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Occurrence and Expression of Gene Transfer Agent Genes in Marine Bacterioplankton[▽]

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Genes with homology to the transduction-like gene transfer agent (GTA) were observed in genome sequences of three cultured members of the marine *Roseobacter* clade. A broader search for homologs for this host-controlled virus-like gene transfer system identified likely GTA systems in cultured *Alphaproteobacteria*, and particularly in marine bacterioplankton representatives. Expression of GTA genes and extracellular release of GTA particles (~50 to 70 nm) was demonstrated experimentally for the *Roseobacter* clade member *Silicibacter pomeroyi* DSS-3, and intraspecific gene transfer was documented. GTA homologs are surprisingly infrequent in marine metagenomic sequence data, however, and the role of this lateral gene transfer mechanism in ocean bacterioplankton communities remains unclear.

Genome-wide comparisons among marine bacterioplankton suggest that taxa such as *Roseobacter* and *Vibrio* have diverse metabolic capabilities supported by large and adaptable genomes, while "passive oligotrophs," like *Prochlorococcus* and *Pelagibacter*, have small but optimized genomes that potentially make them less adaptable (25). Lateral gene transfer via conjugation, transduction, and transformation can confer plasticity on bacterial genomes, but some gene exchange mechanisms are less available to marine bacterioplankton. For example, free DNA is found in low concentration in the pelagic ocean (0.6 to 88 μg liter^{-1}) (14), possibly too dilute for frequent transformation. Further, numerically dominant bacteria, such as *Prochlorococcus* and *Pelagibacter*, lack apparent plasmids and transposons (5, 7), making conjugation an unlikely mechanism of genetic transfer. Transduction, on the other hand, is a mechanism of genetic exchange that may be ecologically relevant in the ocean (11), as free viruses are relatively abundant in seawater (1, 26) and integrated prophages are evident in bacterial genomes (3, 12, 13, 34).

A novel mode of lateral gene transfer was discovered over 3 decades ago in *Rhodobacter capsulatus* (basonym, *Rhodopseudomonas capsulata*) that appeared similar to virus-mediated generalized transduction, yet the transducing agent was not a typical bacteriophage (21). This gene transfer agent (GTA) particle was lighter than any known phage (based on sedimentation), was smaller than most phages with similar morphology, was not inducible by mitomycin C, and did not form plaques (21, 31). Most importantly, GTA particles contained small, random pieces of host DNA rather than a phage genome (39), suggesting that their primary activity was lateral exchange of host DNA. Genetic evaluation of the *R. capsulatus* genes involved in making GTA particles revealed a cluster of 15 genes (designated orfg1 to orfg15) (16–18, 20), including homologs of phage structural genes (capsid, tail, portal, and DNA packaging) but not of self-replication or host lysis genes (e.g., holin).

The discovery of GTA-like genes in the genomes of three members of the marine *Roseobacter* clade (23), as well as other members of the *Alphaproteobacteria* (17), motivated a search for orthologous genes in newly available genome sequences, particularly those from marine bacterioplankton. In this study, we describe the abundance of *R. capsulatus*-like GTA genes in sequenced bacterial genomes, provide evidence for a functional GTA system in the cultured marine bacterioplankter *Silicibacter pomeroyi* DSS-3, and quantify GTA-like genes in natural marine bacterioplankton communities represented in metagenomic data sets.

MATERIALS AND METHODS

Identification of *R. capsulatus*-like GTA orthologs. Amino acid sequences of *R. capsulatus* GTA genes (GenBank accession number AF181080) were used in BLASTp searches (E value ≤ 10^{-4}) against complete and draft bacterial and archaeal genomes, and potential orthologs were compiled in BioEdit and aligned with ClustalW (35). A neighbor-joining tree was constructed by aligning concatenated sequences for orfg3, orfg5, and orfg12 in MEGA (version 3.1) (15) using a percent accepted mutation matrix-weighting model, 100 bootstrap replications, and pairwise deletion correction for gaps. The phylogenetic trees were used to identify sequences evolutionarily related to the experimentally verified *R. capsulatus* GTA genes.

