INTRODUCTION

Marine Group A (MGA) bacteria were first identified in small subunit ribosomal RNA (16S rRNA) gene clone libraries generated from surface waters of the Atlantic and Pacific Oceans (Fuhrman et al., 1993; Gordon and Giovannoni, 1996; Fuhrman and Davis, 1997). MGA, originally referred to as the ‘SAR406 gene lineage’, represents a deeply branching lineage of bacteria related to the genus *Fibrobacter* and the green sulfur bacterium (GSB) phylum, which includes the genus *Chlorobium* (Gordon and Giovannoni, 1996). To date, MGA remains a candidate phylum with no cultured representatives. Modern phylogenetic analyses indicate that the closest cultivated relatives of MGA are *Caldithrix abyssi* and *Caldithrix palaeochoryensis*, both belonging to the phylum *Caldithrix*. These isolates are anaerobic, mixotrophic, thermophiles obtained from hydrothermal vent and sediment environments, respectively (Miroshnichenko et al., 2003, 2010). Although ubiquitous in the dark ocean, MGA are most prevalent and diverse in interior regions of the ocean with distinct oxyclines, such as in oxygen minimum zones (OMZs) and permanent or seasonally stratified anoxic basins (Madrid et al., 2004, 2005; Stevens and Ulloa, 2008; Schattenhofer et al., 2009; Zaikova et al., 2010; Allers et al., 2012; Wright et al., 2012). At present, the metabolic capacity and ecological roles of MGA in OMZs or in the ocean at large remain entirely unknown. Given that OMZs are expanding and intensifying (Emerson et al., 2004; Whitney et al., 2007; Bograd et al., 2008; Stramma et al., 2008; Helm et al., 2011), primarily as a result of global climate change (Keeling et al., 2010), it is of increasing importance to define the metabolic diversity and ecosystem function of dominant microorganisms within these systems in order to predict the systemic impacts of OMZ expansion on ocean ecology and biogeochemistry.

The distribution of certain MGA subgroups was reported as being negatively correlated with the...
concentration of dissolved oxygen (O\textsubscript{2}) in the OMZ of the Northeast subarctic Pacific Ocean (NESAP), suggesting a potential role for O\textsubscript{2} as a driver of MGA habitat selection and metabolic adaption in these waters (Allers et al., 2012). Determining the extent to which 16S rRNA-based patterns of MGA distribution represent ecological types (ecotypes) differentiating in response to selective environmental pressures such as O\textsubscript{2} deficiency requires genome-scale sequence data associated with multiple MGA subgroups to query for changes in genome composition that might promote differential fitness across the oxycline. Here, we explore potential niche partitioning among and within MGA subgroups along oxic to anoxic-sulfidic gradients of dissolved O\textsubscript{2} in the North Pacific Ocean. In the absence of reference genomes representative of the MGA candidate phylum, we use phylogenetic anchor screening to identify 18 large-insert DNA fragments affiliated with various MGA subgroups as a direct route to studying MGA function. We describe and compare the genetic content and organization of these large-insert DNA fragments to gain preliminary insights into MGA metabolism.

Materials and methods

Sample collection and processing in the NESAP

Sampling in the NESAP was conducted via multiple hydrocasts using a Conductivity, Temperature, Depth (CTD) rosette water sampler aboard the CCGS John P. Tully during Line P cruises 2009-09 June 2009 (major stations: P4 (48°39.0′N, 126°40.0′W) - 7 June, P12 (48°58.2′N, 130°40.0′W) - 9 June and P26 (50°N, 145°W) - 14 June; 2009-10 August 2009 (major stations P4 - 21 August, P12 - 23 August, P26 - 27 August; and 2010-01 February 2010 (major stations: P4 - 4 February, P12 - 11 February) (Supplementary Figure S1). At these stations, large volume (20 l) samples for DNA isolation were collected from the surface (10 m), whereas 1201 samples were taken from three depths spanning the OMZ core and upper and deep oxyclines (500 m, 1000 m and 1300 m at station P4; 500 m, 1000 m and 2000 m at station P12). Sampling at Saanich Inlet (SI) station S3 (48°35.30′N, 123°30.22′W) was performed as previously described (Zaikova et al., 2010) as part of a monthly monitoring program aboard the MSV John Strickland. Sample collection and filtration protocols can be viewed as visualized experiments at http://www.jove.com/video/1159/ (Zaikova et al., 2009) and http://www.jove.com/video/1161/ (Walsh et al., 2009), respectively.

Environmental DNA extraction

DNA was extracted from Sterivex filters as described by Zaikova and colleagues (Zaikova et al., 2010) and DeLong and colleagues (DeLong et al., 2006). The DNA extraction protocol can be viewed as a visualized experiment at http://www.jove.com/video/1352/ (Wright et al., 2009).

Phylogenetic analysis and tree construction using MGA 16S rRNA gene sequences

Phylogenetic analysis and tree construction using full-length 16S rRNA gene clone sequences from the NESAP and SI and 16S rRNA gene sequences identified on large-insert DNA fragments was performed as reported previously (Allers et al., 2012); see Supplementary Methods for details.

