Vol. 58: 311–321, 2010
doi: 10.3354/ame01367

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online February 11

Genes for transport and metabolism of spermidine in *Ruegeria pomeroyi* DSS-3 and other marine bacteria

Xiaozhen Mou[1,*], Shulei Sun[2], Pratibha Rayapati[2], Mary Ann Moran[2]

[1]Department of Biological Sciences, Kent State University, Kent, Ohio 44242, USA
[2]Department of Marine Sciences, University of Georgia, Athens, Georgia 30602, USA

ABSTRACT: Spermidine, putrescine, and other polyamines are sources of labile carbon and nitrogen in marine environments, yet a thorough analysis of the functional genes encoding their transport and metabolism by marine bacteria has not been conducted. To begin this endeavor, we first identified genes that mediate spermidine processing in the model marine bacterium *Ruegeria pomeroyi* and then surveyed their abundance in other cultured and uncultured marine bacteria. *R. pomeroyi* cells were grown on spermidine under continuous culture conditions. Microarray-based transcriptional profiling and reverse transcription-qPCR analysis were used to identify the operon responsible for spermidine transport. Homologs from 2 of 3 known pathways for bacterial polyamine degradation were also identified in the *R. pomeroyi* genome and shown to be upregulated by spermidine. In an analysis of genome sequences of 109 cultured marine bacteria, homologs to polyamine transport and degradation genes were found in 55% of surveyed genomes. Likewise, analysis of marine metagenomic data indicated that up to 32% of surface ocean bacterioplankton contain homologs for transport or degradation of polyamines. The degradation pathway genes *puuB* (γ-glutamyl-putrescine oxidase) and *spuC* (putrescine aminotransferase), which are part of the spermidine degradation pathway in *R. pomeroyi*, emerged as suitable targets for molecular-based studies of polyamine processing by marine bacterial communities. The frequency of genes encoding transport and catabolism of spermidine and related polyamines suggests an important role for these compounds in carbon and nitrogen budgets of marine bacterioplankton.

KEY WORDS: Polyamine · Transcriptomic analysis · Microarray · Marine bacteria · Dissolved organic nitrogen

INTRODUCTION

Spermidine and other polyamines are aliphatic organic compounds with multiple amino groups. They are synthesized by organisms across all 3 domains of life, playing vital roles in diverse cellular processes including nucleic acid and protein biosynthesis (Tabor & Tabor 1985, Higashibata et al. 2000, Kusano et al. 2007) and biosilica precipitation in diatom frustule formation (Kroger et al. 2000, Sumper & Kroger 2004). In the cytoplasm of bacteria and marine algae, intracellular soluble polyamine concentrations reach mM levels (Tyms 1989, Marian et al. 2000). As free constituents in seawater, however, they are only found at nM levels (Jorgensen et al. 1993, Lee & Jorgensen 1995, Nishibori et al. 2001, 2003), partly due to their active turnover by marine bacteria (Höfle 1984, Lee & Jorgensen 1995).

Compared to extensive studies on the concentration and fate of other dissolved organic nitrogen compounds in seawater, such as dissolved free amino acids (DFAA), investigations of bacterially-mediated polyamine transformations have been rare (Höfle 1984, Lee & Jorgensen 1995). Yet recent metagenomic and meta-

*Email: xmou@kent.edu

transcriptomic sequencing of marine microbial communities have recovered homologs of genes predicted to be involved in spermidine and putrescine transport and metabolism (Venter et al. 2004, Poretsky et al. 2005, Mou et al. 2008), in some cases showing evidence of differential distribution with ocean depth (DeLong et al. 2006). Furthermore, a recent Sargasso Sea study identified spermidine transporter proteins from SAR11 as an abundant component of the surface seawater metaproteome (Sowell et al. 2009). This sequence-based evidence suggests that polyamines are important substrates for heterotrophic microorganisms in the ocean, with implications for the cycling of both nitrogen and carbon.

In the present study, we focused on spermidine use by the marine Roseobacter member *Ruegeria pomeroyi* DSS-3. Up to now, systematic investigations of spermidine transport and catabolism have been restricted to a few model bacteria with clinical and medical implications, such as *Escherichia coli* K12 (Shaibe et al. 1985) and *Pseudomonas aeruginosa* PAO1 (Lu et al. 2002, Dasu et al. 2006). However, Alphaproteobacteria in the Roseobacter lineage are prevalent in marine surface waters, where they account for up to 20% of the total bacterioplankton in coastal areas and 10% in open oceans (Giovannoni & Rappe 2000). The genome sequence of *R. pomeroyi* contains a number of genes that might be involved in spermidine transport (Moran et al. 2004) and metabolism.

Transcriptional profiling using whole genome microarray analysis (Bürgmann et al. 2007) and qPCR was used to identify key genes for spermidine processing in *Ruegeria pomeroyi*, and to provide insights into transporter specificity. A comprehensive bioinformatic survey of polyamine-related genes in marine bacterial genomes and metagenomes confirmed the numerical abundance of polyamine transport and degradation genes among marine bacterioplankton, and identified 2 candidate genes for monitoring of polyamine transformations *in situ*.

MATERIALS AND METHODS

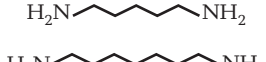
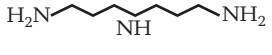
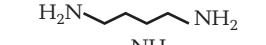
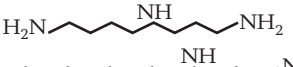
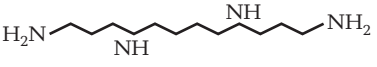
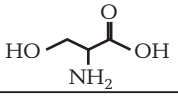
Culture conditions. *Ruegeria pomeroyi* DSS-3 cells were grown in a modified marine basal medium (MBM; Gonzalez et al. 2003) containing spermidine or putrescine as the sole carbon and nitrogen source. Serine was also used as a substrate to provide comparative expression data for an amino acid. The concentration of the 3 compounds was normalized to 3 mM carbon (i.e. 0.43 mM spermidine, 0.75 mM putrescine, or 1 mM serine, Table 1). Cells were grown in 200 ml chemostats at 30°C, a dilution rate of 0.125 h^{-1}, an airflow rate of 1 ml min^{-1}, and a stirring speed of 200 rpm. Chemostat cultures were maintained at a constant cell density of OD_{600} = 0.2 for at least 4 retention times prior to harvesting. To harvest the cells, the outflow pumps were set to 6 ml min^{-1}, and 9 ml of cells were directly collected in chilled tubes containing 1 ml stop solution (5% phenol, 95% ethanol, pH = 8). Chemostat cultures were established in quadruplicate for all 3 compounds in order to provide 4 independent replicates of each treatment for microarray or qPCR analysis.