Amino acid sequences for all 15 GTA genes (*R. capsulatus*; AF181080) and for 6 single-copy bacterial genes (*recA*, *atpD*, *gyrB*, *dnaK*, *rpoB*, and *tufA* of *Escherichia coli* K-12; NC_000913) were used to BLAST against unassembled Global Ocean Sampling (GOS) metagenome sequences (E values < 10^{-20} for GTA and single-copy genes) through the Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis webpage (http://camera.calit2.net/) (29). The numbers of hits for all genes were normalized for gene size (relative to *recA*, 1,062 bp), and the numbers of size-normalized hits for each single-copy gene were averaged per site. The percentage of cells containing GTA genes was then calculated as follows: (number of size-normal-

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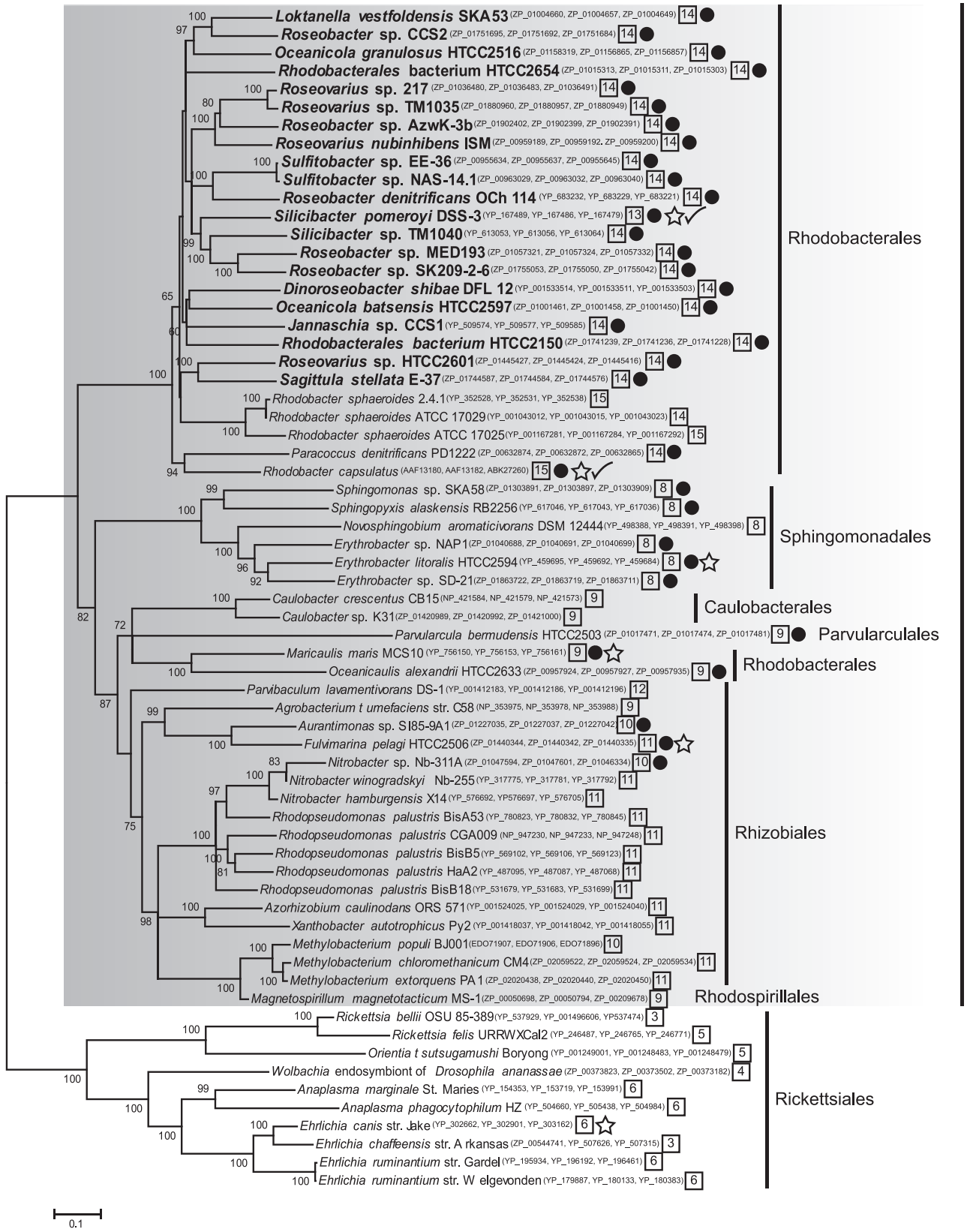

FIG. 1. Phylogenetic tree of concatenated amino acid sequences of orfg3 (portal protein), orfg5 (capsid protein), and orfg12 (unknown protein) homologs constructed with the neighbor-joining algorithm and percent accepted mutation correction for genomic data available as of 1 December 2007. Accession numbers for each concatenated gene are listed in parentheses; the numbers within squares indicate the numbers of *R. capsulatus*-like GTA genes contained within the genome (15 possible); the filled circles indicate marine bacteria, open stars indicate that gene neighborhoods are shown in Fig. 2, checkmarks indicate organisms for which GTA activity has been experimentally verified, and boldface text indicates members of the *Roseobacter* clade. Bootstrap values of >50% are indicated on the nodes. Two nonmarine *Rhizobiales*, *Brucella melitensis* 16 M and *Brucella suis* 1330, contain GTA-like genes but are omitted from this tree because they lack one of the three concatenated homologs (orfg5 and orfg3, respectively).

