Postgenomic Adventures with *Rhodobacter sphaeroides**

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Key Words

microarray, proteomics, gene-regulation, genome architecture, evolution

Abstract

This review describes some of the recent highlights taken from the studies of *Rhodobacter sphaeroides* 2.4.1. The review is not intended to be comprehensive, but to reflect the bias of the authors as to how the availability of a sequenced and annotated genome, a gene-chip, and proteomic profile as well as comparative genomic analyses can direct the progress of future research in this system.

Contents

INTRODUCTION

The purple nonsulfur photosynthetic Eubacterium, *Rhodobacter sphaeroides* 2.4.1 (ATCC number BAA-808), belongs to the α-3 subgroup of the Proteobacteria and is metabolically highly versatile. It grows by either aerobic or anaerobic respiration, photosynthesis, or fermentation. Organic compounds are used as both a source of carbon and reductant for photoheterotrophic and chemoheterotrophic growth, with carbon dioxide used as the sole carbon source under autotrophic growth conditions (89). Hydrogen can be used as the source of reducing power for photoautotrophic or chemoautotrophic growth (118). *R. sphaeroides* 2.4.1 can also utilize dinitrogen as the sole source of organic nitrogen. The ecological niche of *R. sphaeroides* is a measure of its ability to photoassimilate low-molecular-weight organic products into cell material in the presence of light or under conditions of oxygen limitation and anaerobiosis (89). Although *R. sphaeroides* grows under conditions of high O_2 tension, it appears to be best suited to microaerophilic conditions, allowing the organism to easily transition between chemotrophic and phototrophic growth. Movement is carried out by a single subpolar flagellum.

STRUCTURE AND FUNCTION

Structure and Function of the Photosynthetic Apparatus

The formation, function, regulation, and structure of the photosynthetic apparatus have been well described over several years. In response to decreasing oxygen tensions, *R. sphaeroides* develops intracytoplasmic membranes (ICM), which are invaginations of the cytoplasmic membrane. The ICM contains the photosynthetic apparatus including the pigment protein complexes and the photosynthetic electron carriers (123). After the isolation of chromatophores (sealed 50 Å vesicles produced by pinching off the ICM) was first

reported (95), numerous studies have focused on examining the structure and function of their components. This led to breakthroughs in the 1980s and 1990s that defined the structure and function of the reaction center (RC) and the light-harvesting (LH) complexes as well as details of the primary photochemistry of photosynthesis. A high-resolution structure of the RC from *R. sphaeroides* (26) has demonstrated that the RC consists of three major subunits: L, M, and H. Both L and M have five-transmembrane domains and their orientation within the membranes were described as well as the path for electron flow (81) and proton movement (83–86). The RC is surrounded by the core antenna complex LH1 to form the so-called LH1-RC-PufX core complex, with a fixed ratio of approximately 12:1 to 15:1. The LH2 complex surrounds the LH1 complex and the ratio of LH2 to LH1 is variable, with the stoichiometry changing in inverse proportion to the incident light intensity via the differential regulation of the two LH component types (56). See the following references for details on the process of light capture and energy transduction in the photosynthesis (PS) complex and the generation of ATP and proton motive force (pmf) (12, 112, 113).

In *R. sphaeroides*, the photosynthetic and respiratory pathways share a common electron transfer chain. Oxygen represses bacteriochlorophyll and carotenoid (Crt) biosynthesis and prevents the formation of the photosynthetic complexes. However, cytochrome bc_1 and cytochrome c_2 remain, and respiratory electron transfer can use these carriers to transfer electrons to the cytochrome aa_3 terminal oxidase, which has a low affinity for oxygen and is induced by increasing oxygen tension (68). Cytochrome cbb_3 has a high affinity for oxygen and is functional under low oxygen tension (77, 124). The presence of the cbb_3 in the ICM, reported in a recent proteomic study, is in accordance with its role under low oxygen tension (X. Zeng, J.H. Roh, S.J. Callister, C.L. Tavano, T.J. Donohue, M.S. Lipton & S. Kaplan, manuscript submitted).