RNA extraction, purification, and amplification. Cells harvested from the chemostat cultures were immediately centrifuged at 5000 × *g* (10 min) at 4°C; the cell pellets were then frozen at −80°C or used immediately for RNA extraction. Total RNA extraction, mRNA purification, and mRNA amplification to amino-allyl labeled antisense RNA (aa-aRNA) were performed following protocols described previously (Bürgmann et al. 2007).

Microarray hybridization and processing. The *Ruegeria pomeroyi* DSS-3 whole genome microarray was designed on a CombiMatrix Custom Array platform (Bürgmann et al. 2007). Along with probes for quality control, each array contains 12 000 probes that target 4161 out of 4348 identified genes in the *R. pomeroyi* genome (mostly 2 probes per gene; Bürgmann et al. 2007). The genes that were excluded from the array either had close homologs in the genome or did not meet probe design criteria with regard to hybridization temperature.

The aa-aRNA was fluorescently labeled and hybridized to the *Ruegeria pomeroyi* microarray as described previously (Bürgmann et al. 2007), except that a non-competitive hybridization scheme was used,

Table 1. Structure and C:N ratio of 5 polyamine compounds used in the present study and the amino acid serine

Compound	Formula	C:N	Chemical Structure
Cadaverine	$C_5H_{14}N_2$	2.5	
Norspermidine	$C_6H_{17}N_3$	2	
Putrescine	$C_4H_{12}N_2$	2	
Spermidine	$C_7H_{19}N_3$	2.33	
Spermine	$C_{10}H_{26}N_4$	2.5	
Serine	$C_3H_7NO_3$	3	

i.e. aa-aRNA was labeled only with a single dye (AlexaFluor dye 647; Invitrogen). After hybridization, the microarrays were scanned with an Axon GenPix 4000B microarray scanner (Molecular Devices Corporation) at 5 µm resolution. Images were acquired and analyzed using GenePix Pro 6.0 software (Molecular Devices Corporation). The detection limits (DL) were calculated based on reading of the 149 empty spots on the array using the equation: DL = average sum of medians + 2×(SD). Spots with intensity below the DL and those identified as bad, empty, and not meeting quality assurance were excluded from further analysis.

Background corrected expression data from each array were globally normalized by trimmed means (2% from each side) and $\log_2$ transformed prior to being imported into the Acuity 4.0 software (Molecular Devices Corporation). Analysis datasets were created using the conditions (signal-to-noise ratio > 3; circularity > 80; F635% < 2; B635 CV < 50) to exclude probes with features close to background, saturated, with bad circularity, or with highly non-uniform intensities or background.

Gap statistics predicted that the optimal cluster size for the array data was 3. Self Organizing Maps (SOM) cluster analysis was then performed within Acuity using a 1×3 cluster matrix, the Euclidean squared similarity metric, and data centering (Fig. S1 available as supplementary material at www.int-res.com/articles/suppl/a058p311_app.pdf). The 4 experimental replicates were averaged to calculate the fold change between the spermidine samples and serine controls. *t*-test parameters and false discovery rates (FDR) were calculated to determine the significance of observed differences. Upregulated genes were reported when gene probes in spermidine samples showed more than 2-fold increase in expression level than those in serine controls, with *t*-test p values < 0.05, false discovery rate < 10%, and membership in SOM probe clusters that showed increased expression in the spermidine relative to the serine samples. Downregulated genes were reported when genes showed an opposite but equivalent response. The remainder of the genes were designated as non-responding. Microarray data were deposited in the Gene Expression Omibus database (www.ncbi.nlm.nih.gov) under accession number GLP4067.

RT-qPCR. For microarray result verification and transporter preference analysis, 12 genes were analyzed by reverse transcription (RT)-qPCR using mRNA obtained independently from that used for microarray analysis. The genes included each of the polyamine-binding components of 6 complete polyamine ABC transporter systems and another 2 genes randomly selected from each of the upregulated, downregulated, and non-responding genes. Primer sets for each gene were designed using Geneious Pro software (Biomatters) and are listed in Table S1 available as supplementary material at www.int-res.com/articles/suppl/a058p311_app.pdf. The designed annealing temperature for each primer set ranged from 59 to 61°C. The practical annealing temperature for all primer sets was chosen by performing a gradient PCR assay (annealing temperature varied between 57 and 65°C) using the genomic DNA of *Ruegeria pomeroyi* as template, and this converged at 60°C. Both aa-aRNA and non-amplified mRNA extracts that had only been treated for rRNA removal (mRNA only kit; Invitrogen) were used as RT-qPCR templates. RNA samples were quantified by spectrophotometer and were reverse transcribed to cDNA with random hexamer primers at a concentration of 0.3 µg μl^{-1} using iScript (Bio-Rad) according to the manufacturer's instructions. Triplicate qPCR reactions were conducted in 25 µl volumes on an iCycler IQ multicolor Real-Time PCR detection System (Bio-Rad). Each reaction contained 1 µl of cDNA template or standard, forward and reverse primers at a final concentration of 10 pM each, and 1× IQ SYBR Green Supermix (Bio-Rad). Control reactions omitting template or reverse transcriptase were included for each analysis. The qPCR program contained an initial denaturation step (95°C, 5 min) and 45 amplification cycles, each consisting of a denaturation step (95°C, 45 s), annealing step (60°C, 90 s), and a final melting curve analysis. Standards were obtained from a dilution series of PCR amplicons of RNase-treated *R. pomeroyi* genomic DNA over 6 orders of magnitude for each primer set and used for calculating fold changes between treatments. For transporter substrate preference analysis, an identical RT-qPCR procedure was performed except that only the gene sets for the 6 polyamine-binding protein genes were used and mRNA was obtained from both spermidine- and putrescine-grown chemostat cultures.

Phylogenetic analysis. The amino acid sequences of the putative polyamine substrate-binding proteins in *Ruegeria pomeroyi* were aligned based on Blosum62 using ClustalW with the MEGA4 program (Kumar et al. 2004) and checked manually. The resulting distance matrix was used for generating a phylogenetic tree using the minimum evolutionary, neighbor-joining, and UPGMA algorithms with 1000 bootstraps using MEGA4.