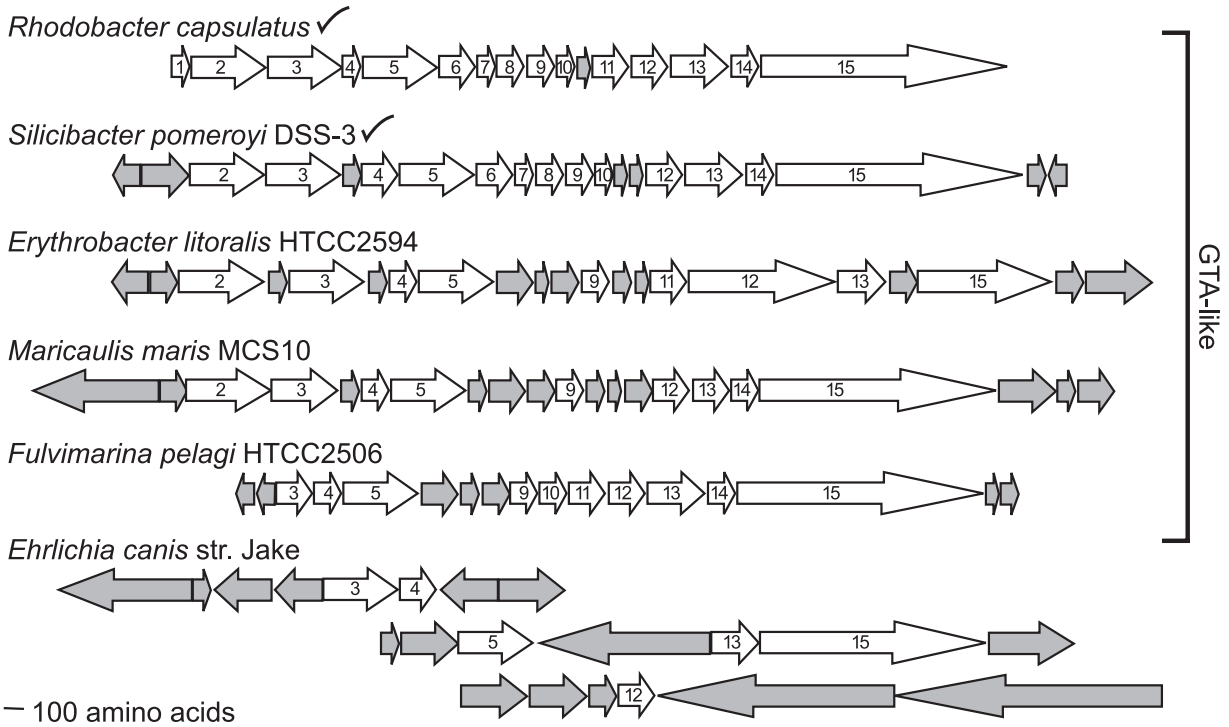

FIG. 2. Gene neighborhoods of *R. capsulatus*-like GTA genes in *R. capsulatus* and one representative bacterium from each clade shown in Fig. 1. The checkmarks indicate organisms for which GTA activity has been experimentally verified. The numbers within the arrows indicate *R. capsulatus*-like GTA gene homologs as defined by BLASTp matches having E values of 10^{-4} or lower. The *R. capsulatus* gene orientation and order were taken from Lang et al. (20). Complete genome sequences are available at NCBI (http://www.ncbi.nlm.nih.gov).

ized GTA hits/average number of size-normalized single-copy gene hits) × 100.

Transmission electron microscopy of *S. pomeroyi* GTA particles. Bacterial cells were removed from a dense *S. pomeroyi* DSS-3 culture (Marine Broth 2216; Difco) by centrifugation (8,000 × *g*; 10 min). GTA was pelleted from the medium (35,000 × *g*; 20 min), and the pellet was resuspended in SM buffer (10 mM NaCl, 50 mM Tris, 10 mM $MgSO_4$, and 0.1% gelatin). One drop of GTA suspension was placed on a carbon-coated grid and, after 10 min, stained for 30 seconds with phosphotungstic acid. The *S. pomeroyi* GTA particles were then visualized on a Philips/FEI Technai 20 (FEI Co., Eindhoven, The Netherlands).