Fosmid library construction and end sequencing

Thirty fosmid libraries (~7680 clones/libraries) were constructed from DNA samples collected from Line P stations P4, P12, and P26 in June and August of 2009, and stations P4 and P12 during February 2010 (Supplementary Table S1, Supplementary Figure S1). An additional 16 fosmid libraries were constructed from DNA samples collected from SI station S3 during the 2006-2007 seasonal stratification and deep-water renewal cycle (Supplementary Table S1, Supplementary Figure S1) (Walsh et al., 2009). Further details on fosmid library construction and sequencing can be found in Supplementary Methods.

Fosmid library screening, preparation, and full-length sequencing

Twenty three of the 46 fosmid end sequenced libraries described above including 7 from Line P and 16 from SI were screened for the presence of 16S rRNA genes using the NAST aligner (DeSantis et al., 2006a) and BLAST using default parameters against the 2008 Greengenes database (DeSantis et al., 2006) (Supplementary Figure S1). After preliminary phylogenetic analyses, 14 fosmid clones containing MGA-affiliated 16S rRNA genes were selected for complete sequencing (8 fosmids from Line P libraries and 6 fosmids from SI libraries; Table 1, Supplementary Figure S1). For sequencing protocols, see Supplementary Methods.

GC content and oligonucleotide frequency analysis

GC content of large-insert DNA fragments (14 fosmids from NESAP and SI in addition to four large-insert DNA fragments from other North Pacific Ocean environments; Table 1) was calculated using gccontent.pl with default parameters, available for download at https://github.com/hallamlab/utilities. Tetranucleotide frequencies were calculated as normalized Z-scores using TETRA (Teeling et al., 2004a,b; http://www.mega.xnet/tetra). Principal component analysis was performed on normalized Z-score profiles for each insert using PRIMER v6.1.13 (Clarke, 1993; Clarke and Gorley, 2006). Principal component analysis was overlaid with clusters determined by Hierarchical Cluster
Table 1 Characterization of large-insert DNA fragments containing MGA 16S rRNA genes

| Fosmid insert size (bp) | GC content (%) | No. of ORFs encoding hypotheticals | Accession number | Sampling location | Sampling coordinates | Collection date | Depth (m) | $\text{O}_2$ $(\mu\text{mol kg}^{-1})$ | Phylogenetic Identity | Syntenic group | Reference |
|------------------------|---------------|-----------------------------------|-----------------|------------------|----------------------|----------------|----------|-------------------------------------|------------------------|----------------|----------|
| **Saanich Inlet**      |               |                                   |                 |                  |                      |                |          |                                     |                        |                |          |
| FPPP_13C3              | 40370         | 32.8                              | 36              | 19               | KF170421             | Station S3     | Nov-06   | 10                                  | 249.0                  | Arctic96B-7 | II       |
| FPPP_33K14             | 27446         | 37.4                              | 17              | 15               | KF170417             | Station S3     | Nov-06   | 10                                  | 249.0                  | A714018       | IV       |
| FGYC_13M19             | 32678         | 43.6                              | 16              | 11               | KF170416             | Station S3     | Feb-06   | 125                                 | 5.0                     | SHBH391       | I        |
| FPPS_57A9              | 32217         | 37.9                              | 21              | 13               | KF170418             | Station S3     | Nov-06   | 100                                 | 15.4                    | SHAN400       | I        |
| FPPU_33B15             | 32230         | 37.0                              | 14              | 12               | KF170419             | Station S3     | Nov-06   | 200                                 | 54.0                    | SAR406        | IV       |
| FPPZ_SC6*              | 41899         | 38.2                              | 36              | 26               | KF170420             | Station S3     | Apr-07   | 200                                 | 1.1                     | Arctic96B-7 | I        |
| **Monterey Bay**       |               |                                   |                 |                  |                      |                 |          |                                     |                        |                |          |
| EBAC750-03B02          | 34714         | 42.0                              | 21              | 15               | AY458631             | Monterey Bay   | Apr-00   | 750                                 | 16.5                    | Arctic95A-2 | II       |
| **Hawaii Ocean Time-series** |           |                                   |                 |                  |                      |                 |          |                                     |                        |                |          |
| HF0010_18O13           | 37088         | 33.0                              | 33              | 17               | GU474850             | Station ALOHA  | Oct-02   | 10                                  | 204.6                   | ZA3312c       | I        |
| HF0500_01L02           | 32792         | 37.7                              | 25              | 19               | GU474916             | Station ALOHA  | Oct-02   | 500                                 | 118.0                   | Arctic96B-7 | I        |
| HF4000_22B16           | 31597         | 43.0                              | 17              | 15               | GU474892             | Station ALOHA  | Dec-03   | 4000                                | 147.8                   | P262000N21   | IV       |
| **NESAP**              |               |                                   |                 |                  |                      |                 |          |                                     |                        |                |          |
| 405006-B04             | 43543         | 42.8                              | 46              | 32               | KF170424             | Station P4     | Feb-10   | 500                                 | 40.4                    | SAR406        | I        |
| 4050020-J15            | 43860         | 39.3                              | 49              | 39               | KF170424             | Station P4     | Feb-10   | 500                                 | 40.4                    | Deferrribacteres-| V        |
| 413004-H17             | 35813         | 39.8                              | 45              | 29               | KF170425             | Station P4     | Feb-10   | 1300                                | 22.7                    | Arctic96B-7 | I        |
| 4130011-I07            | 34533         | 41.5                              | 39              | 27               | KF170426             | Station P4     | Feb-10   | 1300                                | 22.7                    | Arctic95A-2 | II       |
| 413009-K18             | 35438         | 41.4                              | 25              | 23               | KF170413             | Station P4     | Feb-10   | 1300                                | 22.7                    | SAR406        | IV       |
| 125003-E23             | 34144         | 37.3                              | 41              | 28               | KF170423             | Station P12    | Feb-10   | 500                                 | 32.2                    | Arctic96B-7 | I        |
| 1250012-L08            | 34444         | 40.6                              | 35              | 26               | KF170414             | Station P12    | Feb-10   | 500                                 | 32.2                    | Arctic95A-2 | III      |
| 122006-I05             | 35932         | 47.7                              | 45              | 26               | KF170422             | Station P12    | Feb-10   | 2000                                | 59.5                    | P262000D03   | II       |

