-dependent ADCs found in archaea and some bacteria. Pyruvoyl-dependent decarboxylases autocatalytically self-process into α- and β-chains at a serine residue that generates the pyruvoyl cofactor. The pyruvoyl cofactor becomes the N terminus of the α-chain. The pylADC is found predominantly in the euryarchaeota and is structurally and mechanistically similar to pyruvoyl-dependent histidine decarboxylase (21, 39). Some Chlamydia species use a very divergent pylADC as part of an acid resistance system (23, 54); however, the Chlamydia enzymes are unlikely to participate in polyamine biosynthesis. Like the PLP-dependent ADCs, the pylADCs are commonly found in polyamine-related gene clusters/operons (Fig. 5). Among the archaea, there are pylADC orthologues in 64 euryarchaeote genomes (Table 3) but in only one crenarchaeote (Thermofilum pendens Hrk 5 (YP_920276)). One euryarchaeote species, Methanosaeta thermophila PT, uses a typical AR-fold ADC (Figs. 2 and 3 and supplemental Fig. S1) and does not possess a pylADC orthologue. The pylADC appears to be the only route for putrescine biosynthesis in the anoxygenic, photosynthetic Chlorobi phylum (Table 3), and a subset of species of the Bacteroides and Proteobacteria classes possess pylADC orthologues. One genome each in the Actinobacteria, ε-Proteobacteria, Planctomycetales, Elusimicrobia, and Synergistetes phyla and two in the Acidobacteria contain pylADC orthologues, but there is very shallow genome sampling of the Elusimicrobia and Synergistetes, so py-
IADC in principle could be more important in these poorly sampled phyla.

Most of the Crenarchaeota use an AdoMetDC paralogue to decarboxylate arginine for polyamine biosynthesis (22) and thus contain two AdoMetDC paralogues in each genome. This unusual method for arginine decarboxylation appears to be specific to the Crenarchaeota. Three species within the Crenarchaeota use diverged AR-foldADCs although replete with the typical interdomain insertion (two are shown in Figs. 1 and 2 and supplemental Fig. S1). Both Desulfurococcus kamchatkensis 1221n and Staphylothermus hellenicus DSM 12718 of the Crenarchaeota possess an AR-foldADC and only one AdoMetDC orthologue; however, Staphylothermus marinus DSM 12333 F1 possesses two AdoMetDC paralogues and an AR-fold ADC.

The biosynthetic AAT-foldADC is found almost exclusively in the Firmicutes, with 155 genomes containing an orthologue (Table 3). There are also orthologues in two Fusobacteria and two Mollicutes genomes and one Spirochaete. In contrast, the AR-fold ADC is excluded from the Firmicutes except for Clostridium cellulolyticum H10 and Clostridium papyrosolvens DSM 2782. The AR-fold ADC is most prevalent in the $\gamma$- and $\epsilon$- and $\delta$-Proteobacteria, the Cyanobacteria, the Bacteroides class of the Bacteroidetes phylum, the Verrucomicrobia, Deinococcus-Thermi, and Planctomycetales (Table 3). A smaller proportion of the $\alpha$- and $\beta$-Proteobacteria also contain AR-foldADC orthologues. As discussed above, the ancestral, shorter form of the AR-fold ADC lacking the interdomain insertion is limited to members of the Chloroflexi and Bacteroidetes. Almost all terrestrial plants contain long form AR-foldADC orthologues, but they are absent in algae except for one example in Micromonas pusilla. The nucleus-encoded plant AR-foldADC orthologues are derived from the cyanobacterial ancestor of the chloroplast (11, 55). Biosynthetic ADCs remain to be identified in other eukaryotes.

The clear discernable pattern in the phylogenetic distribution of the different ADC forms is that the AR-fold ADC is mostly absent from the Archaea, Firmicutes, and Actinobacteria, which are single-membraned. The Actinobacteria appear to lack any ADC orthologues (except for a pylADC orthologue in Beutenbergia cavernae DSM 12333 (YP_002880881)). The Firmicutes depend on the short AAT-fold biosynthetic ADC, and Archaea use mainly pyruvoyl-dependent ADCs, with the euryarchaeotes possessing the histidine decarboxylase-like pylADC, whereas the Crenarchaeota mostly use the AdoMetDC-derived ADC.

