An Alternative Polyamine Biosynthetic Pathway Is Widespread in Bacteria and Essential for Biofilm Formation in Vibrio cholerae

Polyamines are small organic cations found in all cells, and the biosynthetic pathway is well described in eukaryotes and Escherichia coli. The characterized pathway uses decarboxylated S-adenosylmethionine as the aminopropyl group donor to form spermidine from putrescine by the key enzymes S-adenosylmethionine decarboxylase and spermidine synthase. We report here the in vivo characterization of an alternative polyamine biosynthetic pathway from Vibrio cholerae, the causative agent of human cholera. The pathway uses aspartate β-semialdehyde as the aminopropyl group donor and consists of a fused amine biosynthetic pathway from L-2,4-diaminobutyrate aminotransferase and L-2,4-diaminobutyrate decarboxylase, a carboxynorspermidine dehydrogenase (CANSDH), and a carboxynorspermidine decarboxylase (CANSDC). We show that in V. cholerae, this pathway is required for synthesis of both sym-norspermidine and spermidine. Heterologous expression of the V. cholerae pathway in E. coli results in accumulation of the nonnative polyamines diaminopropane and sym-norspermidine. Genetic deletion of the V. cholerae CANSDC led to accumulation of carboxynorspermidine, whereas deletion of either CANSDC or the putative CANSDH led to loss of sym-norspermidine and spermidine. These results allowed unambiguous identification of the gene encoding CANSDH. Furthermore, deletion of either CANSDH or CANSDC led to a 50–60% reduction in growth rate of planktonic cells and severely reduced biofilm formation, which could be rescued by exogenously supplied sym-norspermidine but not spermidine. The pathway was not required for infectivity in a mouse model of V. cholerae infection. Notably, the alternative polyamine biosynthetic pathway is widespread in bacteria and is likely to play a previously unrecognized role in the biology of these organisms.
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(SCHEME 1. Synthetic scheme for the chemical synthesis of carboxynorspermidine.

Vibrio cholerae is the causative agent of the human diarrheal disease cholera (30). We demonstrate that the asparagine-β-semialdehyde-based alternative polyamine biosynthetic pathway is responsible for norspermidine and spermidine biosynthesis in V. cholerae, and by genetic deletions of the CANSDH and CANSDC, we demonstrate that the alternative polyamine biosynthetic pathway is essential for normal biofilm formation. Extracellularly supplied sym-norspermidine but not spermidine is able to rescue biofilm formation in the null strains. Depletion of norspermidine in the V. cholerae CANSDH and CANSDC null strains did not attenuate infection in an infant mouse model of infection. Bioinformatic analysis suggests that the alternative polyamine biosynthetic pathway, based on asparagine-β-semialdehyde as an aminopropyl group donor and the CANSDH and CANSDC enzymes, is widely distributed in bacteria.

EXPERIMENTAL PROCEDURES

Materials—Chemical reagents were purchased from Sigma unless otherwise noted. Infinity™ carbon dioxide detection reagent was from Thermo Electron Corp. (Louisville, CO). The AccQ-Fluor reagent kit for labeling amino acids was purchased from Waters (Milford, MA). Genomic DNA from Vibrio vulnificus CMCP6 was kindly provided by Dr. Joon Haeng Rhee (Chonnam University, Gwangju, Korea).

Chemical Synthesis of Carboxynorspermidine and Carboxyspermidine—The reaction is shown in Scheme 1.

Reductive Amination Step—Boc-L-2,4-diaminobutyric acid (2.5 g, 11.4 mmol), methanol (30 ml), and water (4 ml) were added to a 100-ml round bottom flask. Triethylamine (1.6 ml, 11.4 mmol) was then added, and the reaction was cooled to ~5 °C using an ice bath and stirred until a homogeneous solution was obtained. To this was added 3-[(benzyloxy-carbon-yl)amino]propionaldehyde (2.37 g, 11.4 mmol), followed by the slow addition of sodium triacetoxylborohydride (2.42 g, 11.4 mmol) as a solid portion-wise. The reaction was monitored by liquid chromatography/mass spectrometry until >95% of the Boc-2,4-diaminobutyric acid was consumed. Solvent was removed by roteovepaporation, and the product was purified by silica gel column chromatography using a gradient of 0–20% methanol in dichloromethane. Combination of the appropriate fractions yielded the product as a clear oil, which was then further purified by crystallization from ethyl acetate/hexane at 4 °C to give 450 mg (~9% yield) of the desired reductive amination product.