Abbreviations: MGA, Marine Group A; ORF, open reading frames.

This fosmid was derived from an $\text{H}_2\text{S}$-containing sample.
Analysis of normalized Z-scores using a Euclidean distance matrix (also performed in PRIMER).

Global nucleotide similarity analysis
Global nucleotide similarity in large-insert DNA fragments was determined by performing pairwise blastn comparisons between all fragments using onecircos.pl with default settings for all parameters except percent_identity (−p), which was calculated at 50%, 80%, 90% and 95% in separate analyses. onecircos.pl is available for download at: https://github.com/hallamlab/utilities and is based on Circos (http://circos.ca/; Krzywinski et al., 2009).

Open reading frame prediction and gene annotation
Open reading frames (ORFs) were predicted and annotated using the in-house MetaPathways pipeline (Konwar et al., 2013), available for download at: http://hallam.microbiology.ubc.ca/MetaPathways/. Briefly, primary nucleotide sequences from large-insert DNA fragments were quality controlled for ambiguous bases and file-format errors. ORFs were predicted using Prodigal (Hyatt et al., 2010). ORFs shorter than 60 amino acids in length were removed and were annotated using Protein BLAST (Altschul et al., 1990) (bit-score ratio > 0.4 (Rasko et al., 2005), e-value = 1e−5) against the RefSeq (Pruitt and Maglott, 2001), KEGG (Kanehisa and Goto, 1999), COG (Tatusov et al., 2001) and MetaCyc (Karp et al., 2000) databases. Annotations were assigned to predicted ORFs based on the following four criteria: (i) the BLAST hit with top e-value was selected from each database; (ii) each BLAST hit was assigned an ‘information score’ based on the sum of distinct and shared enzymatic words (prepositions, articles and auxiliary verbs were removed) with a preference for Enzyme Commission numbers (+10 score); (iii) the annotation with the highest score was selected and assigned to the respective ORF; (iv) ORFs with no hits were assigned the annotation ‘hypothetical protein’.

Amino acid similarity analysis
Predicted amino acid similarity of large-insert DNA fragments was plotted in Trebol (available for download at: http://bioinf.udec.cl/trebol) using tblastx with a minimum bit-score cutoff of 50. COG categories present on large-insert fragments were plotted using tblastn of COG proteins against large-insert DNA fragments with a minimum e-value cutoff of 1e-4.

Fragment recruitment of fosmid end sequences
Coverage plots relating fosmid end sequences from individual NESAP and SI fosmid end libraries to large-insert DNA fragments were generated using the Nucmer program implemented in MUMmer 3.23 (Kurtz et al., 2004) as cited in (Hallam et al., 2006). Further details on fragment recruitment can be found in Supplementary Methods.

Phylogenetic analysis of PsrABC
Protein sequences (including predicted protein sequences for PsrA, PsrB, and PsrC identified on fosmids FPPP_13C3 and 122006-I05) were aligned using MUSCLE v3.6 with default parameters (Edgar, 2004). For the purposes of this analysis, the PsrBC fusion proteins encoded by psrBC on fosmid FPPP_13C3 and on certain reference sequences were divided into PsrB and PsrC subunits and analyzed in separate trees. Phylogenetic analyses were performed using PHYML (Guindon et al., 2005) using a WAG model of amino-acid substitution, where the parameter of the G distribution and the proportion of invariable sites were estimated for each data set. The confidence of each node was determined by assembling a consensus tree of 100 bootstrap replicates. The presence of TAT signal sequences on PsrA proteins was predicted using TatP 1.0 (Bendtsen et al., 2005), available at: http://www.cbs.dtu.dk/services/TatP/.