Arginine Decarboxylase Is the Dominant Route for Polyamine Biosynthesis in the Human Gut Microbiota—The genomes of the 55 most common bacterial species found in the human gut microbiota (43) were screened in silico for orthologues of the AR- and AAT-fold ODCs. No ODC orthologues from either fold were found in these species. In contrast, biosynthetic AR- and AAT-fold ADC genes were found in the majority of species, although 11 species appeared to be auxotrophic for polyamine biosynthesis (Table 4). The Firmicutes species contained the AAT-fold biosynthetic ADC, whereas the Bacteroidetes contained the AR-foldADC with the exception of Bacteroides capillosus and B. pectinophilus, which contain horizontally acquired AAT-foldADCs. The Bacteroidetes species A. putredinis contains an ancestral short AR-fold
ADC. Genes encoding AIH and NCPAH are always clustered except for the presence of isolated AIH-encoding genes in the firmicute Enterococcus faecalis and the actinobacterium Collinsella aerofaciens. At least in the case of E. faecalis, the AIH-encoding gene is involved in agmatine catabolism (56).

**DISCUSSION**

Arginine decarboxylation for polyamine biosynthesis has received scant attention due to its absence from non-plant eukaryotes. However, arginine decarboxylation in prokaryotes and plants provides a useful model for assessing the molecular processes shaping metabolic pathway evolution. Prominent mechanisms involved in pathway evolution are gene duplication and gene loss. The PLP-dependent AR-fold family of basic amino acid decarboxylases most probably evolved by gene duplication from DAPDC, the last step in the lysine biosynthetic pathway. One of the AR-fold decarboxylases, the bifunctional L/ODC, is able to decarboxylate the product of DAPDC, ATCC 8482 (B) YP_001298648 Absent Absent YP_001300474 YP_001300475

**TABLE 4**

Arginine pathway polyamine biosynthetic genes in 55 frequently found genomes of the human gut microbiota

Species names are followed by B for Bacteroidales, F for Firmcutes, or A for Actinobacteria. The table below shows the presence or absence of selected polyamine biosynthetic genes in various bacterial species.