Polyamine growth survey. *Ruegeria pomeroyi* DSS-3 cells were grown in MBM containing the following single polyamine compounds as sole carbon and nitrogen sources: spermidine, putrescine, cadaverine, norspermine, and spermine. Serine provided comparative data for an amino acid. The concentration of each compound was normalized to 3 mM carbon (i.e. 0.43 mM spermidine, 0.75 mM putrescine, 0.6 mM

cadaverine, 0.5 mM norspermidine, 0.3 mM spermine, and 1 mM serine; Table 1). Cells were grown in the dark at 30°C with shaking at 200 rpm. Biomass was measured as the optical density of cells at a wavelength of 600 nm ($OD6_{00}$) at regular intervals until the cells reached a stationary phase. Batch cultures were established in triplicate.

Bioinformatic analysis. Polyamine transport systems (gene designation: *pot*) were putatively identified from sequenced marine bacterioplankton genomes in the Moore Microbial Genome sequencing database (data freeze date: 1 October 2008; https://moore.jcvi.org/moore/) based on key word searches. A blastp assay was also performed using identified polyamine-binding proteins in the genome of *Escherichia coli* K12 (*potD* and *potF*), with an E value cutoff of $<10^{-20}$. The candidates from the 2 procedures were combined and then manually inspected for orthology to polyamine transporters and to ensure that each identified *pot* system contained consecutive genes that encoded at least 1 copy of each of the 4 components, which is required for a polyamine transporter to function. Polyamine degradation genes do not require consecutive gene systems to function. A reciprocal best hit methodology with an E value cutoff of $<10^{-30}$ was used to identify orthologs to *puuB* and *spuC* in the sequenced marine bacterioplankton genomes in the Moore genome database.

Homologs to experimentally confirmed polyamine-binding proteins (*potD* and *potF*) were identified in the Global Ocean Survey (GOS) dataset using blastp with an E value cutoff of $<10^{-20}$. The 10 GOS hits at the boundary of the cutoff were blasted back to the NCBI Refseq database (www.ncbi.nlm.nih.gov/RefSeq/) and the *Escherichia coli* and *Ruegeria pomeroyi* genomes. If 2 or more hits were not to the correct functional category, the E value cutoff was decreased by 5 orders of magnitude for a subsequent blast. The same procedure was adopted for the identification of homologs to *R. pomeroyi* SPO3465 (predicted as *puuB*) and SPO3473 (predicted as *spuC*) in the GOS dataset. The final cutoff for all analyses converged on an E value of $<10^{-30}$. The acetylornithine aminotransferase gene (*argD*) homolog in *R. pomeroyi* (SPO0962) has a related but distinct function to *spuC.* The blast hits that had a higher bit score for *argD* than *spuC* in blastp analyses were removed from the final list of *spuC* homologs. Paired read sequences in GOS data were only counted once.

Homologs to polyamine synthesis genes in *Ruegeria pomeroyi* were obtained by blasting known genes from *E. coli* K12 (Blattner et al. 1997, http://ecoli.naist.jp/GB6/search.jsp) and *Pseudomonas aeruginosa* PAO1 (Stover et al. 2000, www. pseudomonas.com) to the *R. pomeroyi* genome sequence using blastp through the RoseoBase website (www.roseobase.org). Gene orthologs were reported when the reciprocal best hit E value was $<10^{-30}$.

RESULTS

Microarray analysis and quality control

Ruegeria pomeroyi cells were grown on spermidine or serine under steady-state conditions in a chemostat with fixed cell growth rates, temperature, pH, and air flow. This effort minimized gene expression artifacts due to factors other than substrate differences. Of the 4161 genes that were arrayed, significant increases in mRNA levels were found for 92 genes in spermidine treatments relative to serine treatments (i.e. ≥2-fold higher normalized fluorescence; *t*-test, $p < 0.05$; Table 2); these were designated as upregulated genes (Fig. 1). About one-third of the upregulated genes

Table 2. *Ruegeria pomeroyi.* Upregulated genes hypothesized to be involved in spermidine transport and degradation by *R. pomeroyi.* Complete lists of up- and downregulated genes are provided in Tables S2 & S3 available as supplementary material at www.int-res.com/articles/suppl/a058p311_app.pdf

Gene locus tag	COG	Gene name	Annotation
SPO1300	COG0174		Glutamine synthetase family protein
SPO1301	COG0518		Glutamine amidotransferase class I
SPO1302	COG0174		Glutamine synthetase family protein
SPO2659	COG0765	*gltJ*	Glutamate/aspartate ABC transporter, permease protein
SPO3465			Conserved hypothetical protein
SPO3466	COG1177	*potI*	Putrescine ABC transporter, permease protein
SPO3467	COG1176	*potH*	Putrescine ABC transporter, permease protein
SPO3468	COG3842	*potG*	Putrescine ABC transporter, ATP-binding protein
SPO3469	COG0687	*potF*	Putrescine ABC transporter, periplasmic substrate-binding protein
SPO3471	COG0161		Aminotransferase class III
SPOA0273			DNA-binding protein, putative