Deprotection Step—The product (450 mg, 1.1 mmol) obtained from the above procedure was dissolved in 4 N HCl in dioxane (5 ml) at room temperature, and the resulting Boc deprotection was monitored by liquid chromatography/mass spectrometry. Once all of the starting material was consumed (~6 h), the reaction was filtered to collect product as its HCl salt (330 mg). This solid was taken directly into the benzoylcarbonyl deprotection step by dissolving it in methanol (20 ml), followed by the addition of catalytic amounts of Pd/C. The reaction was then placed under an atmosphere of hydrogen (via balloon) and allowed to stir at room temperature over 16 h. Water (1 ml) was added then the reaction mixture was filtered through a 0.22-μm filter, followed by solvent removal via rotary evaporation to give carboxynorspermidine as its HCl salt (200 mg).1H NMR (400 MHz, D2O) δ 3.65 (t, 1H), 3.2–2.9 (m, 6H), 2.1 (m, 2H), 1.9 (m, 2H). Carboxyspermidine was synthesized analogously, yielding its HCl salt.1H NMR (400 MHz, D2O) δ 3.60 (t, 1H), 2.85–3.00 (m, 6H), 1.81–2.00 (m, 2H), 1.43–1.80 (m, 4H). Details are provided in the supplemental material.

Gene Cloning and Construction of E. coli Expression Plasmids—A list of cloning primers and plasmid constructs used in the studies are provided in Tables S1 and S2.

For the V. vulnificus CANSDC expression plasmid, the VV1_3048 ORF from V. vulnificus CMCP6 was amplified by PCR from genomic DNA using primers VvCANSDC_F and VvCANSDC_R and ligated into pET-15b (Novagen) at NdeI and Xhol sites, resulting in the plasmid pET15:VvCANSDC. For the V. cholerae CANSDH expression plasmid, an XbaI site in pET-15b was mutated to Sall by the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primers QpET15F and QpET15R to generate qET-15b plasmid. The VC1623 ORF of V. cholerae El Tor biotype C6709 was amplified by PCR from genomic DNA using primers pET1623F and pET1623R and ligated into qET-15b (Novagen) at the NdeI and Xhol sites, yielding plasmid qET15:1623. An NdeI site in the V. cholerae VC1623 ORF was removed by QuikChange™ mutagenesis using the primers Q1623F and Q1623R before the final cloning step.

For the V. cholerae DABA AT/DC and CANSDH expression plasmids, the following steps were taken. The V. cholerae PCR-amplified VC1625 ORF (primers pET1625F and pET1625R) and VC1624 ORF (primers pET1624F and pET1624R) were cloned into qET-15b at the NdeI and Xhol sites, yielding the plasmids qET15:1625 and qET15:1624, respectively.
Expression of the recombinant proteins was induced by containing ampicillin (pET15:V. cholerae ornithine decarboxylase (27, 32)).

Cells were isolated by centrifugation and resuspended in

buffer A (50 mM HEPES, pH 7.0, 300 mM NaCl, 0.02 mM pyridoxal 5’-phosphate, 0.015% Brij) containing lysozyme (1 mg/ml). Cells were lysed by freezing and thawing followed by sonication, and the extract was clarified by centrifugation (150,000 × g for 1 h). Protein extracts were applied to Ni²⁺-nitrilotriacetic acid affinity resin (Qiagen) in buffer A containing 20 mM imidazole. The column was washed in buffer A containing 60 mM imidazole to remove contaminating proteins, and His-tagged protein was eluted in buffer A with 200 mM imidazole. For CANSDC, the pooled fractions were buffer-exchanged on an Amersham Biosciences Hi-prep™ 16/10 desalting column equilibrated with buffer B (50 mM HEPES, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 0.03% Brij, 1 mM dithiothreitol, and 10% glycerol). The protein was concentrated by ultrafiltration using Centricon YM-10 (Amicon). DABA AT/DC was also affinity-purified in the same way, except that buffer B contained 50 mM HEPES pH 8.0. Protein purity was analyzed by SDS-PAGE, and the protein concentration was determined by absorbance measurements at 280 nm. All enzyme preparations were used in assays without cleavage of the His₆ tag.