Results
Physicochemical characteristics of the NESAP and SI
This study was conducted along the Line P transect of the NESAP (Supplementary Figure S1), beginning in SI, Vancouver Island, British Columbia (SI, Station S3: 48°58’N, 123°50’W) and ending at Ocean Station Papa (also referred to as station P26: 50°N, 145°W) (Freeland, 2007). Owing to strong stratification and sluggish circulation of the interior NESAP waters, a large region of O₂-deficient (<90 µmol kg⁻¹) water containing dysoxic (20–90 µmol kg⁻¹) and suboxic (1–20 µmol kg⁻¹) compartments spans from ~400 m to 2000 m in depth resulting in a persistent OMZ (O₂ < 20 µmol kg⁻¹). The OMZ is centered at 1000 m, wherein dissolved O₂ concentrations typically drop to ~9 µmol kg⁻¹ (Whitney et al., 2007). During the past 50 years of oceanographic observation, O₂ concentrations in the OMZ of coastal to open-ocean regions of the NESAP have not been observed to reach anoxic (<1 µmol kg⁻¹) levels. However, interior and basin waters of SI typically experience seasonal periods of anoxia and sulfide accumulation on an annually recurring basis (Anderson and Devol, 1973; Lilley et al., 1982; Ward et al., 1989). Physicochemical data from basin (S3), coastal (P4), transition (P12) and open-ocean (P26) stations measured along the Line P transect relevant to the present study are provided in Table 1 and Supplementary Table S1.

Taxonomic diversity of MGA in the NESAP and SI
To identify 16S rRNA genes affiliated with MGA inhabiting SI waters, we screened 19 previously published bacterial 16S rRNA gene clone libraries...
(containing a total of 6645 sequences) generated from samples traversing the water column during the 2006-2007 seasonal stratification and deep-water renewal cycle and during the spring stratification in 2008 at Station S3 (Supplementary Table S1; Walsh and Hallam, 2011). A total of 415 16S rRNA gene sequences affiliated with MGA were recovered from SI clone libraries. These sequences were added to a data set containing 290 MGA 16S rRNA sequences previously reported from Line P stations P4, P12, and P26 (Allers et al., 2012) and clustered at 97% identity, forming 156 distinct operational taxonomic units (OTUs), 120 of which contained only singletons. Representative sequences were obtained for each non-singleton OTU and placed in phylogenetic context with relevant reference sequences from other locations (Supplementary Figure S2). Five out of 10 previously defined MGA subgroups were recovered in SI clone libraries (ZA3648c and ZA3312c (Fuchs, unpublished); Arctic96B-7 (Bano and Hollibaugh, 2002); SAR406 (Gordon and Giovannoni, 1996); and A714018 (Allers et al., 2012), and three novel subgroups were identified (SHBH1141, SHBH391, and SHAN400) (Supplementary Figure S2). These novel subgroups were found exclusively in SI and contained the most abundant OTUs identified in this location (Supplementary Figures S2, S3).

As described by Allers and colleagues (Allers et al., 2012), MGA sequences identified in coastal and open ocean waters of the NESAP comprised 0.7 ± 0.84% of 10 m clone libraries and 11.2 ± 3.9% of clone libraries from O₂-deficient waters, with a maximum of 16.4% at P26 1000 m. The most abundant MGA OTUs present in these locations comprised between 1% and 4% of clone libraries and belonged to subgroups Arctic95A-2, ZA3312c, Arctic96B-7, SAR406, and HF770D10, in order of decreasing OTU abundance (Supplementary Figures S2, S3). In comparison, MGA OTUs identified in SI comprised 1.6 ± 0.81% of 10 m clone libraries and 7.1 ± 3.6% of clone libraries from O₂-deficient waters. The most abundant OTUs present in SI comprised between 1% and 5% of clone libraries, and belonged to subgroups SHBH391, SHAN400, SHBH1141, ZA3312c, SAR406 and Arctic96B-7, in order of decreasing OTU abundance (Supplementary Figures S2, S3).

Characterization and phylogenetic assignment of large-insert DNA fragments

To connect 16S rRNA-based patterns of distribution across the oxycline in the NESAP and SI to genomic information associated with specific MGA subgroups, we screened 23 end sequenced fosmid libraries for the presence of clones containing 16S rRNA gene sequences (Supplementary Figure S1). Collectively, fosmid end libraries contained a total of 164 736 genomic clones representing 255.3 Mb of environmental genomic DNA (Supplementary Table S1). Screening of fosmid end sequences for 16S rRNA genes uncovered 14 fosmid inserts containing partial or full-length 16S rRNA gene sequences affiliated with MGA (Table 1; Supplementary Figure S1). These 14 fosmid inserts were fully sequenced (Materials and methods) for downstream analyses, generating ∼540 kb of DNA sequence linked to MGA. In addition, four large-insert DNA fragments from Hawaii Ocean Time-series Station ALOHA (DeLong et al., 2006; Rich et al., 2011) and Monterey Bay (Suzuki et al., 2004) harboring MGA 16S rRNA gene sequences were identified in public databases and used in comparative analyses (Table 1; Supplementary Figure S1).

To identify subgroup affiliations, all 18 MGA 16S rRNA gene sequences identified on large-insert fragments from North Pacific Ocean environments were placed into the MGA reference tree described above (Supplementary Figure S2). Seventeen out of 18 16S rRNA gene sequences identified on large-inserts grouped with 10 defined MGA subgroups. The remaining 16S rRNA gene (on fosmid 4050020-J15) appeared to group outside of MGA and was most closely affiliated with sequences in the phylum Deferribacteres. We chose to include this fosmid in downstream analyses to represent a close relative of MGA.