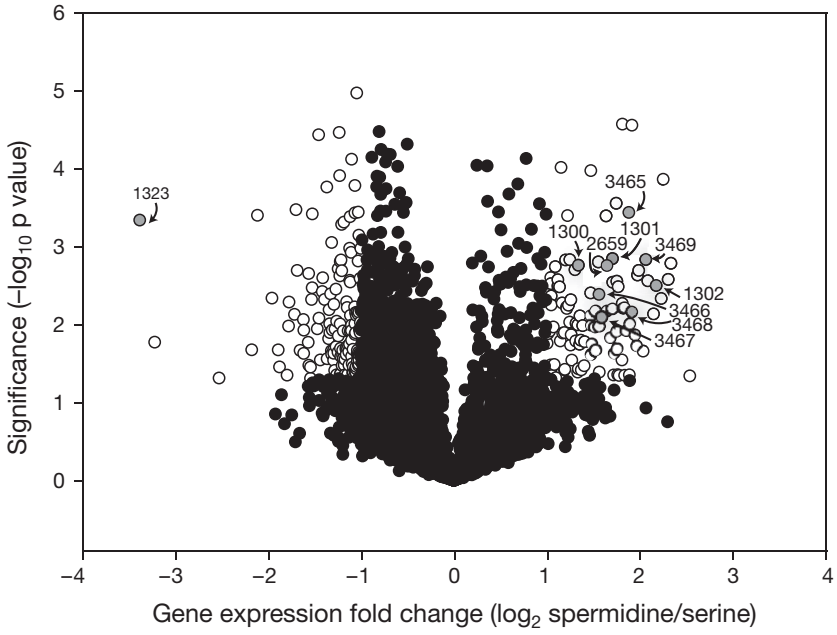


Fig. 1. *Ruegeria pomeroyi.* Transcriptional response of genes grown on spermidine relative to serine shown on a volcano plot. Up- or downregulated genes (≥2 fold expression change; $p < 0.05$) in spermidine treatments relative to the serine treatments are illustrated as open or gray circles, with locus tag coding of the gray circles the same as in Tables 2, S2, & S3. Genes showing no significant transcriptional changes are illustrated as black circles

(29 genes) had sequences too divergent to allow annotation of even general function (Table S2 provided as supplementary material at www.int-res.com/articles/suppl/a058p311_app.pdf). Most of the remainder represented functions involved in substrate transport (12 genes), nitrogen metabolism (12), carbon metabolism (10), and protein regulation (10). As expected, the L-serine ammonia-lyase gene (*sdaA*), a gene for serine degradation, was downregulated in the spermidine treatment relative to serine (*t*-test, $p < 0.05$), as were other serine metabolism-related genes (Table S3). RT-qPCR using original mRNA extracts and amino-allyl labeled antisense RNA (aa-aRNA, the same form as the microarray template) agreed well with each other and both agreed with the microarray data (Fig. 2).

Transport genes upregulated by spermidine

In bacteria, exogenous polyamines are thought to be mainly transported by ATP-binding cassette transport systems (ABC-type transporters; Tabor & Tabor 1985). Each polyamine transport system (gene designation: *pot*) typically consists of 4 indispensable components that are encoded by contiguous genes, i.e. 1 periplasmic substrate- binding protein, 2 hydrophobic integral membrane proteins (permeases), and 1 hydrophilic peripheral membrane ATP-binding protein. The genes of the substrate-binding proteins are the most divergent, while the genes of the ATP-binding proteins are the most conserved (Tam & Saier 1993, Saurin & Dassa 1994).

In the *Ruegeria pomeroyi* genome, 6 complete sets of 4-component *pot* systems have been predicted based on sequence homology (Moran et al. 2004). Only 1 set, SPO3466–SPO3469, was upregulated by exogenous spermidine in the microarray analysis (Table 2) and,

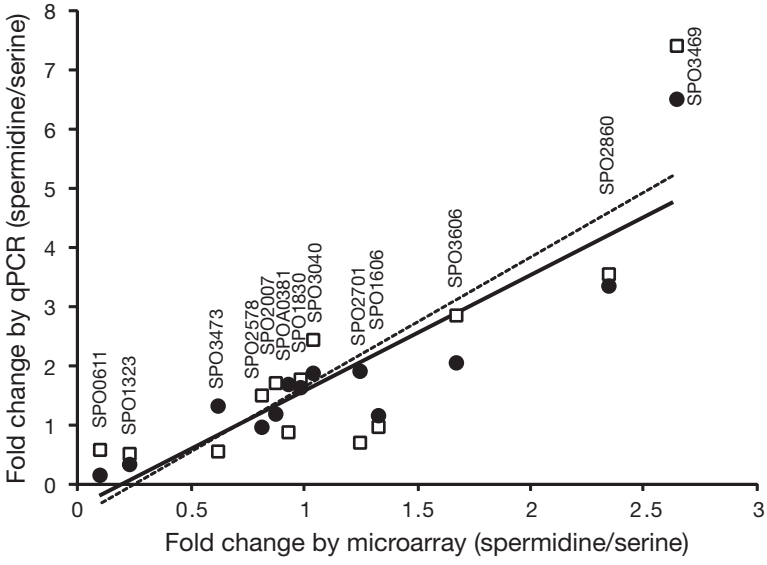


Fig. 2. *Ruegeria pomeroyi.* Validation of microarray data shown by correlations with RT-qPCR-based analysis of expression level changes relative to serine. Closed circles, solid trend line: mRNA; open squares, dashed trend line: amino-allyl labeled antisense RNA. Gene locus tags are labeled adjacent to corresponding data points (see Tables 2, S2 & S3)

unexpectedly, this system has been annotated as a putrescine-specific transport system with gene designations *potFGHI*. Comparing the amino acid sequence of the substrate-binding protein of the upregulated transporter system, SPO3469, to experimentally confirmed substrate-binding proteins in other bacteria, we found high similarity to both putrescine and spermidine-binding proteins. SPO3469 was 50% identical in amino acid sequence to *potF* (experimentally shown to be specific for putrescine binding in *Escherichia coli*), 30% identical to *potD* (experimentally shown to bind both spermidine and putresine in *E. coli*, but with a preference for the former; Igarashi & Kashiwagi 1999), and 51% and 47% identical to *spuD* and *spuE*, the 2 preferential spermidine-binding proteins in a 5-component *pot* system (SpuDEFGH) of *Pseudomonas aeruginosa* (Lu et al. 2002).

Amino acid sequence alignments were used to assess residue conservation and phylogenetic relatedness of the binding protein upregulated in response to spermidine (SPO3469), the other 5 putative *pot* system binding proteins in *Ruegeria pomeroyi*, and the experimentally confirmed spermidine- and putrescine-binding proteins in *Escherichia coli* and *Pseudomonas aeruginosa*. SPO3469 and another putative *pot* binding protein gene from *R. pomeroyi* (SPO1606) consistently clustered with *potD* and *potF* from *E. coli* and *spuD and spuE* from *P. aeruginosa* (Fig. 3). However, SPO3469 is the only polyamine-binding protein in the *R. pomeroyi* genome that is conserved for all 3 amino acid residues experimentally demonstrated to be the most critical to spermidine binding by *potD* in *E. coli* (Igarashi & Kashiwagi 1999; Fig. S2).