Expression of the sym-Norspermidine Biosynthetic Operon in E. coli—Cells of E. coli BL21(DE3) were transformed with a single expression plasmid pET28:1625 that expresses VcDABA AT/DC or a co-expression plasmid pET28:1625/1624/1623 that co-expresses VcDABA AT/DC, putative VcCANSDH, and VcCANSDC. An empty pET28a plasmid was used as a control. The transformed cells were grown at 37 °C in LB medium containing kanamycin, and overexpression of the recombinant proteins from a T7 promoter was induced by 200 μM isopropyl-1-thio-β-D-galactopyranoside at 16 °C overnight to facilitate soluble protein production. Overexpression of the proteins in soluble form was confirmed by comparing total cell lysate and soluble fraction by SDS-PAGE analysis.

Enzyme Steady-state Kinetic Analysis—CANSDC and DABA DC activity were assayed using the Infinity™ CO₂ detection kit as described (27, 32). The optimal enzyme concentration was determined to ensure that the reaction rate was proportionally dependent on the enzyme concentration. Final assay conditions for the reported kinetic constants were as follows: [CANSDC] = 30 nM for carboxynorspermidine of 0.1–1.5 mM; [CANSDC] = 200 nM for carboxysspermidine of 0.3–3 mM; [DABA AT/DC] = 50 nM for 0.05–1 mM DABA; [DABA AT/DC] = 1.5 μM for 2–40 mM l-Orn.

Polyamine Analysis of E. coli and V. cholerae by HPLC—Cell cultures grown in LB or M9 minimal medium (0.5–0.75 ml of 1.2–1.8 A₆₀₀₉₉₉₉) were pelleted at 4000 × g. The cell pellets were washed in 1 ml of 1× phosphate-buffered saline (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) three times and resuspended in 25 μl of lysis buffer (20 mM MOPS, pH 8.0, 10 mM NaCl, 4 mM MgCl₂). The resuspended cells were lysed by three cycles of freeze thaw in liquid nitrogen. Protein was precipitated by the addition of trichloroacetic acid (7.5 μl of 40%) followed by incubation on ice for 5 min. The cell lysate was cleared of cell debris by centrifugation at 13,000 × g for 3 min at room temperature and stored at −20 °C until analysis.

Cellular polyamines were labeled with AccQ-Tag Reagent Kit (Waters, Milford, MA), the fluorescent reagent (6-aminoquinolyl-n-hydroxysuccinimidyl in acetonitrile) in 5% sodium tetraborate as described previously (33, 34). Labeled polyamines were analyzed by HPLC using AccQ-Tag C₁₈ column (Waters) with fluorescence detection. Briefly, 5 μl of polyamine containing sample was reacted with 20 μl of the fluorescent
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reagent in a 100-μl reaction at 55 °C. Cleared culture media were also analyzed to measure secreted polyamines. Six different labeled polyamines (carboxynorspermidine, 1,3-diaminopropane, putrescine, cadaverine, sym-norspermidine, and spermidine) and 1,7-diaminoheptane as an internal standard were clearly separated in the following gradient system with the buffer A (450 mM sodium acetate (diluted from 1 M stock pH 4.5), 17 mM TEA, pH adjusted to 4.75 with NaOH) and buffer B (60% acetonitrile and 0.01% acetonitrile): 1–5 min, 0–20% B; 5–50 min, 20–50%; 50–55 min, 50–100%; 55–57 min, 100%. A modified buffer A containing 140 mM sodium acetate, pH 4.28, was used to resolve 1,3-diaminopropane and carboxynorspermidine.

Bacterial Strains and Growth Conditions—Features of bacterial strains used in this study are described in Table S2. E. coli was grown at 37 °C in Luria-Bertani broth, and V. cholerae was grown at 37 °C or 30 °C in LB or M9 minimal medium (M9MM) containing 0.4% glycerol except in the growth assays in which 0.4% glucose-containing M9MM was used. Appropriate antibiotics were added as needed: streptomycin (30 μg/ml), chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), ampicillin (50 μg/ml), and carbenicillin (50 μg/ml). L-Arabinose was added at 0.2% (w/v) in the culture as an inducer for the P_{BAD} promoter.