| Bacteroides uniformis (B) | AR-fold ADC |  |  |  |
|---------------------------|-------------|---|---|---|
| ZP_02069527               | Absent      | Absent | ZP_02070604 | ZP_02070605 |
| Alistipes putredinis (B)  | ZP_0242406a | Absent | ZP_02425128 | Absent  |
| Parabacteroides merdae (B)| ZP_02034148 | Absent | ZP_02032161 | Absent  |
| Dorea longicatena (F)     | Absent      | ZP_01005018 | Absent      | Absent  |
| Ruminococcus sp. L2-63 (F)| Absent     | ZP_158037 | Absent      | Absent  |
| Bacteroides cacceae (B)  | ZP_01961811 | Absent | ZP_01958999 | ZP_01958999 |
| Clostridium sp. SS2/1 (F)| Absent      | ZP_02420164 | Absent      | Absent  |
| Bacteroides thetaiotaomicron VPI-5482 (B)| Absent | ZP_812306 | Absent | Absent  |
| Eubacterium rectale (F)  | Absent      | ZP_03715546 | Absent      | Absent  |
| Ruminococcus torques L2-14 (F)| Absent | CBL25540 | Absent | Absent  |
| Unknown sp. SS3 4         | NA          | NA      | NA          | NA      |
| Ruminococcus sp. SR 1/5 (F)| Absent     | CBL18584 | CBL18586   | Absent  |
| Faecalibacterium prausnitzii SL3/3 (F) | Absent | CBL22567 | ZP_0451473 | Absent  |
| Ruminococcus lactaris (F) | Absent      | ZP_03167723 | Absent      | Absent  |
| Collinsella aerofaciens (A)| Absent     | ZP_02235921 | Absent      | Absent  |
| Dorea formigenicenae (F)  | Absent      | ZP_01029604 | Absent      | Absent  |
| Bacteroides vulgatus ATCC 8482 (B)| Absent | CBL08498 | Absent | ZP_000300474 |
| Rodobacter intestinalis M50/1 (F)| Absent | ZP_02424069 | Absent | YP_001300475 |
| Bacteroides sp. 2_L_7 (B) | Absent      | ZP_02235921 | Absent      | Absent  |
| Eubacterium siraeae 70/3 (F)| Absent | CBK97421 | Absent | ZP_02423330 |
| Parabacteroides distasonis ATCC 8503 (B)| Absent | ZP_04599006 | Absent | YP_001032561 |
| Bacteroides sp. 9_1_42FAA (B)| Absent | ZP_06616985 | Absent | YP_001030561 |
| Bacteroides ovatus (B)    | ZP_02535666 | Absent | ZP_02545800 | ZP_0454799 |
| Bacteroides sp. 2_4_47FAA (F)| Absent | ZP_04559006 | Absent | YP_001030561 |
| Eubacterium rectale M104/1 (F)| Absent | CBK93443 | Absent | CBK93439  |
| Bacteroides xylanisolvens XB1A (F)| CBK67757 | CBK65746 | CBK93438 |  |
| Coprococcus comes (F)    | Absent      | ZP_03081366 | Absent      | Absent  |
| Bacteroides sp. D1 (B)   | ZP_04547174 | Absent | ZP_0453700 | ZP_0453701 |
| Bacteroides sp. D4 (B)   | ZP_04555452 | Absent | ZP_0455588 | ZP_0455577 |
| Eubacterium ventriosi (F)| Absent      | ZP_02050949 | Absent      | Absent  |
| Bacteroides dorei (B)    | ZP_03300857 | Absent | ZP_03031417 | ZP_03031418 |
| Ruminococcus obeum A2–162 (F)| Absent | CBL22837 | CBL22839 | Absent  |
| Sphingobacterium variabile (F)| Absent | ZP_0581888 | ZP_0581890 | Absent  |
| Bacteroides capillos (B) | Absent      | ZP_02036374 | ZP_02036372 | Absent  |
| Streptococcus thermophilus LMD-9 (F)| Absent | ZP_02080046 | ZP_02080049 | Absent  |
| Clostridium leptum (F)   | Absent      | Absent | Absent      | Absent  |
| Holdemadrum nitroformis (F)| Absent | ZP_02432642 | Absent | Absent  |
| Bacteroides stercoris (B) | Absent      | Absent | Absent      | Absent  |
| Coprococcus euctactus (F)| Absent      | Absent | Absent      | Absent  |
| Clostridium sp. M62/1 (F)| Absent      | ZP_06347603 | ZP_06347601 | Absent  |
| Bacteroides egerthii (B) | Absent      | ZP_03459356 | Absent      | Absent  |
| Butyribrio crosses (F)   | Absent      | ZP_05792554 | Absent      | Absent  |
| Bacteroides finegoldii (B)| ZP_05414660 | Absent | ZP_05414556 | ZP_05414556 |
| Parabacteroides johnsonii (B)| ZP_03477317 | Absent | ZP_03476338 | ZP_03476338 |
| Clostridium hirumi (F)   | Absent      | Absent | Absent      | Absent  |
| Clostridium nesle (F)    | Absent      | ZP_03289957 | ZP_03289955 | Absent  |
| Bacteroides pectinophilus (B)| Absent | ZP_03461361 | Absent | ZP_03461425 |
| Anaerotruncus colihominis (F)| Absent | ZP_0243066 | Absent | ZP_03461425 |
| Ruminococcus atheroxyti (F)| Absent | ZP_02043066 | ZP_02043062 | Absent  |
| Bacteroides intestinalis (B)| Absent | ZP_03013854 | Absent | ZP_03014439 |
| Bacteroides fragilis 3_1_12 (B)| ZP_02528264 | Absent | ZP_02528150 | ZP_02528150 |
| Clostridium asparagiforme (F)| Absent | Absent | Absent      | Absent  |
| Enterococcus faecalis TX0104 (F)| Absent | Absent | ZP_0497749 | Absent  |
| Clostridium scindens (F) | Absent      | ZP_05853960 | ZP_05853962 | Absent  |