Atomic force microscopy has developed into a powerful tool, generating high-resolution images of native ICM (3, 44, 96, 97). The ICM is composed of numerous photosynthetic domains in which linear arrays of dimeric LH2 and LH1-RC-PufX are clustered and arranged. The peripheral LH2 complexes are either grouped as 10–20 molecules to form light-capture domains that are directly associated with linear arrays of dimers of core complexes (RC-LH1-PufX), or are clustered outside these arrays, with no direct contact of these arrays to the core complexes. However, where are the F_0F_1 ATP synthase and other components of the cyclic electron carriers, such as the bc_1 complex? Numerous biochemical and recent proteomic studies (17, 29) have confirmed that these proteins are present in the ICM.

The Proteomic Analysis of *R. sphaeroides*

In recent proteomic studies, peptide mass fingerprinting was widely used to identify proteins from sample mixtures (16). The accurate mass and time tag approach has been utilized to identify various proteins and their subcellular localization in aerobically and photosynthetically grown *R. sphaeroides* (9, 10). A total of 8300 peptides were identified with high confidence (>0.7) and 1514 proteins (35% of proteins encoded by the genome) were sequenced by matching the genome data of *R. sphaeroides* 2.4.1. Of the proteins predicted to be localized in a unique subcellular fraction by the program PSORTb, 81% agree with the proteomic analysis.

The proteomics approach has been utilized to analyze the protein components of intracytoplasmic vesicles, an initial result coming from purified ICM vesicles of *Rhodopseudomonas palustris* (28). In *R. sphaeroides* 2.4.1, the ICM vesicles contain 609 proteins identified by matching the annotation data obtained from the genome project of *R. sphaeroides* 2.4.1 (**http://www.rhodobacter.org/**), but only

ICM: intracytoplasmic membrane

Reaction center (RC): a pigment-protein structure found in the membrane where photons of light are trapped and their energy converted into a chemical form

LH: light harvesting

PS: photosynthesis or photosynthetic

Proteomics: the global study, often at different times and under different conditions, of the structure, function, and expression of proteins in a cell

153 proteins had at least one peptide identified in all replicates of the ICM proteomic data (X. Zeng, J.H. Roh, S.J. Callister, C.L. Tavano, T.J. Donohue, M.S. Lipton & S. Kaplan, manuscript in preparation). All the subunits for the photosynthetic complexes including RC, LH1, and LH2 exist in the ICM vesicles uniquely; the bc_1 complex subunits, a possible alkane hydroxylase, and two soluble proteins, spheroidene monooxygenase and a generic methyltransferase, were also anchored in or associated with the ICM at relatively high levels. Another surprise observed for the ICM proteome is the possible presence of 42 inner-membrane-enriched proteins related to ATP synthesis, ABC transporter/translocation, respiratory electron transfer, as well as numerous membrane-associated proteins (3, 33, 44, 73, 98, 114).

Quorum sensing: a special kind of intraspecies chemical signaling that bacteria use to monitor their population density and respond to its changes as a community

Exopolysaccharide: a sugar polymer that forms the mucilage secreted from a cell which is of relevance in biofilm formation

ORF: open reading frame

Chemotaxis: a guided motility in which bacteria move away from harmful or toward favorable chemical conditions

COMMUNITY LIVING AND MOBILITY

Quorum Sensing

Planctonic cultures of *R. sphaeroides* 2.4.1 grown under standard laboratory conditions produce one acyl-homoserine lactone signal, 7,8-*cis*-*N*-(tetradecenoyl)-homoserine lactone (93), which is the *R. sphaeroides* autoinducer (AI). Its formation is catalyzed by the enzyme CerI, a homolog of LuxI. Inactivation of CerI abolishes AI production and leads to the clumping of cells in liquid cultures because of increased exopolysaccharide production. Probable contributors to the AI mutant phenotype are the *opgGIHC* genes found downstream of the *cer* locus (15). Although no evidence appears to connect this locus to quorum sensing, recent experiments suggest that a second *mdoG* homolog, RSP3187, may be the source of the excess exopolysaccharide. Because of its homology to the LuxR family of genes, an open reading frame (ORF) upstream of *cerI* was proposed to encode the cognate regulator and therefore designated *cerR*. Sequence homologies predict at least four more quorum-sensing regulatory elements in the completed genome, two of them, RSP6095 and RSP6096, are positioned upstream of *cerRAI*. The protein sequence deduced for RSP6095 contains only an AI-binding domain, suggestive of a modulator function.