Genomic content and organization of large-insert DNA fragments derived from MGA

Four criteria were used to determine the extent to which large-insert DNA fragments partitioned into groups consistent with shared environmental context or phylogenetic association, including GC content, tetranucleotide frequency, global nucleotide similarity, and amino acid similarity of predicted ORFs. The size of the large-insert fragments containing MGA 16S rRNA genes ranged from 27.4 kb to 43.5 kb with a GC content ranging from 32.8% to 47.7% (Table 1). Large-insert fragments did not differentiate into discrete groups based on similar GC content (Table 1) or tetranucleotide frequency (Supplementary Figure S4). To further investigate potential similarities among fragments associated with nucleotide arrangement, pairwise blastn analyses were performed between all fragments (Figure 1). Bit-scores for pairwise blastn analyses ranged between 0 and 4.5 × 10⁴ for non-identical fragments. Large-insert fragments from Monterey Bay (EBAC750-03B02) and the NESAP (1250012-L08 and 4130011-I07), affiliated with subgroup Arctic95A-2, were most similar to one another and formed a distinct group based on global nucleotide similarity (Figure 1). The remaining inserts did not form distinct groups based on global nucleotide similarity, but displayed a gradient of similarity, with bit scores for pairwise blastn analyses averaging (2.2 ± 1.5) × 10³. Fosmid 122006-I05, affiliated with subgroup P262000D03, was most unique at the nucleotide level.

To investigate potential similarities among large-insert fragments at the protein-coding level, ORFs
were predicted and annotated (Materials and methods). The number of predicted ORFs per insert ranged from 14 to 49, and the number of ORFs on each fragment annotated as ‘hypothetical protein’ ranged from 11 to 39 (51–92% of ORFs per insert) (Table 1).

Four groups with shared but not identical amino-acid sequences of predicted ORFs surrounding the 16S rRNA gene were identified (groups I–IV), whereas the \textit{Deferribacteres}–affiliated fosmid (4050020-J15) did not show significant similarity to any other fragments at the protein-coding level and was placed in its own group (group V) (Figure 2). These groups did not uniformly correlate with shared environmental origin or 16S rRNA sequence identity at the level of defined subgroups (Table 1, Supplementary Figure S2). In some cases it was clear that fosmid groups represented different flanking regions of the rRNA operon (that is, groups I and II; Figure 2, Supplementary Figure S5).

Four out of eight fosmids in group I were affiliated with the Arctic96B-7 subgroup, whereas the remaining four fosmids were affiliated with ZA3312c, SHBH391, SAR406 and SHAN400. Fosmids in group I contained a conserved gene cluster with genes encoding glucosamine-fructose-6-phosphate aminotransferase (involved in glucosamine biosynthesis), GMP synthase (involved in purine nucleotide biosynthesis) and acetyl-coenzyme A carboxylase carboxyl transferase subunits alpha and beta (potentially involved in fatty acid biosynthesis or CO$_2$ fixation) (Figure 2, Supplementary Figure S5). SI fosmid FPPZ.5C6 also contained a gene encoding RNA polymerase sigma-70 factor (rpoE), known to have a role in high temperature and oxidative stress response (Hild et al., 2000).

Fosmid HF0010_18O13 contained the conserved cluster of genes found in group I fosmids as well as a cluster of cytochrome c oxidase subunit genes present in Fe(II) oxidation and three pentose phosphate pathway genes also found in group III fosmids (ribulose-phosphate 3-epimerase, ribose-5-phosphate isomerase b and transketolase).

Group II fosmids were affiliated with Arctic96B-7 and P262000D03. Both fosmids in this group (FPPP.13C3 and 122006-105) contained a cluster of genes encoding enzymes involved in the pentose phosphate pathway of carbon metabolism, including ribulose-phosphate 3-epimerase, ribose-
5-phosphate isomerase b, and in one case, transketolase. Both fosmids also contained an operon encoding an enzyme complex related to polysulfide reductase (Psr). The operon on fosmid 122006-I05 contained three genes encoding homologs of the three Psr subunits: PsrA, a molybdopterin oxidoreductase; PsrB, a [4Fe-4S]-binding subunit; and PsrC, a membrane anchor subunit carrying the site of quinol oxidation, whereas the operon on fosmid FPPP_13C3 contained two genes encoding PsrA and a PsrBC fusion protein. Fosmid FPPP_13C3 contained additional neighboring genes encoding molybdobenam cofactor and molybdopterin biosynthesis proteins potentially associated with the assembly of the molybdobenam and molybdopterin guanine dinucleotide-containing subunit PsrA. Fosmid FPPP_13C3 also contained a gene for glutamate synthase, often involved in nitrogen assimilation (Vanoni and Curti, 2008), and a gene for rubrythrin, involved in oxidative stress protection in some anaerobic bacteria and archaea (deMaré et al., 1996; Sztukowska et al., 2002). Fosmid 122006-I05 contained a gene encoding a rhodanese-like protein, belonging to a superfamliy of sulfur transferases (Cipollone et al., 2007), upstream of the Psr operon.