Substrate preference of *Ruegeria pomeroyi pot* systems

The high sequence divergence of polyamine-binding protein genes among the 6 *pot* systems in the *Ruegeria pomeroyi* genome (averaging only 19% sequence identity; Fig. 3) suggests differing substrate preferences. This idea is consistent with the high redundancy of *pot* transporters (6 sets) in the *R. pomeroyi* genome, as well as the apparent expression of only 1 of these in response to growth on spermidine. To obtain additional information on transporter specificity, the relative transcriptional response of the 6 polyamine-binding genes was compared by RT-qPCR for chemostat-grown cells with spermidine or putrescine as the substrate. SPO3469 had 6.5-fold increased transcription during growth on spermidine, in accordance with the microarray data. SPO3473 and SPO1606 showed no upregulation in response to spermidine, but had 3.2- and 4.5-fold increased transcription during growth on putrescine (Table 3). SPO2007, SPO2701, and SPOA0381, the putative polyamine-binding protein genes in 3 other *pot* systems in the *R. pomeroyi* genome (Fig. 3), were not significantly upregulated by either spermidine or putrescine, and likely transport other polyamines or related compounds. Batch culture assays indicated that at least 3 additional polyamines (cadaverine, norspermidine, and spermine; Table 1) could be transported and metabolized by *R. pomeroyi* (Fig. S3 available as supplementary material at www.int-res.com/articles/suppl/a058p311_app.pdf).

Catabolic genes upregulated by spermidine

Three pathways have been hypothesized previously to mediate bacterial spermidine degradation (Fig. 4).

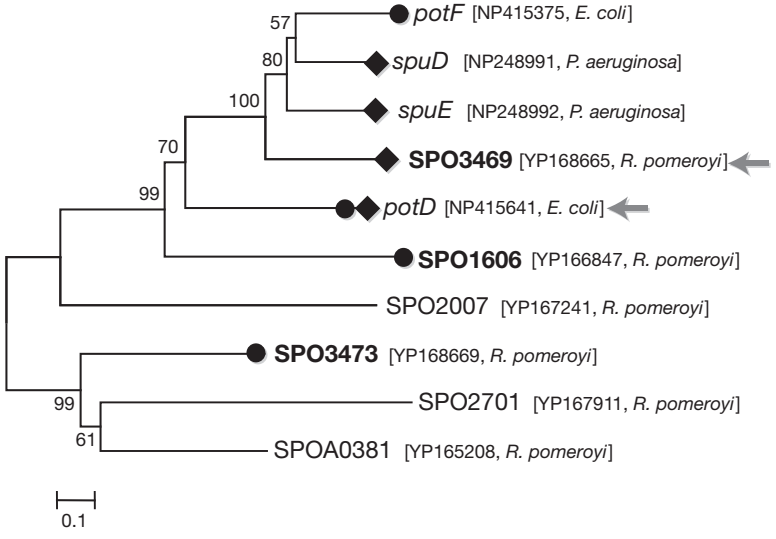


Fig. 3. *Ruegeria pomeroyi.* Phylogenetic tree based on amino acid sequences of polyamine-binding proteins in experimentally confirmed *pot* systems and in 6 putative *pot* systems in the genome of *R. pomeroyi*. The tree was constructed using minimum evolution with 1000 bootstrap replications, and bootstrap values >50% are indicated at the branch nodes. Scale bar indicates the amount of genetic change in terms of the number of amino acid substitutions per site. GenBank accession numbers and source organisms are shown in brackets. Bold font indicates *R. pomeroyi* spermidine and putrescine transporter genes identified in the present study. Black circles and diamonds indicate binding proteins shown experimentally to transport putrescine and spermidine, respectively. Gray arrows indicate binding proteins with 3 conserved amino acids hypothesized to be critical for spermidine binding (Igarashi & Kashiwagi 1999). Other tree-building algorithms (UPGMA, neighbor joining) resulted in nearly identical branching patterns

Table 3. *Ruegeria pomeroyi.* Average fold changes in abundance of transcripts for polyamine-binding protein genes of *R. pomeroyi* based on RT-qPCR of chemostat-grown cells in polyamine treatments relative to serine treatments. Each fold-change result was an average of 9 sets of data (3 RT-qPCR technical replicates × 3 chemostat samples)

Samples	SPO1606	SPO2007	SPO2701	SPO3469	SPO3473	SPOA0381
Putrescine mRNA[a]	3.2	0.9	1.8	1.4	4.5	1.1
Spermidine mRNA[a]	1.1	1.2	1.9	6.5	1.3	1.7
Spermidine aa-aRNA[b]	1.1	1.7	0.7	7.4	0.6	0.9

[a]mRNA used as template; [b]amino-allyl-labeled antisense RNA used as template

Two of them channel spermidine through putrescine degradation pathways, and these are further divided into the transamination (Lu et al. 2002) and γ-glutamylation routes (Kurihara et al. 2005). The third pathway involves oxidative cleavage of spermidine into 4-aminobutyraldehyde and 1, 3-diaminopropane before further degradation to cellular intermediates (Dasu et al. 2006). All 3 routes produce intermediates for the tricarboxylic acid (TCA) cycle and other cellular processes. The microarray data suggested that 2 of these pathways are operational in *Ruegeria pomeroyi*. All key genes of the transamination and γ-glutamylation routes have putative homologs in the genome of *R. pomeroyi*, and as detailed below, many were upregulated during growth on spermidine (Table 2, Fig. 4).

Upregulated genes SPO3465 and SPO3471 are located on either side of the upregulated *pot* transport system SPO3466–SPO3469 (Fig. 5). SPO3465 shares 30% identity with the γ-glutamyl-putrescine oxidase gene of *Escherichia coli* (*puuB*; Kurihara et al. 2005) in the γ-glutamylation route of putrescine degradation (Fig. 3). SPO3471 shares 60% identity with the putrescine aminotransferase gene (*spuC*), which catalyzes the removal of 1 of the 2 amino groups from putrescine (Lu et al. 2002; Fig. 4). The adjacent gene SPO3470 had a higher expression during growth on spermidine compared to serine (although it did not meet the significance criteria) and is annotated as a GntR-type transcriptional regulator. The 7 consecutive genes SPO3465– SPO3471 together appear to form a cluster that functions in both spermidine uptake and degradation (Fig. 5).