* Short ancestral form of AR-fold ADC.
* Probably a yaaO orthologue.
thought that ADC was anomalous within the AR-fold decarboxylase family because it is considerably longer than the other members. This is primarily due to a single large insertion. The size of the insertion is conserved across prokaryotic phyla, but the amino acid sequence of the insertion is much less conserved (shown in Fig. 3), suggesting a structural rather than catalytic role for the insertion domain. Recently, the insertion domain in the AR-fold ADC x-ray crystal structure has been shown to be a four-helix bundle that is responsible for the AR-fold ADC being a tetramer rather than a dimer like the rest of the AR-fold decarboxylase family (20). The shorter form of AR-fold ADC present in the Chloroflexi and Bacteroidetes lacks the four-helix bundle domain insertion and thus represents an ancestral form of AR-fold ADC. It is the same size as DAPDC and may represent a form intermediate between DAPDC and the AR-fold long ADC. The four-helix bundle is located precisely between the N-terminal β/α domain and the C-terminal β-barrel domain. AR-fold decarboxylases are obligate homodimers with two identical active sites, each formed from residues across the dimer interface between the N-terminal β/α domain of one monomer and the C-terminal β-barrel domain of the other. The position of the four-helix bundle insertion is probably the only place in the enzyme where an insertion would not disrupt enzymatic function. The related ODC enzyme can be split at this same position, and the resulting N- and C-terminal domains can be co-expressed and a functional ODC enzyme can be reconstituted in vivo from the two separated domains (58). We do not know whether the short ADC lacking the four-helix bundle was widespread in bacteria and was then subsequently replaced by the long form or whether the long form ADC arose early in bacterial evolution. The Chloroflexi and Sphingobacteria/Flavobacteria are not thought to be phylogenetically close. If there was not a widespread loss of the short form ADC, then there was probably horizontal transfer of the short form ADC between the two phyla. It appears that the long form ADC is present in all land plants and that this is a case of endosymbiotic gene transfer from the cyanobacterial ancestor of the chloroplast.

In the case of the pyridoxal 5’-phosphate-dependent AAT-fold basic amino acid decarboxylases and in particular the acid-inducible ADC, gene fusion was a key mechanism in the evolution of biological function. The most plausible evolutionary scenario is that the short biosynthetic AAT-fold ADC, typically found in firmicutes, such as *B. subtilis*, *C. difficile*, and *S. ruminantium*, was the ancestral form of the acid-inducible AAT-fold ADC enzyme. In *S. ruminantium*, the short form AAT-fold ADC is a dimer (49). A response regulator receiver domain protein was then fused to the N terminus end of the short biosynthetic AAT-fold ADC, thereby forming the wing domain of the acid-inducible AAT-fold ADC. The wing domain is responsible for decamer formation (a pentamer of homodimers) of the acid-inducible ADC due to the role of the wing domain in forming interactions between the homodimers (51). In the *E. coli* acid-inducible ADC, which has the N-terminal wing domain, decamer formation is required for enzyme activity in acidic conditions. At neutral pH, the negative charge associated with the wing domain causes dissociation of the decamer and loss of enzymatic activity. In contrast, at acidic pH, the negative charge of the wing domain is neutralized, leading to decamer formation and catalytic activation (51). Only the decamer form is active, and the dimer is inactive until decamer formation. The wing domain can therefore be seen to be acting as a typical response regulator; the presence of the receiver domain in the acid-inducible AAT-fold ADC confers environmental responsiveness (i.e. acid inducibility) to the output domain (i.e. the ADC activity).

Besides the AR-fold and AAT-fold ADCs, there are also the two pyruvoyl-dependent ADCs, the pylADC and the AdoMetDC-like ADC. Thus, there are at least four different protein folds and two different cofactors representing the catalytic activity EC 4.1.1.19 (ADC). There are three forms of the AR-fold ADC: the ancestral short form described herein, the long form with four-helix bundle insertion, and the chlorovirus ADC which has evolved from the AR-fold ODC (24, 25). In addition, there are two forms of the AAT-fold ADC, the firmicute biosynthetic ADC and the *E. coli* acid-inducible ADC; there are biosynthetic and acid-inducible forms of the pylADC (21, 23, 39) and the one known form of the AdoMetDC-like ADC. There are thus four protein folds and eight different enzyme forms represented by the description EC 4.1.1.19. This is a rather extreme form of what have been recently described as, “non-homologous isofunctional enzymes” (8), enzymes carrying out the same chemistry but from entirely different evolutionary origins. The multiplicity of ADC forms indicates the importance of arginine decarboxylation and polyamine biosynthesis for life because different enzymatic solutions for arginine decarboxylation have arisen independently multiple times during evolution.