V. cholerae Growth Assay—Wild-type and mutant strains (ΔVC1623 and ΔVC1624) of V. cholerae were grown at 37 °C in LB overnight and washed twice in M9MM. They were diluted to an initial A_{600} of 0.02 in fresh M9MM or LB ± 250 μM spermidine and/or 250 μM sym-norspermidine and incubated at 37 °C with shaking at 250 rpm. The A_{600} was measured from each sample at the times indicated. A culture grown for 24 h in M9MM was used to inoculate fresh M9MM, and after four subcultures, growth was monitored.

Measurement of Static Biofilm Formation—A microtiter static biofilm assay was modified from the standard method described in Ref. 35. In brief, overnight bacterial cultures were washed twice in M9MM and diluted 1:100 in LB or M9MM, yielding an initial A_{600} of 0.02–0.04. Diluted culture (0.1 ml) was incubated in quadruplicate wells of a microtiter plate at 30 °C for 24 h, and the planktonic cell density was determined by measuring A_{600}. Planktonic cells were removed, and plates were washed in distilled water. Remaining biofilm was stained with 0.1% crystal violet solution (0.125 ml) and washed twice in distilled water. Crystal violet stain was solubilized in DMSO (0.2 ml) for 10 min, and 0.125 ml of each well was transferred to a visible flat bottom 96-well plate for determination of absorbance at 595 nm.

Bacterial Conjugation—Overnight cultures of E. coli SM10APir grown in LB medium and transformed with pCVD1623 or pCVD1624 as a donor and wild-type V. cholerae as a recipient were diluted in LB medium 1:10 and 1:100, respectively, and grown without antibiotics for 1 h. Equal volumes of E. coli and V. cholerae culture were mixed, inoculated on LB agar plates, and incubated for 8 h at 37 °C for conjugation. Conjugated cells were plated on LB medium without NaCl plus 5% sucrose plus streptomycin plus either chloramphenicol (ΔVC1623) or kanamycin (ΔVC1624) and incubated at 30 °C until colonies grew large enough for patching on ampicillin plates, which were used for selection of ampicillin-sensitive mutants with gene replacement.

Genetic Complementation of Null Strains—Complementation was carried out by introduction of the VC1623 and VC1624 ORFs under P_{BAD} promoter control. The VC1623 (primers pBAD1623F and pBAD1623R) and VC1624 (primers pBAD1624F and pBAD1624R) ORFs were PCR-amplified from V. cholerae genomic DNA and cloned into pBAD/Myc-HisA at XhoI and HindIII sites. The resulting plasmids pBAD1623 and pBAD1624 were electroporated in ΔVC1623 and ΔVC1624, respectively, and the resulting complemented mutant strains were named c ΔVC1623 and c ΔVC1624 (Table S2).

V. cholerae Mouse Model Competition Assays—Assays were performed using the infant mouse model of infection as previously described (36). Briefly, ~25 colonies of each strain were scraped from 37 °C overnight LB plates into 1 ml of phosphate-buffered saline, and the A_{600} was adjusted to 0.01 (~10^7 colony-forming units/ml). Inocula were prepared by mixing each mutant strain with the wild-type strain in a 1:1 ratio. Groups of 5-day-old CD-1 mice were anesthetized by inhalation of 2.5% isoflurane and were inoculated intragastrically with 50 μl. After 24 h, mice were euthanized, and the small intestines were removed, mechanically homogenized, and plated to determine the output ratios of wild-type and mutant strain. The competitive index was calculated as the output ratio of mutant/wild-type divided by the input ratio.