All three genomic inserts belonging to group III were affiliated with subgroup Arctic95A-2 and were derived from Monterey Bay and the NESAP (Table 1, Figure 2, Supplementary Figure S6). These three fosmids also formed a discrete group based on global nucleotide similarity analysis (Figure 1). The main organizational feature shared by these inserts was a set of genes encoding transporters, including an ABC-type multidrug transporter, ATPase component, ABC-2 permease and a TonB-dependent receptor. Group III inserts also contained genes encoding succinyl-diaminopimelate desuccinylase, involved in lysine biosynthesis. Monterey Bay insert EBAC750-03B02 contained a gene affiliated with methionine sulfoxide reductase (msrB). In Escherichia coli, MsrB has been shown to have sulfoxide and dimethyl sulfoxide reductase activity (Grimaud et al., 2001). This insert also contained a gene encoding a rhodanese-like protein.

Fosmids in group IV were affiliated with subgroups P262000N21, SAR406, and A714018, and primarily contained genes encoding hypothetical proteins except for two conserved genes encoding an ATP-dependent protease Clp ATPase subunit and protease subunit. Group IV fosmid HF4000_22B16...
was assembled as two unordered pieces, as such, it contained a break point within the 23S rRNA gene (Supplementary Figure S6).

The only fosmid in group V (4050020-J15; most closely related at the 16S rRNA gene sequence level to members of the phylum *Deferribacteres*) did not exhibit much protein similarity to any of the MGA-affiliated fosmids. This fosmid contained genes for NADH-ubiquinone and quinone oxidoreductase involved in energy metabolism, a major facilitator superfamily transporter, a dihydroorotate dehydrogenase, a cell wall associated hydrolase, and a tRNA nucleotidyltransferase, in addition to genes encoding a number of hypothetical proteins.

**Population structure of MGA syntenic groups**

To determine the prevalence and distribution of MGA subgroups represented by large-insert DNA fragments detected in this study, the proportion of fosmid end sequences from each NESAP and SI library recruiting to large-insert fragments was determined (Figure 3). The largest proportions of sequences recruiting to large-insert fragments were derived from depths \( \geq 500 \) m in the NESAP and \( \geq 100 \) m in the SI. A very small proportion of end sequences were recruited from Aug-09 P26 libraries, which could be due to the relatively small size of these libraries (Supplementary Table S1). End sequences from NESAP libraries generally recruited to large-insert fragments in larger numbers and with a higher degree of nucleotide similarity than end sequences from SI libraries, even for large-insert fragments derived from SI (Figure 3). End sequences similar to group III fragments were most highly and consistently represented in NESAP fosmid end libraries, followed by end sequences similar to several group I fragments. End sequences similar to the *Deferribacteres*-like fosmid 4050020-J15 were also well represented and very similar to sequences derived from oxic through suboxic (but not anoxic) NESAP and SI libraries.

**Phylogenetic analysis and distribution of Psr**

To gain insight into the evolutionary history of *psrA*, *psrB*, and *psrC* genes detected on MGA fosmids, phylogenetic trees of their predicted protein products were constructed. Phylogenetic analysis of the catalytic subunit, PsrA, confirmed that predicted PsrA homologs detected on MGA fosmids were most closely related to Psr and thiosulfate reductase (Phs) of the dimethyl sulfide reductase family of molybdenum-containing enzymes (Supplementary Figure S7). Predicted PsrA homologs from MGA fosmids were \( \sim 63\% \) similar to one another, and...
most closely related to proteins encoded on fosmids from the Mediterranean Sea and Monterey Bay derived from Marine Group II euryarchaeota (Figure 4a). Predicted MGA proteins were less similar to canonical PsrA proteins originally characterized in *Wolinella succinogenes* (Krafft et al., 1995). Phylogenetic trees of predicted PsrB with PsrB-like respiratory proteins containing [4Fe-4S]-binding-subunits and of predicted PsrC with PsrC-like membrane anchor subunits indicated similar phylogenetic relationships (Figures 4b and c). Predicted PsrA proteins encoded on MGA fosmids did not contain any obvious signal sequences (for example, twin-arginine translocation (TAT) signal sequences) suggesting that these proteins are located in the cytoplasm, similar to PsrA proteins detected in most green sulfur bacteria (GSB) (Frigaard and Bryant, 2008). PsrA encoded by *W. succinogenes*, by comparison, encodes a TAT signal sequence and is translocated into the periplasm (Krafft et al., 1995).

In contrast to the organization of the psrABC operon originally described in *W. succinogenes* (Krafft et al., 1995), the ORFs encoding PsrB and PsrC homologs on both MGA fosmids were located upstream of ORFs encoding PsrA homologs (Figure 2). Also in contrast to the *W. succinogenes* psrABC operon, the genes encoding PsrB and PsrC on fosmid FPPP_13C3 appeared to form a gene fusion (psrBC), a feature also detected in several PSR-containing GSB (Frigaard and Bryant, 2008) and other PSR-containing bacteria and archaea. The psrBCA format of operon organization detected on MGA fosmids was also detected on Marine Group II fosmids and several PSR-containing GSB in addition to *Sulfurimonas denitrificans* DSM 1251, *Caldilinea aerophila* DSM 14535, *Chloroflexus aggregans* DSM 9485, and *Haladaptatus paucihalophilus* DX253 (Figures 4b and c). A third format of operon organization (psrACB) was detected in *Sulfurimonas gotlandica* GD1 and *Sulfurihydrogenibium azorense* Az-Fu1.