The upregulated genes SPO1300 and SPO1302 are both similar to the γ-glutamyl-putrescine synthase gene *puuA* (both 35% identical), which catalyzes the first step of the γ-glutamylation pathway in putrescine degradation (Kurihara et al. 2005; Fig. 4). This observed duplication of *puuA* homologs is consistent with the *Pseudomonas aeruginosa* genome (*spuB* and *spuI*, both of which have been shown experimentally to function in spermidine degradation; Lu et al. 2002). SPO1301 is annotated as a glutamine amidotransferase (GATase), which mediates the biosynthesis of a variety of organic nitrogen compounds including nucleotides. Putative GATases in *Escherichia coli* (designated *puuD*) and *P. aeruginosa* (designated *spuA*) have been shown to be involved in spermidine degradation (Lu et al. 2002, Kurihara et al. 2005), but both have low identities to SPO1301 (~15% identical in each case). While SPO1300–SPO1302 are located distantly from

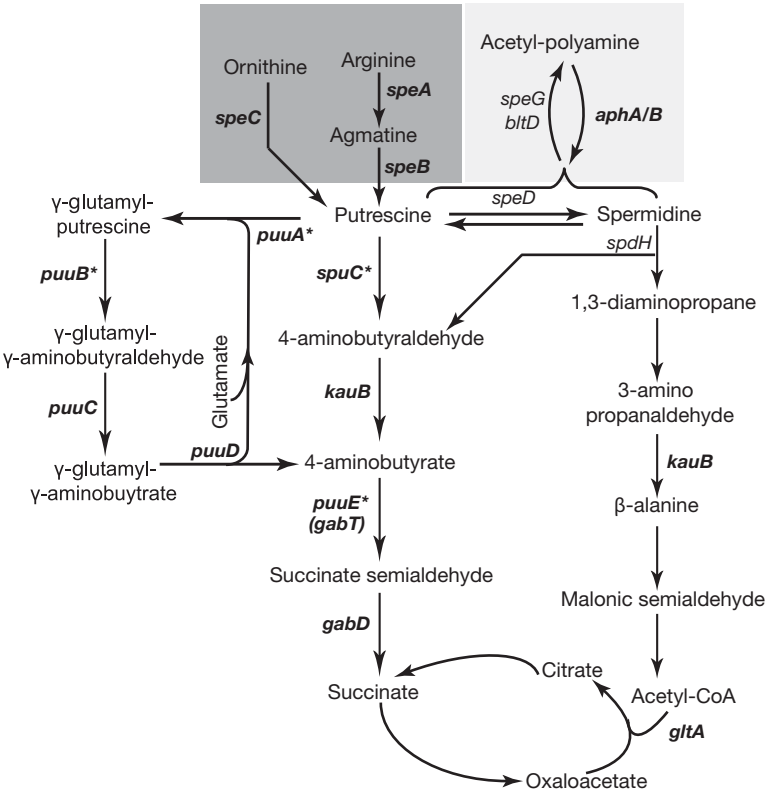


Fig. 4. Hypothesized spermidine degradation pathways in bacteria. Gene names with normal font: no homolog in *Ruegeria pomeroyi*; bold font: homolog in *R. pomeroyi*; bold font and asterisk: homolog upregulated by spermidine in *R. pomeroyi*; arrow with no gene label: gene has not yet been identified (modified after Dasu et al. 2006, Chou et al. 2008)

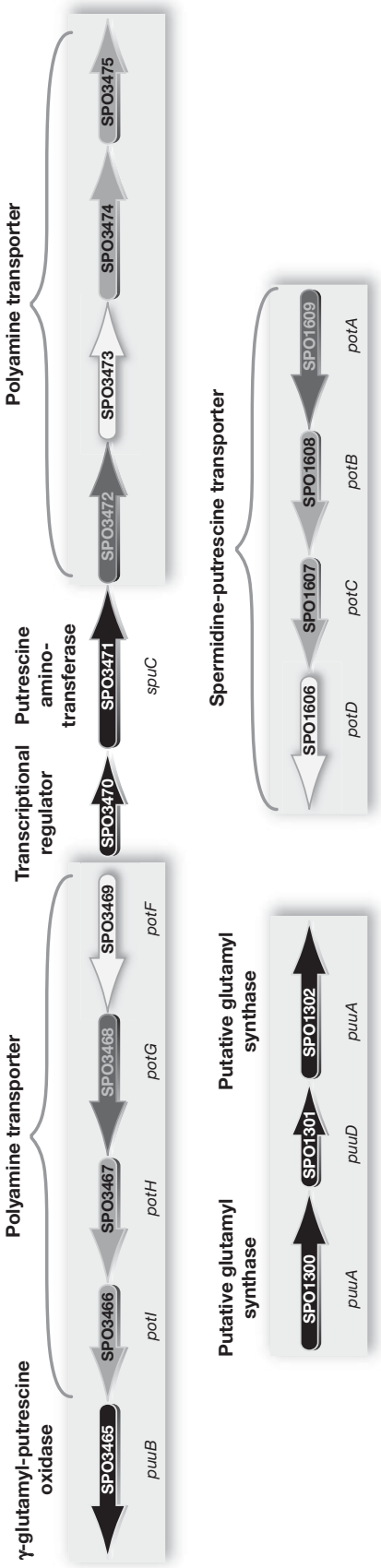


Fig. 5. *Ruegeria pomeroyi*. Organization of the spermidine- and putrescine-related gene clusters in the *R. pomeroyi* genome. Raised boxes indicate predicted operons. Within the clusters that contain genes to transport polyamines, white color coding indicates polyamine-binding protein genes, light gray indicates permease genes, and dark gray indicates ATP-binding protein genes. These genes occur in 3 separate clusters in the *R. pomeroyi* genome (SPO3465 through SPO3475; SPO1300 through SPO1302; and SPO1606 through SPO1609)

SPO3465–SPO3471 in the genome of *Ruegeria pomeroyi* (Fig. 5), orthologs have been found in a single cluster in other genomes (Lu et al. 2002, Kurihara et al. 2005). Upregulation of SPO2659, a putative glutamate transport gene (*gltJ*), might indicate a higher demand for glutamate in response to increased activity of the γ-glutamylation degradation route.