Polyamine-related gene clusters encoding either the AR-fold, AAT-fold, or pyruvoyl-dependent ADCs are commonly found in prokaryotic genomes. These gene clusters exhibit a property that has been noted generally for gene clusters and operons (i.e. there is a tendency for the genes of the cluster to be arranged in the biochemical reaction order, known as co-linearity) (59, 60). In the ADC-containing gene clusters, there is a marked tendency for the *speA* ORF to be at the beginning of the cluster (Figs. 4 and 5). Another phenomenon that is revealed in the ADC-containing gene clusters is the modularity of polyamine biosynthesis. Not only are there alternative ADC enzymes, but each form of *speA* involved in polyamine biosynthesis can be clustered with either agmatine ureohydrolase (agmatinase) or with agmatine deiminase and *N*-carbamoylpurrescine amidohydrolase, as part of a putrescine biosynthetic module. The clusters may also include the carbamoyl(nor)sperrmidine dehydrogenase/CANSDC pairs or the AdoMetDC/spermidine synthase pairs for the spermidine biosynthetic module. Some of the clusters contain a deoxypurine synthase-like ORF that is probably an alternative homospermidine synthase (10).

The phylogenetic distribution of the different forms of ADC establishes a distinct pattern. Both the short and long form AR-fold ADCs are limited to double-membraned bacteria except for two Clostridia species where the genes have been acquired by horizontal transfer. Biosynthetic AAT-fold ADC is limited to the Firmicutes (single-membrane bacte-
ria). Actinobacteria (single-membraned bacteria) do not contain ADC except for one genome. Pyruvyl-dependent ADC is the predominant ADC of the Euryarchaeota (single-membraned), although it is found in some bacterial genomes as well, and the AdoMetDC-like ADC is found only in the Crenarchaeota (single-membraned). Prominent hypotheses for the root of the tree of life place the root near or in the Chloroflexi (61); between clades consisting of actinobacteria/double-membraned bacteria and firmicutes/archaea (62); or nowhere, due to insufficient support for a tree of life but with the major prokaryotic division being between bacteria and archaea (63).

The phylogenetic distribution of the different ADC forms recapitulates the major divisions of life: bacteria, archaea, and eukaryotes. Except for plants, eukaryotes do not possess a polyamine biosynthetic ADC. Double-membraned bacteria are distinguished from single-membraned bacteria by different ADC forms. In single-membraned bacteria, Firmicutes are distinguished from Actinobacteria. The Euryarchaeotes are distinguished from Crenarchaeotes, and the archaea are distinguished from bacteria. Bacteria mainly use PLP-dependent ADCs, and Archaea predominantly use pyruvyl-dependent ADCs. The alternative route for putrescine biosynthesis, ODC, is of limited phylogenetic distribution in prokaryotes, mainly in Proteobacteria, a few Actinobacteria, and Lactobacilli. Decarboxylation of arginine is the dominant mode of polyamine biosynthesis in bacteria and archaea. This dominance of the ADC pathway for polyamine biosynthesis in bacteria is exemplified by the human gut microbiota, where ADC is the only route for polyamine biosynthesis among the 55 most common gut microbiota species. Because human cells contain only the ODC pathway for polyamine biosynthesis, pharmacological manipulation of ADC activity might permit reorganization of the gut microbiota population.

Although polyamines are found in almost all bacteria, it is not clear what the core physiological requirements for polyamines are. We have shown that ADC and, therefore, polyamines are essential for biofilm production in the single-membraned bacterium *B. subtilis*. It is already known that polyamines are required for biofilm production in the double-membraned bacteria *Y. pestis* and *V. cholerae*. However, in the *Y. pestis* polyamine biosynthetic knock-out strain, putrescine and not spermidine restored biofilm formation, whereas in *V. cholerae*, sym-norspermidine but not spermidine was required. In *B. subtilis*, spermidine is able to restore biofilm formation. Because 90% of spermidine is bound to RNA in *E. coli* (64), it is interesting to note that in *B. subtilis* biofilm formation is dependent upon spermidine and is controlled by a regulatory RNA mechanism (65).

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