RESULTS AND DISCUSSION

Heterologous Biosynthesis of sym-Norspermidine in E. coli—Our bioinformatic analysis indicated that all sequenced genomes of Vibrio species lack orthologues of the known polyamine biosynthetic genes S-adenosylmethionine decarboxylase and spermidine synthase, consistent with the observed absence of corresponding S-adenosylmethionine decarboxylase and spermidine synthase enzymatic activities (25). However, a cluster of three genes is present in Vibrio species (Fig. 1B), potentially encoding a novel fusion protein of DABA AT and DABA DC (VC1625 in the V. cholerae El Tor strain), CANSDC (VC1623), and an ORF variously annotated as a conserved hypothetical protein, saccharopine dehydrogenase, or CANSDH (VC1624). There is no supportive data or publication for the unattributed annotation in a few species of VC1624 homologues as a CANSDH; however, its presence between the putative DABA AT/DC fusion and CANSDC ORFs in Vibrio species suggests that VC1624 could be a CANSDH and that the three-gene cluster could encode an entire alternative polyamine biosynthetic pathway. Heterologous expression of the cluster as an operon using pET28 in E. coli produced three proteins (Fig. 1B) corresponding in size to the predicted CANSDC (387 amino acids), putative CANSDH (414 amino acids), and DABA AT/DC fusion (961 amino acids). Analysis of polyamines in E. coli cells expressing all three ORFs revealed the novel accumulation of sym-norspermidine (Fig. 1C), which is not a native polyamine of E. coli, confirming that the three ORFs encode an entire sym-norspermidine biosynthetic pathway. Expression in E. coli of only the DABA AT/DC fusion protein (VC1625) led to the novel accumulation of the nonnative diaminopropane (Fig. 1C). This is the
first demonstration of heterologous biosynthesis of either diaminopropane or sym-norspermidine in any organism. Purified recombinant His-tagged DABA AT/DC fusion protein was assayed in vitro and was found to decarboxylate L-2,4-diaminobutyrate and L-ornithine among tested substrates and exhibited a preference for L-2,4-diaminobutyrate over L-ornithine by 1000-fold (Table 1).

Deletion of Putative CANSDH or CANSDC Abolishes sym-Norspermidine Biosynthesis in V. cholerae—Wild-type V. cholerae strain C6709 cells grown in M9 minimal medium contain diaminopropane, putrescine, and sym-norspermidine and a low level of spermidine (Fig. 2), and at stationary phase, putrescine is the most abundant polyamine. Deletion of either VC1624 (putative CANSDH) or VC1623 (CANSDC) abolished sym-norspermidine and spermidine accumulation and resulted in substantially increased accumulation of diaminopropane (Figs. 2 and 3), whereas putrescine levels remained unaffected, suggesting that putrescine levels are regulated independently of sym-norspermidine biosynthesis. Deletion of VC1623 resulted in the disappearance of norspermidine and the novel accumulation of carboxynorspermidine (Fig. 3), confirming VC1623 as the functional CANSDC. This is the first demonstration of a carboxypolyamine intermediate in vivo. Carboxynorspermidine was detectable in ΔVC1623 cells grown in LB medium but not M9 minimal medium, probably because the faster rate of growth in rich medium required elevated polyamine biosynthesis. Both the carboxynorspermidine and sym-norspermidine peaks were absent in the ΔVC1624 mutant (result not shown), confirming VC1624 as the functional CANSDH. Genetic complementation of the ΔVC1624 and ΔVC1623 mutant strains with overexpressed wild-type copies of the corresponding genes (c_ΔVC1624 and c_ΔVC1623, respectively) restored norspermidine biosynthesis (Fig. 2 and Table S3).
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Overexpression of the CANSDH in the cΔVC1624 mutant strain resulted in a 2.8-fold decrease in the level of putrescine, a 32% decrease in diaminopropionate, and an 18-fold increase in spermidine levels relative to the wild-type strain. In the V. cholerae wild-type strain, spermidine content was 15% that of sym-norspermidine; however, after overexpression of the CANSDH (in the cΔVC1624 strain), spermidine content rose to 296% that of sym-norspermidine, concomitant with the decrease in putrescine level (Fig. 2 and Table S3). These data suggest that the CANSDH enzyme is in excess, it increasingly uses putrescine as a substrate and forms carboxyspermidine, which is then converted to spermidine by CANSDC. The data show that the CANSDH/CANSDC alternative polyamine biosynthetic pathway is able to synthesize both sym-norspermidine and spermidine (Fig. 2).