Increased expression of many homologs of genes with demonstrated function in putrescine degradation indicates that spermidine is converted to putrescine in *Ruegeria pomeroyi* (Table 2, Fig. 4), as has been hypothesized in a *Pseudomonas* strain (Padmanabhan & Kim 1965). We found no homologs for the third pathway for spermidine degradation involving direct oxidative cleavage that has been demonstrated in *P. aeruginosa* (Dasu et al. 2006; Fig. 4). Overall, the microarray and qPCR data indicate that *R. pomeroyi* transports exogenous spermidine into the cell by a dedicated ABC-type transporter, degrades it to putrescine, and then ultimately to the TCA cycle intermediate succinate. Ammonia, alanine, and glutamate are also generated along the degradation pathway (Fig. 4), satisfying both carbon and nitrogen demands of the cell.

Polyamine genes in marine bacterial genomes and metagenomes

A bioinformatic survey of 109 marine heterotrophic bacterioplankton genomes identified a total of 196 complete polyamine transport systems in 74 genomes from many major bacterioplankton groups (Table 4). Complete transporter gene sets are found in all Actinobacteria, Rhizobiales, Roseobacter, SAR11, Oceanospirillales, and Vibrionales genomes (2, 4, 2, 2, and 10 genomes, respectively). Groups with few or no transporters include Bacteriodetes and Planctomyces (15 and 2 genomes, respectively). Multiple *pot* systems are often found in the same genome. For example, roseobacters average >4 *pot* systems per sequenced genome. Some bacterioplankton, such as the 2 SAR11 representatives, are equipped with only a single *pot* system. Because polyamine-binding proteins may not cluster according to substrate specificities in phylogenetic analyses (Fig. 3), the substrates of these marine bacterioplankton *pot* transporters could not be identified further.

pot systems were also found with high frequency in the GOS metagenomic dataset, based on blastp queries using experimentally-confirmed polyamine-binding protein genes (Venter et al. 2004, Rusch et al. 2007; Table 5). Ratios of putative *pot* binding proteins to universal single-copy genes (Howard et al. 2008) indicate that as many as 32% of surface marine bacte-

Table 4. Frequency of genes for polyamine transport (*pot*) and degradation (*puuB* and *spuC*) in 109 sequenced marine bacterioplankton genomes. Table entries show the number of multi-gene systems (*pot*) or single genes (*puuB* and *spuC*) per genome. Only taxa with positive results are listed. The notation in parenthesis shows the number of positive genomes/total number of genomes surveyed for each group.

Bacterioplankton taxa	*pot*	*puuB*	*spuC*
Alphaproteobacteria			
Roseobacter (23/23)			
Alpha proteobacterium HTCC2255	3	1	1
Loktanella vestfoldensis SKA53	1	1	
Oceanicola batsensis HTCC2597	3	1	1
Oceanicola granulosus HTCC2516	2	1	
Oceanibulbus indolifex HEL-45	3	1	1
Octadecabacter antarcticus 307	4	1	
Phaeobacter gallaeciensis 2.10	3	1	1
Phaeobacter gallaeciensis BS107	2	1	1
Rhodobacterales bacterium HTCC2150	2	1	1
Rhodobacterales bacterium HTCC2654	2	1	1
Roseobacter litoralis Och 149	6	1	1
Roseobacter sp. AzwK-3b	3	1	1
Roseobacter sp. CCS2	2	1	1
Roseobacter sp. MED193	2	1	1
Roseobacter sp. SK209-2-6	4	1	1
Roseovarius nubinhibens ISM	7	1	1
Roseovarius sp. 217	8	1	1
Roseovarius sp. HTCC2601	3	1	1
Roseovarius sp. TM1035	5		1
Ruegeria pomeroyi DSS-3	6	1	1
Sagittula stellata E-37	6	1	1
Sulfitobacter sp. EE-36	2	1	1
Sulfitobacter sp. NAS-14.1	2	1	1
Other Rhodobacterales (2/3)			
Stappia aggregata IAM 12614	5	1	1
Stappia alexandrii DFL-11	2	1	1
SAR11 (2/2)			
Pelagibacter ubique HTCC1002	1	1	
Pelagibacter sp. HTCC7211	1	1	
Rhizobiales (4/4)			
Aurantimonas sp. SI85-9A1	1	1	
Fulvimarina pelagi HTCC2506	1	1	
Hoeflea phototrophica DFL-43	2	1	1
Nitrobacter sp. Nb-311A	1		
Other alpha (1/7)			
Alpha proteobacterium BAL199	4	1	1
Gammaproteobacteria			
Alteromonadales (6/9)			
Alteromonas macleodii Deep ecotype		1	
Marinobacter algicola DG893	2		
Marinobacter sp. ELB17	4	1	
Moritella sp. PE36	1		
Pseudoalteromonas tunicata D2	1	1	
Shewanella benthica KT99	1		1
Oceanospirillales (3/3)			
Marinomonas sp. MED121	3	1	1
Oceanobacter sp. RED65	2	1	1
Oceanospirillum sp. MED92	2	1	1
Vibrionales (10/10)			
Photobacterium profundum 3TCK	2	1	
Photobacterium sp. SKA34	3	1	
Vibrio alginolyticus 12G01	4	1	
Vibrio angustum S14	4	1	
Vibrio campbellii AND4	2		
Vibrio fischeri MJ11	2		
Vibrio shilonii AK1	4	1	1
Vibrio sp. MED222	2	1	
Vibrio splendidus 12B01	2	1	
Vibrionales bacterium SWAT-3	2	1	
Other gamma (3/6)			
Congregibacter litoralis KT71		1	1
Marine gamma HTCC2080		1	1
Reinekea sp. MED297	1		1
Actinobacteria (2/2)			
Janibacter sp. HTCC2649	1		1
Marine actinobacterium PHSC20C1	2		1
Firmicutes (3/4)			
Bacillus sp. B14905	1		
Bacillus sp. NRRL B-14911	1		
Carnobacterium sp. AT7	1		
Bacteroidetes			
Flavobacteria (1/12)			
Psychroflexus torquis ATCC 700755			1
Other bacteroidetes (1/3)			
Microscilla marina ATCC 23134			1
Cyanobacteria (2/10)			
Lyngbya aestuarii CCY9616	2		
Prochlorococcus marinus MIT 9211		1	
Planctomyces (0/2)			
Other (3/10)			
Caminibacter mediatlanticus TB-2	1		
Marinitoga piezophila KA3	1		
Plesiocystis pacifica SIR-1	1		

rioplankton cells could contain a *pot* system. This number is an overestimate of per-cell frequency if multiple *pot* systems are present in some bacterioplankton genomes, as is the case for cultured roseobacters (Table 4), or if 1 *pot* system contains >1 polyamine-binding protein, as is the case for *Pseudomonas aeruginosa* (Lu et al. 2002). This frequency is lower than that found for *pot* systems in cultured marine bacterioplankton (68%; Table 4).