Motility

Although both structurally and functionally similar to the paradigm *E. coli*, the signaling system that controls the single, subpolar flagellum of *R. sphaeroides* is more complex (115). Three chemotaxis operons (*cheOp*) (46, 92), an unlinked methyl-accepting chemotaxis protein (MCP), and a transducer-like protein are encoded by the large chromosome of *R. sphaeroides* 2.4.1. The small chromosome encodes six MCPs, one of them forming the *McpG* locus with an ORF encoding the response regulator CheY4 (101). MCPs and most of the chemosensory proteins encoded by *cheOp2* form large clusters at the poles of the cell, while transducer-like proteins along with the proteins encoded by *cheOp3* occupy discrete regions in the cytoplasm (116, 117). Correct positioning of the cytoplasmic clusters requires PpfA, a protein encoded by *cheOp3* (110). On the basis of sequence homologies to known components of flagella biosynthesis pathways, 62 genes in the *R. sphaeroides* 2.4.1 genome have been identified as probable participants in this process. Thirty-seven of these genes are located in the vicinity of *cheOp3*. The rest (located independently of the three chemotaxis operons) are encoded by a region on the large chromosome and by a short operon on pRS241a. In *R. sphaeroides* 2.4.1, 19 flagellum-biosynthesis-related proteins, including flagellin, are encoded by more than one gene. In a recently proposed model (90) the master regulator FleQ initiates the flagella biogenesis of *R. sphaeroides*, and the genes participating in the process are expressed sequentially, forming a four-tiered hierarchy.

MICROARRAY DATA

Transcriptome Analysis of *R. sphaeroides* 2.4.1 Using the Affymetrix GeneChip

The *R. sphaeroides* 2.4.1 DNA microarray (GeneChip) is composed of 4490 probe sets from genes and 816 probe sets from both strands of intergenic regions. There are also 25 probe sets from other *R. sphaeroides* strains. Seven probe sets from *Escherichia coli*, 2 probes sets from bacteriophage P1, and 15 probe sets from *Bacillus subtilis* were included as controls. To date, the *R. sphaeroides* GeneChip has been used for two purposes: to investigate directly the effects of growth conditions and mutations on transcription (2, 6, 70, 94, 109, 128) and to predict indirectly the DNA-binding sequences for the transcriptional regulators FnrL, PpsR, and PrrA (64).

Transcriptome patterns using independent, triplicate cultures from seven different growth conditions (21 samples) were compared using the hierarchical clustering algorithms within dChip software (51). As shown in **Figure 1*a***, clustering revealed distinct global transcriptional patterns. Transcriptome profiles from cells grown under low- or medium-light (3 W/m^2 or 10 W/m^2) photosynthetic conditions were substantially different from fully aerobic (30% O_2) cultures or high-light (100W/m^2) grown photosynthetic cells (**Figure 1*a***). As reported previously (87, 94), the genes encoding the aerobic energy-generating system (RSP1035–1038) and metabolic pathways (e.g., tricarboxylic acid cycle enzymes) showed increased expression under aerobic conditions. In addition, expression of the genes for the anaerobic metabolic pathways such as CO_2 and N_2 fixation as well as photosystem formation are increased under 3 W/m^2 and 10 W/m^2 conditions (87, 94).The transcriptome patterns from aerobic (30% O_2) and 100 W/m^2 grown photosynthetic cells show a similarity to one another (94) that we would not have predicted a priori.