To determine the prevalence of predicted MGA psr genes in NESAP and SI fosmid end sequenced libraries, the proportion of fosmid end sequences that recruited to the psrBC operon on fosmids FPPP_13C3 and 122006-I05 was calculated for each end sequenced library (Supplementary Figure S8). The majority of end sequences recruiting to psr genes were derived from ≥500 m depth in the NESAP and ≥100 m depth in SI, and psr homologs were most consistently present throughout O2-deficient waters of the NESAP in August 2009 at station P4.

**Discussion**

The 17 large-insert DNA fragments containing MGA 16S rRNA genes derived from North Pacific Ocean metagenomic libraries were affiliated with seven previously defined and two novel MGA subgroups, whereas the 16S rRNA gene on an 18th insert was more closely related to the phylum *Deferribacteres*.
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associated with adaptation to O2-deficiency and mental origin, O2 concentration, or 16S rRNA gene was not generally consistent with shared environmental similarity of predicted ORFs. Group membership DNA fragments were obtained from multiple environments (Supplementary Figure S2). Although large-insert fragments did not clearly coalesce into coherent groups based on GC content, tetranucleotide frequency, or global nucleotide similarity. However, fragments did coalesce into five syntenic groups based on shared amino acid similarity of predicted ORFs. Group membership was not generally consistent with shared environmental origin, O2 concentration, or 16S rRNA gene sequence identity (Table 1). These observations could be explained in several ways. MGA subgroups may contain multiple unlinked copies of the 16S rRNA operon (Acinas et al., 2004). Alternatively, large-insert fragments may be derived from flanking regions of the same 16S rRNA operon, as observed for syntenic groups I and II. It is also possible that subgroups ZA3312c through A714018 actually represent one subgroup of MGA, evidenced by a lack of bootstrap support for nodes encompassing these subgroups within the MGA 16S rRNA gene tree (Supplementary Figure S2).

Recruitment of fosmid end sequences from Line P and SI libraries to large-insert DNA fragments reflected 16S rRNA-based patterns of MGA distribution in that the proportion of MGA sequences was maximal in waters >500 m depth in the NESAP and >100 m depth in SI (Figure 3). MGA sequences comprised a much larger proportion of NESP (open ocean) than SI (coastal basin) end libraries, a pattern also reflected in MGA 16S rRNA distribution and representative of the overall higher proportion of MGA detected in the NESAP than in SI microbial communities (Zaikova et al., 2010; Allers et al., 2012). The proportion of SI end sequences recruiting to large-insert fragments was maximal in dysoxic and suboxic samples from Nov 2006 and April 2007, supporting the hypothesis that dominant MGA subgroups are adapted to O2-deficiency in this location. The largest proportion of end sequences from NESAP libraries recruited to group III fragments affiliated with subgroup Arctic95A-2 supporting 16S rRNA-based observations that Arctic95A-2 is a dominant subgroup in the NESAP open-ocean. Group I fragments affiliated with subgroups Arctic96B-7 and SAR406 also recruited a relatively large proportion of NESAP end sequences. A reasonable proportion of end sequences from the NESAP and SI libraries also recruited to Deferribacteres-like fosmid 4050020-J15, with a pattern of distribution suggesting adaptation to suboxic and dysoxic, but not anoxic, conditions.

Although large-insert fragments did not clearly partition into ecologically distinct groups based on O2 concentration, predicted protein-coding genes associated with adaptation to O2-deficiency and sulfur-based energy metabolism were detected on multiple fosmids. With respect to adaptation to O2-deficiency, a gene encoding rpoE RNA polymerase sigma-70 factor, known to have a role in oxidative stress response, was detected on SI fosmid FPPZ_5C6, obtained from an anoxic-sulfidic 200 m sample. A gene encoding rubrerythrin, also involved in oxidative stress response in some anaerobic prokaryotes, was detected on SI fosmid FPPP_13C3, obtained from an oxic 10 m sample. With respect to sulfur-based energy metabolism, a gene encoding methionine sulfoxide reductase (MsrB) was detected on Monterey Bay insert EBAC750-03B02, which in E. coli has been shown to have sulfoxide and dimethyl sulfoxide reductase activity (Grimaud et al., 2001). In addition, four fosmids encoded rhodanese-like proteins, affiliated with a superfamily of sulfur transferases. Perhaps most interestingly, a psr operon was detected on SI fosmid FPPP_13C3 and on NESAP fosmid 122006-I05, obtained from a dysoxic 2000 m sample. Sequences similar to psr genes encoded on these fosmids were also detected in a number of fosmid end sequenced libraries derived from >500 m depth in the NESAP and >100 m depth in SI, suggesting that these genes are associated with O2-deficient environments (Supplementary Figure S8). In the anaerobic epsilonproteobacterium W. succinogenes, PSR and hydrogenase or formate dehydrogenase allows respiration on polysulfide (Sn) using H2 or formate as an electron donor, with concomitant production of H2S (Jankielewicz et al., 1995). The PSR complex isolated from W. succinogenes has also been documented to catalyze sulfide oxidation to polysulfide by dimethylphthaloquinone, however, with much lower efficiency (Hedderich et al., 1999). The identification of proteins homologous to PSR on two fosmids suggests that specific MGA subgroups may have the capacity to generate energy via dissimilatory polysulfide reduction to hydrogen sulfide (H2S) or via dissimilatory H2S oxidation (Schröder et al., 1988; Klimmek et al., 1991; Kraft et al., 1992, 1995; Jormakka et al., 2008).