Enumerating *S. pomeroyi* GTA particles. To assess the temporal patterns of GTA particle formation, we enumerated GTA particles in *S. pomeroyi* cultures using the Sybr gold stain. While GTA particles contain only a few kilobases of DNA (less than a typical phage genome), Sybr gold proved sufficiently sensitive for counting in culture medium. We set up experiments by growing *S. pomeroyi* DSS-3 cells in 250 ml Marine Broth 2216 at 25°C for 14 days with shaking (200 rpm). Aliquots were prepared for GTA particle enumeration by adding 950 μl of TM buffer (10 mM Tris, 5 mM MgCl, pH 7.5) to 50 μl of paraformaldehyde-fixed culture, filtering the mixture through a 0.2-μm-pore-size cellulose membrane (Gelman), treating the filtrate with DNase I (0.2 U ml^{-1}) and RNase A (10 μg ml^{-1}) for 1 hour at room temperature, and then filtering the particles onto 0.02-μm-pore-size Al_2O_3 Anodisc membrane filters (Whatman). To monitor host cell density variation during the incubation, 1 to 50 μl of fixed sample was brought up to a final volume of 1 ml with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and filtered onto a 0.22-μm-pore-size black polycarbonate filter (Osmonics). Duplicate filters from each culture were stained with 1× Sybr gold (Molecular Probes, Inc.). Because Sybr gold intercalates into single- or double-stranded DNA or RNA, it distinguishes nucleic acid-containing particles and cells from membrane vesicles (30) and other lipid- or protein-containing particles. Cells and GTA particles were visualized with epifluorescence microscopy under blue excitation (485 nm) on a Zeiss Axioplan epifluorescence microscope (Zeiss). At least 200 bacterial cells or GTA particles were counted per sample on 20 randomly chosen fields.

GTA gene expression. *S. pomeroyi* DSS-3 cultures were incubated in the dark at 30°C with shaking (200 rpm) until they reached stationary phase (1/2 YTSS medium [2.5 g yeast extract, 4 g tryptone, 20 g Sigma sea salts per liter]). Aliquots were periodically collected for RNA extraction and immediately mixed with 1/10

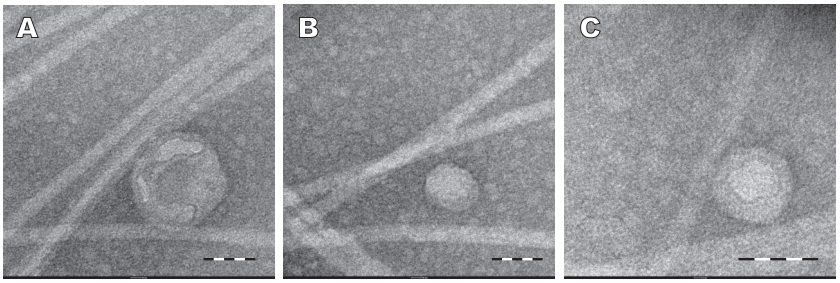

FIG. 3. Transmission electron micrograph of GTA-like particles filtered from *S. pomeroyi* DSS-3 cultures. The long filamentous structures are most likely flagellar filaments (8). Bars = 50 nm.