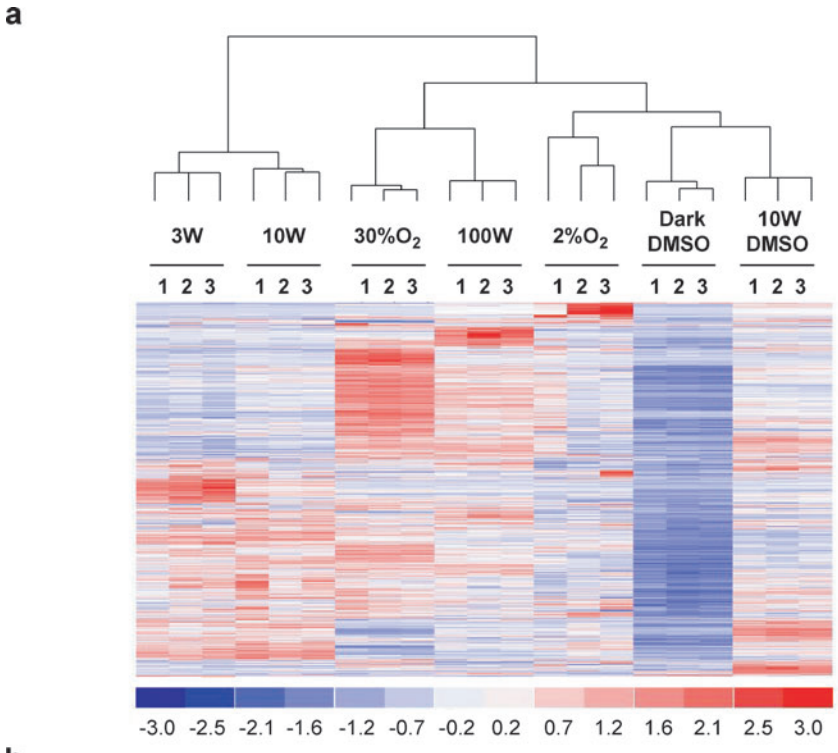
Approximately 38% of the genes in the genome (2070 probe sets) are called present across all seven culture conditions, whereas approximately 18% of the genes (947 probe sets) are not expressed (called absent). The list of all present and all absent genes under the seven growth conditions are available in **Supplemental Table 1** (follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**) and at **http://www.rhodobacter.org/**. One hundred fifty-eight genes are either present (94 genes) or absent (64 genes) exclusively under aerobic conditions, i.e., these same genes are either absent (94 genes) or present (64 genes) under six other growth conditions. Under aerobic conditions the 64 absent genes include the PS genes and genes for CO_2 (RSP3266, RSP3267, RSP3268, and RSP3270) and N_2 fixation (RSP0428 and RSP0547). The expression pattern of these genes is consistent with previous studies (87, 94) and thus provides increased credibility for other genes identified using the transcriptome profiles obtained from the seven different growth conditions. Of particular importance, approximately 30% (1240/4105 ORFs) of the genes that are annotated as hypothetical can be divided into several groups by their expression pattern under the seven different growth conditions (see **Supplemental Table 2**).

In *R. sphaeroides* 2.4.1, flagella- and chemotaxis-related genes are located at four major loci (61). Chemotaxis operon I (RSP2432–RSP2444, between 1066180 bp and 1078567 bp), operon II (RSP1582–RSP1589, between 174089 bp and 181519 bp), and operon III (RSP42–RSP49, between 1747265 bp and 1757246 bp) are present on chromosome I. Chemotaxis operon III is part of a 56-kb flagella biosynthesis gene cluster (RSP0032–RSP0088, between 1736252 bp and 1799246 bp on chromosome I). Expression of the chemotaxis operon II and operon III with the adjacent flagella biosynthesis gene cluster showed significantly increased gene expression in both semiaerobic and 10 W/m^2

Transcriptome: the global collection of mRNAs found within a cell. The transcriptome profile varies in response to environmental conditions that alter gene expression patterns

GeneChip: a collection of oligonucleotides fixed to a solid support that represents the genes in the genome used to detect changes in mRNA levels