In situ probes for polyamine degradation

Homologs to *Ruegeria pomeroyi* spermidine degradation genes SPO3465 (*puuB*) and SPO3473 (*spuC*) (putrescine transamination and γ-glutamylation pathways, respectively) were found with high frequency in sequenced marine bacterioplankton genomes and the GOS metagenomic dataset (Tables 4 & 5). Of the 109 genomes examined, 47 *puuB* and 36 *spuC* orthologs were identified (reciprocal best hits in blastp analyses, Table 4). These putative orthologs are distributed widely among major marine bacterial taxa, and are particularly prevalent in the Roseobacter lineage. The 2 representative bacteria in the SAR11 clade each had a single ho-

Table 5. Frequency of genes for polyamine transport (substrate-binding proteins *potD and potF*) and degradation (*puuB* and *spuC*) in the Global Ocean Sampling (GOS) metagenomic data, expressed as homolog number (and % of cells ± SD), assuming no more than one gene copy per cell

GOS sites[a]	Genomes sampled[b]	*potD/F*	*puuB*	*spuC*
Coastal (n = 19)	1222	409 (32 ± 12%)	108 (10 ± 7%)	189 (17 ± 11%)
Open ocean (n = 13)	1334	500 (32 ± 11%)	183 (11 ± 7%)	340 (24 ± 16%)
Hypersaline (n = 1)	298	44 (15%)	17 (6%)	40 (13%)
Estuary and Other (n = 10)	962	206 (21 ± 14%)	39 (5 ± 7%)	66 (10 ± 11%)

[a]GOS data also include 6 pilot Sargasso Sea datasets (Sites 2 to 7)
[b]Genome equivalents sequenced at each GOS site are taken from Howard et al. (2008) and are based on numbers of homologs of the single-copy gene *recA*

molog of these genes (Table 4). Assuming a single copy per cell, *puuB* and *spuC* homologs are present in 10% and 17% of bacterioplankton cells in coastal metagenomic libraries, and 11% and 24% in open ocean libraries (Table 5; frequencies are not significantly different between coastal and open ocean GOS sites; *t*-test, $p > 0.05$). For most marine bacterial genomes, *puuB* and *spuC* co-occur with *pot* system genes for transport of exogenous polyamines into the cell (Table 4).

DISCUSSION

Intracellular polyamines are found in virtually all living organisms at cellular concentrations in the micromolar range. While this source of organic carbon and nitrogen should be valuable to marine heterotrophic bacteria, whose growth in seawater is often limited by carbon or nitrogen, bacterially-mediated polyamine transformation has been studied in only a few cases. Results generated from the early studies are somewhat contradictory in terms of the fate of the carbon in exogenous polyamines. In one study, exogenous ^{14}C-putrescine was preferentially respired to CO_2 (~85%) rather than incorporated in biomass (~6%) by assemblages of marine bacterioplankton (Höfle 1984). In another, the same polyamine appeared to be largely incorporated into cell biomass by bacterial assemblages in a coastal salt pond (40 to 75% in an oxic zone; 60 to 100% in an anoxic zone; Lee & Jorgensen 1995). In *Ruegeria pomeroyi*, spermidine is degraded to intermediates that feed into the tricarboxylic acid cycle, from which both energy generation and biosynthesis are possible, and growth rates and biomass yields are similar to those on the amino acid serine. The high concentrations used in the present study (up to 1 mM) confirm that polyamines are not toxic to *R. pomeroyi*, as was shown by Höfle (1984) for a marine bacterial assemblage. Whether the fate of polyamine-derived carbon and nitrogen (i.e. incorporation versus regeneration) is affected by the concentration of polyamines and the supply of other labile DOM is yet to be determined.

The substrate specificity of polyamine transporters in marine bacterial genomes and metagenomes is hard to decipher at present. Whole-genome microarray and RT-qPCR data indicate that *Ruegeria pomeroyi* transporter systems may have narrow substrate specificities. This is further supported by the low identity (~19%) of amino acid sequences among predicted polyamine-binding proteins (Fig. 3). The clustering pattern of binding proteins cautions against the use of sequence identity alone as an indicator of substrate specificity among the *R. pomeroyi*, *Escherichia coli*, and *Pseudomonas aeruginosa* binding proteins analyzed here (Fig. 3). Because of their high sequence divergence, polyamine-binding proteins are not good targets for the design of general qPCR primer sets for environmental studies; even orthologs in genomes of 2 closely related strains for which 16S rRNA genes are >96% identical (e.g. *R. pomeroyi* DSS-3 and *Ruegeria* sp. TM1040) presented a significant challenge for primer design. Our analyses suggest instead that the well-conserved degradation genes *puuB* and *spuC* are more robust targets for *in situ* probing of polyamine processing by natural bacterial communities.

Studies of ecologically-relevant model bacteria that are designed and interpreted in the context of metagenomic data from natural bacterial communities can significantly enhance our understanding of biogeochemical processes. Despite recognition of the importance of spermidine and other polyamines in seawater over 2 decades ago, limited information had been generated on the biological processing of these compounds. This study provides new details on the function and distribution of genes for transport and metabolism of spermidine in a model marine bacterium. Moreover, it shows a high frequency and wide taxonomic distribution of homologs to polyamine transport genes (*pot*) and degradation genes (*puuB* and *spuC*) in marine bacterioplankton communities (Tables 4 & 5), arguing for increased attention to the role of polyamines in the marine carbon and nitrogen cycles. Functional characterization of these and other ecologically relevant

bacterial genes is a critical task for postgenomic environmental microbiology.

Acknowledgements. We thank J. Henriksen and W. Ye for assistance with the chemostats; R. Poretsky, S. Gifford, and J. Rinta-Kanto for helpful discussions on microarray data analysis; C. English for graphic support; and G. Burnham and C. Sloan for technical assistance. This research was supported by a National Science Foundation Grant to the Sapelo Island Microbial Observatory (MCB0702125) and the Gordon and Betty Moore Foundation.

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Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA

Submitted: June 29, 2009; Accepted: August 19, 2009
Proofs received from author(s): January 18, 2010