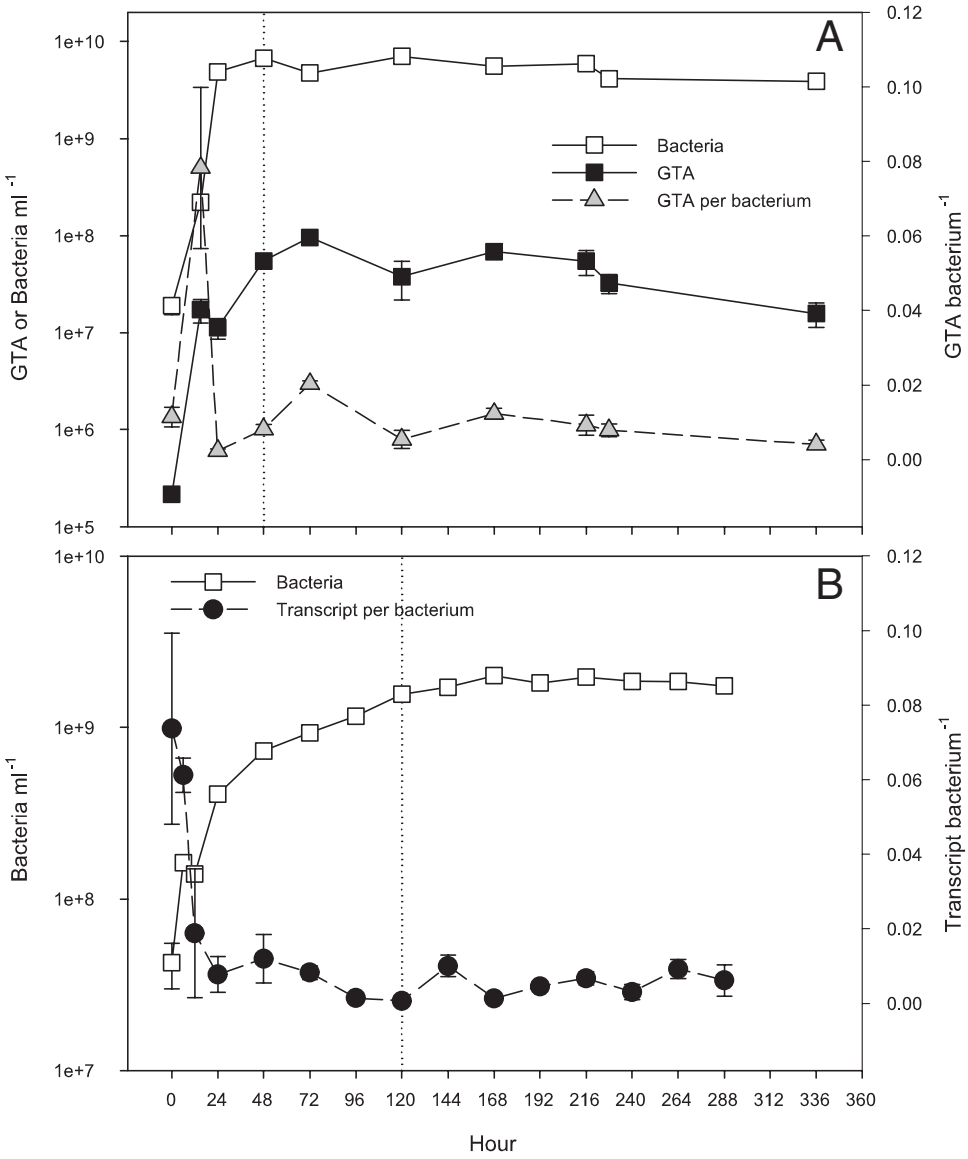

FIG. 4. Production of GTA particles in *S. pomeroyi* DSS-3 cultures. (A) Detection of GTA production by epifluorescence microscopy. (B) Expression of an *S. pomeroyi* DSS-3 GTA gene (orfg3; portal) ($n = 4 \pm$ standard deviation [SD]) as a function of the cell number ($n = 2 \pm$ SD). The dotted line indicates the transition between log growth phase and stationary phase.

(vol/vol) cold stop solution (95% ethanol, 5% phenol). After centrifugation, the pellet was stored at −80°C until RNA extraction (RNaqueous kit; Ambion) and subsequent DNA removal (Turbo DNA-free; Ambion). We designed primers SPg3f (GCTATGAGACGCACCCGATG) and SPg3r (AACAGCAGTTGCCC GAACAG) with online software (Primer3 [http://frodo.wi.mit.edu/cgi-bin /primer3/primer3_www.cgi]) to amplify a 97-bp product from the *S. pomeroyi* portal protein orfg3 (SPO2264; YP_167489). Primer SPg3r was used for reverse transcription (Omniscript; Qiagen).

Quantitative PCR was performed in 25-μl reaction mixtures in a 96-well plate using 1 μl cDNA, 300 nM (final concentration) of each primer, and 12.5 μl iQ Sybr green Supermix (Bio-Rad). Samples were run in duplicate on an iCycler (Bio-Rad) with the following conditions: 3 min at 95°C and 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by melting-curve analysis. A duplicate eight-point standard curve was run in parallel using cloned PCR products from the same strain (Topo TA cloning kit; Invitrogen). The cloned plasmids were extracted with a GenElute Plasmid Mini Prep kit (Sigma), linearized using HindIII (Roche), and quantified (NanoDrop). Tenfold dilutions of linearized plasmid were used for the standard curve. The initial transcript copy number for each reaction was calculated by using 660 g mol^{-1} as the average molecular mass of 1 base pair.

Gene transfer experiments. Two spontaneous *S. pomeroyi* antibiotic-resistant mutants (Rif^r_{75}, with rifampin resistance of >75 μg ml^{-1}; $Strep^r_{50}$, with streptomycin resistance of >50 μg ml^{-1}) were generated and incubated individually and in mixed culture in liquid medium (1/2 YTSS medium) for 8 days in the dark at 30°C without shaking. Every 24 h, aliquots were removed and plated on 1/2 YTSS amended with rifampin alone (75 μg ml^{-1}), streptomycin alone (50 μg ml^{-1}), and both rifampin and streptomycin. Colony numbers on agar plates containing both antibiotics were compared for mutants grown individually versus in mixed cultures to estimate the rates of lateral gene transfer, after subtraction of any spontaneous double mutants.