To characterize the CANSDC enzyme activity, we achieved novel complete chemical syntheses of the substrates carboxynorspermidine and carboxyspermidine. The purified recombinant enzyme was assayed in vitro (for technical reasons, the CANSD orthologue from V. vulnificus was used) and revealed that carboxynorspermidine was the primary substrate and was decarboxylated 20-fold more efficiently than carboxyspermidine (Table 1). The CANSDC also exhibited some activity with L-ornithine but showed no activity with L-arginine, L-lysine, and meso-diaminopimelate.

Norspermidine Biosynthesis Is Essential for Biofilm Formation—Planktonic growth rate of the V. cholerae CANSDH and CANSDC null strains was reduced by 50–60% in chemically defined minimal medium (Fig. 4A, with sym-norspermidine and spermidine fully depleted in the deletion strains. Extracellular sym-norspermidine has been reported to increase biofilm formation in V. cholerae (14); thus, we assessed the ability of the ΔVC1624 and ΔVC1623 mutant strains to form biofilms by employing static growth conditions in LB medium with a microtiter assay. In wild-type V. cholerae cells, the addition of sym-norspermidine (0.5 mM) to the growth medium increased biofilm formation more than 2-fold, whereas spermidine had no significant effect (Fig. 4B), confirming the previous report (14). Both CANSDH and CANSDC were found to be essential for biofilm formation in minimal and rich media, and the defective biofilm formation of both mutants could be rescued by sym-norspermidine (0.5 mM) in the growth medium but not by spermidine (Fig. 4B and Table S4). Karatan et al. (14) proposed that extracellular sym-norspermidine is sensed by NspS, a putative periplasmic spermidine-binding protein, through which a signal is propagated that modulates the function of MbaA and increases cellular levels of dibutyl cyclic GMP, resulting in increased biofilm formation. They found that deletion of nspS decreased biofilm formation by 2-fold, and the biofilm defect could be rescued by extracellular sym-norspermidine but not by spermidine. The decrease in biofilm formation (>10-fold) with the CANSDH and CANSDC deletions was considerably greater than the defect caused by the ΔnspS mutant. This suggests that intracellular sym-norspermidine may be the key factor for biofilm formation, and it is formally possible that NspS may have an additional function as a periplasmic sym-norspermidine-binding protein for a polyamine uptake transporter, facilitating uptake of exogenous sym-norspermidine. In
the plague bacterium Yersinia pestis, genetic deletion of arginine and ornithine decarboxylases (speA and speC) abolished putrescine and spermidine biosynthesis (the native polyamines of Y. pestis), reduced planktonic growth by 60%, and greatly decreased biofilm formation (15). The defective biofilm formation could be rescued by putrescine but not by agmatine or spermidine. Thus, in Y. pestis, the spermidine precursor putrescine and not spermidine itself is of primary importance for biofilm formation, which is in sharp contrast to V. cholerae, where sym-norspermidine is the critical polyamine. In V. cholerae, unlike in Y. pestis, sym-norspermidine is used as the backbone scaffold in the synthesis of the siderophore vibriobactin (37). The link between vibriobactin biosynthesis and biofilm formation has not been assessed. It is notable that siderophore production is required for biofilm formation in Mycobacterium smegmatis and Pseudomonas aeruginosa (38, 39).

Rather than rescuing the defective biofilm formation, the genetically complemented CANSDH and CANSDC deletion strains overexpressed the corresponding wild-type genes exhibited an even greater defect in biofilm formation (Fig. 4B) than the original deletion strains. The biofilm defect was not rescued to wild-type levels by exogenous sym-norspermidine. However, exogenous sym-norspermidine increased relative biofilm formation in the genetically complemented deletion strains by the same degree as in the original knock-out strains (6–7-fold; Fig. 4B and Table S4). The inability of the genetically complemented deletion strains to restore biofilm formation to wild-type levels suggests that biofilm formation is sensitive either to perturbations in polyamine flux caused by overexpression of the biosynthetic enzymes or to the downstream physiological effects of these changes. Analysis of polyamines in the biofilm assay cells at the time of biofilm quantification indicated that in the absence of exogenous sym-norspermidine, spermidine was readily accumulated, but when sym-norspermidine was present, it outcompeted spermidine for accumulation (Fig. S1), suggesting that the polyamine transporter prefers sym-norspermidine.