The PSR complex of W. succinogenes is encoded by the psrABC genes and consists of two periplasmic subunits (a catalytic molybdopterin-containing PsrA subunit and a [4Fe-4S]-binding PsrB subunit) and a membrane-anchoring PsrC subunit (Kraft et al., 1992). Predicted PsrA proteins detected on MGA fosmids were only distantly related to isolated PsrA from W. succinogenes but more closely related to PsrA homologs encoded on Marine Group II euryarchaeotal fosmids derived from the Mediterranean Sea and Monterey Bay. PsrA proteins detected on MGA fosmids were also similar to PsrA homologs found in the GSB Prosthecoclorhis aestuarius DSM 271, Chlorobium chlorochromatii CaD3, Chlorobium luteolum DSM273, Chlorobium limicola DSM 245, and Chlorobium phaeobacteroides DSM 266; the halophilic euryarchaeon Haladapactus paucihalophilus DX253; the thermophilic Chloroflexi strain Caldilinea aerophila DSM 14535; the thermophilic Aquificales strain Sulfurhydrogenibium azorense Az-Fu1; and the sulfur-oxidizing Epsilonproteobacteria Sulfurimonas gotlandica GD1 and Sulfurimonas denitrificans DSM 1251. Interestingly, in
GSB, the phylogeny of PsrA homologs is congruent with a number of phylogenetic anchor genes, suggesting that PSR was present in the last common ancestor of PSR-containing GSB (Gregersen et al., 2011). Given the proximal phylogenetic relationship of MGA and GSB based on 16S rRNA gene sequences (Supplementary Figure S2), it is possible that MGA inherited this operon from a common ancestor. The psrBC genes on MGA fosmid 122006-I05 were encoded by separate ORFs (psrB and psrC), whereas in fosmid FPPP_13C3, these genes were fused (psrBC). A psrBC gene fusion has been described previously in members of the PSR-containing GSB (including P. aestuarii, C. chlorochromatii, and C. luteolum; (Frigaard and Bryant, 2008)), and was detected in Marine Group II fosmids from the Mediterranean Sea and Monterey Bay in addition to H. paucihalophilus and C. aerophila. The broad phylogenetic origins of psrABC genes similar to those detected on MGA fosmids are consistent with multiple lateral transfer events across phyla and domains.

Although direct evidence for the role of PSR in sulfur-based energy metabolism has only been obtained from W. succinogenes, many cultivated reference strains encoding PSR are capable of generating energy using sulfur compounds. The PSR sequences derived from several such reference strains, including S. azorense Az-Fu1 and the GSB, branched with predicted PSR homologs detected on MGA fosmids. S. azorense Az-Fu1 is capable of growth by coupling reduction of elemental sulfur (S²⁻) to hydrogen oxidation, although polysulfide was not directly tested as an electron acceptor (Aguiar et al., 2004). S. azorense Az-Fu1 has also been documented to oxidize S²⁻ and sulfate (SO₄²⁻) (Aguiar et al., 2004). Similarly, the cytoplasmic PSR complex found in many GSB (including P. aestuarii, C. chlorochromatii, C. luteolum, C. limicola and C. phaeobacteroides) has been proposed to oxidize sulfite produced by the dissimilatory sulfate reduction (Dsr) system (Gregersen et al., 2011). Although the actual substrate of PSR cannot be determined based on sequence similarity alone, the phylogenetic position of MGA PSR homologs provides a circumstantial link between MGA and sulfur cycling in the environment.

Oxygen-deficient marine systems, including OMZs and permanent or seasonally stratified anoxic basins, are known to harbor active sulfur cycles that have been linked to the activities of sulfur-oxidizing gamma and epsilonproteobacteria (Walsh et al., 2009; Canfield et al., 2010; Grote et al., 2012). Although this study provides only a glimpse into the metabolic diversity that is likely contained within the MGA candidate phylum, the presence of PSR homologs on MGA-affiliated genome fragments suggests a potential role for MGA in the cryptic sulfur cycle of O₂-deficient marine systems, where the abundance of these bacteria is concentrated. Process rate measurements linking sulfur chemistry with MGA activity are required to support this hypothesis (Milucka et al., 2012). Given the lack of cultivated representatives of MGA, the application of single-cell genomics could aid in providing the genome-wide information needed to fully describe the metabolic capacity of defined MGA subgroups residing in distinct locations (Woyke et al., 2009; Swan et al., 2011; Stepanauskas, 2012). Such high-resolution genomic data may provide additional clues as to the evolutionary history and biogeochemical roles of these widely distributed marine bacteria.

Accession numbers

Fosmid end sequenced libraries reported in this study were deposited in the Genome Survey Sequences (GSS) Database with the accession numbers LIGBSS_039072–LIGBSS_039117 (individual sequences are also available in GenBank with accession numbers KGO89956–KGO19837). Fully sequenced fosmids reported in this study were deposited in GenBank with the accession numbers KF170413–KF170426.

Conflict of Interest

The authors declare no conflict of interest.

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