To assess the requirement for cell-to-cell contact, wild-type (WT) *S. pomeroyi* DSS-3 and a kanamycin-resistant strain (Kan^r_{150}, with kanamycin resistance of >150 μg ml^{-1}) (isolate 41-H6 [9]) were grown in 1/2 YTSS medium with shaking at 200 rpm for 5 days at 30°C. The cultures were centrifuged (4,500 × *g*; 10 min), and the supernatant was filtered twice (0.22-μm-pore-size polycarbonate membrane filter) to remove bacterial cells. The filtrate from WT or Kan^r_{150} cells was

added to WT cells (collected by centrifugation at 4,500 × *g* for 10 min) or to culture medium without any cells. The cultures were shaken at 200 rpm in the dark at 30°C for 1 h before an aliquot was plated on Marine Broth 2216 plates amended with 150 μg ml^{-1} kanamycin.

RESULTS AND DISCUSSION

***R. capsulatus*-like GTA genes in cultured bacteria.** Organisms meeting the following criteria were classified as positive for GTA genes: possession of homologs to at least 8 of the 15 *R. capsulatus*-like GTA structural genes, colocalization of GTA-like genes in the genome, and conservation of GTA gene order and transcript orientation. Similar to recent findings (19), GTA-like genes were found exclusively in members of the *Alphaproteobacteria* (Fig. 1). Fifty-five of 102 sequenced *Alphaproteobacteria* genomes (54%) contained GTA-like genes (Fig. 1 and 2), including members of the *Rhodobacterales*, *Caulobacterales*, *Parvularculales*, *Rhodospirillales*, *Rhizobiales*, and *Sphingomonadales*. Marine bacteria account for the majority of bacteria with complete or nearly complete suites of *R. capsulatus*-like GTA genes (34 of 55; 62%), even though marine bacteria make up only 34% of available alphaproteobacterial genome sequences (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

All but 1 of the 22 available genome sequences in the marine *Roseobacter* clade have a GTA-like gene cluster (Fig. 1), with the draft genome sequence of *Rhodobacterales* bacterium HTCC2255 the only exception. Further, GTA genes are among the core genes previously identified for the *Roseobacter* clade (SPO2250 to SPO2266) (supplemental file 3 from reference 23). In contrast, GTA-like genes are not harbored by *Pelagibacter ubique* HTCC1062 and HTCC1002, cultured members of the ecologically dominant marine alphaproteobacterial SAR11 clade, nor are they found in other nonmarine members of the *Rickettsiales* based on our three criteria (however, see reference 17). GTA transduction, if similar to viral transduction, is likely to be most efficient for bacteria associated with particle surfaces, biofilms, and other high-cell-density habitats (28, 36, 38), which is typical of many marine roseobacters (2) but not of *P. ubique*. Further, the 15 genes required for assembling and releasing GTA particles may be ecologically costly for an organism such as *P. ubique*, whose small genome (1.3 Mb) is thought to represent the minimal set of genes necessary for survival in the ocean (7). The fact that one sequenced *Roseobacter* isolate does not contain any of the 15 GTA genes (*Rhodobacterales* bacterium HTCC2255) may simply reflect the incomplete sequencing of this genome. However, the conditions of isolation (4), along with its small genome size (≥2.3 Mb) and poor growth under laboratory conditions, suggest that HTCC2255 may represent an oligotrophic member of the *Roseobacter* group for which GTA genes are not typical.

GTA-like systems have been identified in two other bacteria, but they do not appear homologous to the *R. capsulatus* system. *Brachyspira hyodysenteriae* (phylum *Spirochaetes*) produces GTA-like particles called VSH-1 (10, 22, 33), but the genes encoding VSH-1 are not *R. capsulatus* GTA homologs (22). Similarly, GTA-like particles described in *Desulfovibrio desulfuricans* (27) (class *Deltaproteobacteria*) are not homologous to *R. capsulatus* GTA genes.

***S. pomeroyi* produces GTA particles.** Since GTA genes are nearly ubiquitous within *Roseobacter* clade members, we chose *S. pomeroyi* DSS-3 for our experimental studies. *S. pomeroyi* contains 13 out of 15 *R. capsulatus* GTA genes yet lacks any integrated prophages that could complicate the interpretation of experimental results.