Considering their effect on biofilm formation, the ΔVC1623 and ΔVC1624 mutant strains were tested for virulence by performing competition assays against the wild-type parental strain in the infant mouse model of infection (36). However, neither strain was attenuated for infectivity as indicated by competitive indices of 1.3 and 1.2 for VC1623 and VC1624, respectively. Thus, although each gene is required for biofilm formation in vitro, neither gene is required for colonizing the small intestine in this animal model of infection. This suggests that either the ability of V. cholerae to form biofilms is irrelevant for colonization or there is significant content of sym-norspermidine in the gut.

**Phylogenetic Distribution**—The V. cholerae polyamine pathway consists of two subsystems: putrescine and polyamine (norspermidine and spermidine) biosynthesis. Based on the reverse genetic and biochemical analyses herein and in silico genome inspection, a representation of the norspermidine/spermidine biosynthetic pathway of V. cholerae can be constructed (Fig. 5). The DABA AT/DC fusion protein is responsible for dianmonopropane production. A clustered gene pair of arginine decarboxylase (VCA0815, speA) and agmatine ureohydrolase (VCA0814, speB), which together synthesize putrescine from arginine, and an ornithine decarboxylase (VCA1063 speC), which forms putrescine directly from ornithine, are both likely to contribute to putrescine biosynthesis. Asparagine β-semialdehyde plays a key role in the Vibrio pathway as an aminopropyl group donor in the formation of sym-norspermidine and spermidine from dianmonopropane and putrescine, respectively, by CANSDH and CANSDC. It is also involved in the formation of 2,4-diaminobutyrate from glutamate for dianmonopropane biosynthesis.

The DABA AT/DC fusion protein is currently found only in the genomes of Vibrio and Photobacterium species and Allivibrio salmonicida (all within the Vibrionaceae). Clustering of DABA AT/DC, CANSDH, and CANSDC is also found only in Vibrio and Photobacterium species and Allivibrio salmonicida. The DABA AT, DABA DC, and CANSDC enzymes belong to...
mechanistically diverse superfamilies, so unequivocal functional assignment of orthologues is difficult without experimental support. In some species, DABA AT produces L-2,4-diaminobutyrate for incorporation into the compatible solute ectoine (40) or the siderophore pyoverdine (41). The presence of both DABA AT and DABA DC genes in a genome for diaminopropane biosynthesis does not necessarily mean that diaminopropane will be used for sym-norspermidine biosynthesis, because some species use diaminopropane for siderophore production, such as rhizobactin in Sinorhizobium meliloti (42). However, the presence of clustered gene pairs of DABA AT/DABA DC and CANS DH/CANS DC is reasonable support for the presence of diaminopropane and norspermidine/spermidine biosynthetic pathways in that genome.

The diaminopropane pathway, supported by a clustered pair of DABA AT and DABA DC genes, is present in species of the Firmicutes, Cyanobacteria, Actinobacteria, and beta-Proteobacteria phyla and in species of the euryarchaeote genus Halobacterium (Fig. 6). The alternative polyamine biosynthetic pathway represented by a clustered pair of CANS DH and CANS DC genes is present in species from all divisions of the Proteobacteria, the Firmicutes, and candidate phylum TG-1 (Fig. 7). For the DABA AT/DABA DC gene pair cluster, DABA AT is always upstream, whereas the relative positions of the genes in the CANS DH/CANS DC clustered gene pairs are variable. In only two species currently, Bacillus halodurans and S. meliloti are both clustered gene pairs present albeit unlinked physically. Although the aspartate semialdehyde pathway was proposed to function in the leguminous plant Lathyrus sativa (43), no sequences homologous to CANS DC or CANS DH are present in any plant genome or expressed sequence tag collection. Widespread absence of DABA AT and DABA DC in bacterial species where a clustered pair of CANS DH and CANS DC genes is present suggests that the alternative, aspartate semialdehyde-based pathway is widely used for spermidine rather than norspermidine biosynthesis. The alternative polyamine biosynthetic pathway is likely to play an important and as yet unappreciated role in the biology of diverse environmental, commensal, and pathogenic bacterial species.

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