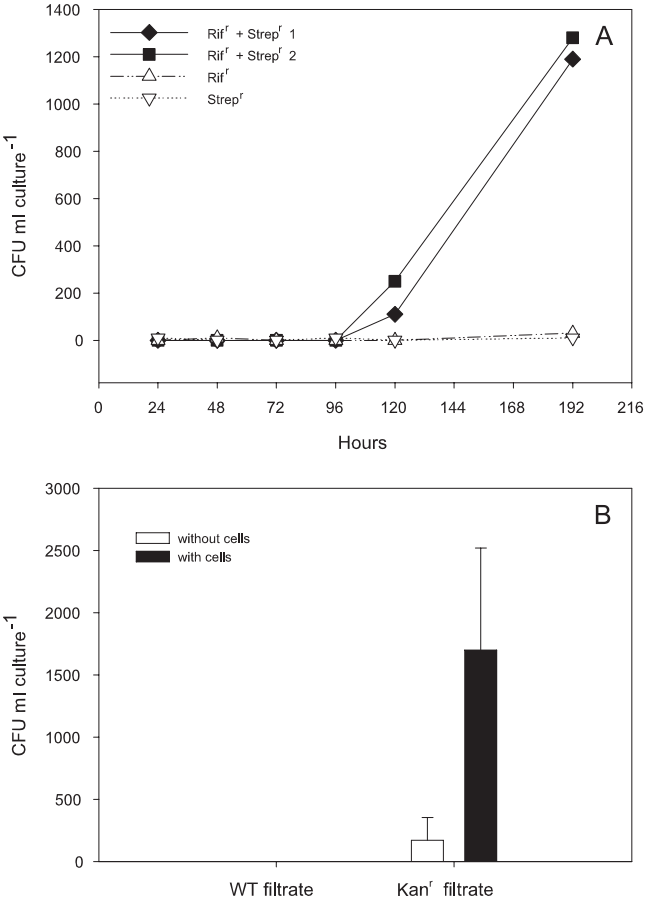

FIG. 5. Transfer of genetic markers between mutant strains of *S. pomeroyi* DSS-3. (A) Cultures of DSS-3 spontaneous mutants (Rif^r or Strep^r) were grown together or individually and plated on double-antibiotic plates. CFU of double mutants when grown together (Rif^r + Strep^r; replicates 1 and 2) provide an estimate of GTA-mediated gene transfer, since spontaneous double mutants in individually grown cultures (Rif^r or Strep^r) occur at very low numbers. (B) CFU from Kan$^r{}_{150}$ *S. pomeroyi* DSS-3 filtrate incubated with WT cells provide evidence for GTA activity. The values are averages (n = 2 plus standard deviation).

S. pomeroyi produces GTA particles that are small (~50 to 70 nm), similar to *R. capsulatus* GTA particles (39) (Fig. 3). Unlike *R. capsulatus* GTA particles, however, *S. pomeroyi* GTA particles do not appear to have tails. In active culture, the GTA particles were ~2 orders of magnitude less abundant than *S. pomeroyi* cells, but particle abundance was correlated with cell numbers (Fig. 4A). In contrast, *R. capsulatus* GTA particle production occurs primarily during the transition between log and stationary phases (32).

S. pomeroyi expressed the orfg3 homolog throughout incubation, with the highest expression per cell occurring during early log growth and the lowest during late log growth (Fig. 4B). In contrast to previous work with *R. capsulatus* (32), high cell densities are not required for GTA gene expression in *S. pomeroyi*.

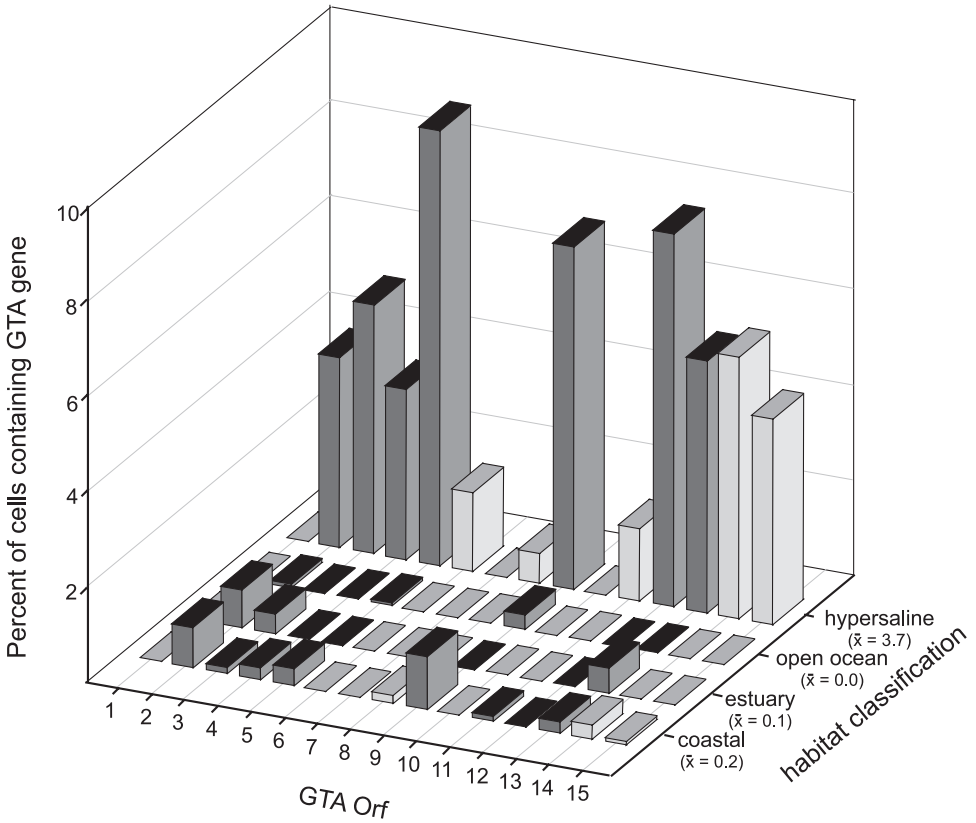

FIG. 6. Presence of GTA genes in the GOS metagenomic library (29) calculated as percentages of cells carrying homologs for each of the 15 GTAs. The dark-gray bars represent "diagnostic genes" (i.e., genes present in >90% of GTA-containing organisms as listed in Fig. 1). The light-gray bars represent "nondiagnostic genes" (i.e., those less common in GTA-containing organisms). The mean abundances across all 15 homologs are given in parentheses.

Evidence of GTA-mediated gene transfer in *S. pomeroyi* DSS-3. GTA activity is expected to mediate the transfer of small, random fragments of the host genome to other (conspecific) cells (21, 32). We tested this by growing two *S. pomeroyi* mutants together, each with resistance to a different antibiotic (Rif^r_{75} and $Strep^r_{50}$), and then assaying for the appearance of double mutants. After 8 days, doubly resistant *S. pomeroyi* cells reached ~12,000 CFU ml^{-1} in mixed cultures but only ~10 CFU ml^{-1} in the individual mutant controls (Fig. 5A). Similarly, resistant colonies were evident on plates containing kanamycin after WT cells were grown in the presence of Kan^r_{150} filtrate, confirming that filtrate alone is sufficient for the transfer of kanamycin resistance and cell-to-cell contact is not required (Fig. 5B). A similar filterable particle conferring genetic transfer was originally found in *R. capsulatus* (21, 31, 32), and a previous study documented that free DNA (outside the protection of a GTA capsule) was not effective in transfer (21).

Interrogation of the GOS metagenome for GTA homologs. To determine whether GTA systems are similarly common among uncultured members of marine alphaproteobacterial groups, we searched the GOS metagenomic data (29) for GTA homologs. Very few bacteria (<0.2% average abundance across the 15 homologs) in surface waters from sites classified as estuarine ($n = 3$), coastal ($n = 18$), or open ocean ($n = 14$) contained GTA gene homologs (Fig. 6). Some of these apparent GTA genes are likely to be paralogous viral genes, since the gene neighborhood and gene order criteria used to identify GTA-like genes in cultured bacteria could not be applied here. Therefore, 0.2% is likely an overestimation of GTA abundance in marine surface ocean bacteria.

Ecological implications. Roseobacters and other alphaproteobacterial taxa (marine *Sphingomonadales* and marine *Rhizobiales*) (Fig. 1) that contain GTA together make up a significant proportion of marine bacterioplankton communities (2, 24). Thus, GTA-mediated gene transfer has the potential to be a major mechanism for gene flow and lineage adaptation in the ocean. However, the low abundance of GTA genes in the surface ocean metagenomic data could indicate that most cultured strains of marine alphaproteobacteria that carry GTA genes are not typical of their uncultured relatives, perhaps because selection for high-density growth on solid media, used in most culturing protocols, also preferentially selects for cells with GTA. *Rhodobacterales* bacterium HTCC2255, the only sequenced *Roseobacter* without GTA homologs, was isolated from seawater by dilution to extinction, not on solid plates (4). Alternatively, the bias against larger bacterioplankton cells during GOS sampling (most sequences are from the 0.1- to 0.8-μm size fraction) (29), may select against larger and aggregate-associated cells that are more likely to harbor GTA homologs. In a single hypersaline habitat included in the GOS survey that had a significant signal of GTA-containing taxa (i.e., 15% of 16S rRNA genes from this site were *Rhodobacte-*

rales, *Sphingomonadales*, *Caulobacterales*, *Parvularculales*, *Rhizobiales*, or *Rhodospirillales*), we estimated that up to 4% of the cells harbored GTA genes (mean abundance across the 15 homologs) (Fig. 6).

It has been suggested that GTA evolved from a prophage that was present before the divergence of *Proteobacteria* (19). Since viral transduction is important for the homogenization of genes within host populations (6), GTA activity may similarly facilitate gene homogenization among bacteria, particularly since GTA particles contain only host genome (21, 39). The extent to which GTAs can operate across taxonomic boundaries is not yet clear, however, as this and previous studies (21, 32, 37) have demonstrated enhanced gene transfer only between strains of the same species. While the importance of GTA activity in the ocean remains to be determined, it could provide a mechanism for maintaining adaptable genomes with diverse metabolic capabilities in marine alphaproteobacterial taxa.

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