Hierarchical clustering: in the context of gene-chips, genes showing similar expression patterns are grouped to reflect their similar expression profiles



Present	ORF	58–64	54–61	65–71	68–72	57–70	57–65	59–60
	GAP	(31–38)	(28–30)	(34–41)	(39–44)	(25–33)	(30–34)	(29–31)
Absent	ORF	32–39	35–41	25–31	25–29	26–40	32–38	36–37
	GAP	(58–65)	(65–68)	(54–61)	(51–56)	(64–71)	(47–66)	(65–67)
Marginal	ORF	3–4	4–5	3–4	3–4	4	3–4	3–4
	GAP	(4–5)	(4–5)	(5–6)	(4–5)	(4–5)	(5–6)	(4)

Figure 1

(*a*) Hierarchical clustering of the 4286 probe sets from the GeneChip after the exclusion of the 947 unexpressed genes (probe sets) under seven growth conditions. Each column represents triplicate samples prepared from each growth condition, and each row represents a gene. At the top and bottom of the hierarchical clustering data is a sample clustering tree and color scale, respectively. Red and blue indicate positive and negative ratios of expression level of genes across all samples, respectively. The expression level for each gene is standardized to have a mean of 0 and a standard deviation of 1. The dChip software performs the standardization and clustering as described (20, 36). (*b*) The ratio of present/absent/marginal probe sets under the seven growth conditions. The Present/Absent/Marginal ratios are indicated by the lowest and highest percentage difference over triplicate samples.

with dimethyl sulfoxide (DMSO) conditions, while expression of chemotaxis operon I was constant.

Genes for N_2- and CO_2-fixing enzymes as well as for photosystem formation are required for anaerobic growth and energy generation under photosynthetic conditions. Their expression is regulated mainly by oxygen levels, i.e., their expression is induced under hypoxic or anaerobic conditions (either

DMSO: dimethyl sulfoxide

presence or absence of light), but not under highly aerobic conditions (87, 94). For example, expression of the PS genes is regulated directly or indirectly by the PpsR protein (70) and repressed by blue-light irradiation (6) and the addition of hydrogen peroxide (128) under semiaerobic conditions. The effect of blue-light irradiation and hydrogen peroxide on PS gene expression is mediated by the AppA/PpsR antirepressor/repressor system (6), and the anaerobic transcriptional regulator FnrL was also involved in the downregulation of the PS genes by the addition of hydrogen peroxide (128).

Analysis of the transcriptome data could help to characterize genes presently designated unknown, as well as redefine the function of known genes. Although we cannot show all the data, readers are encouraged to look to the supplementary material (**Supplemental Table 1** and **Table 2**) to investigate the expression patterns for either known or unknown genes under the seven standard growth conditions. Although additional molecular and biochemical experiments are required to determine the function of each predicted gene, global approaches using the GeneChip allow us to track these genes as a function of growth.

GENE REGULATION

Substitutive Global Regulators: Who Gets the Job Done in *R. sphaeroides*?

The use of hierarchical clustering to detect genes that share similar expression patterns in *R. sphaeroides* 2.4.1 has made possible the identification of DNA-binding sites for the transcriptional regulators PrrA, PpsR, and FnrL, as well as for two as yet unidentified regulators (64). The interplay of global regulators and cell physiology has been observed in the Kaplan and many other laboratories (53). To further study basic aspects of gene regulation, we have attempted to compare the suite of global regulators in *R. sphaeroides* with the global regulators found in *E. coli* using BLAST. This analysis revealed the absence of three major global regulators in *R. sphaeroides*, RpoS, Lrp, and Fis, as well as other critical regulatory proteins (18, 50, 54, 99). Homologues for SoxRS, the oxidative damage regulators (91), were also absent. However, OxyR, the other major oxidative stress regulator, was found. The MukBEF proteins involved in the structural maintenance of chromosomes are also absent in *R. sphaeroides*. The MukBEF proteins have not been classified as global regulators, but they are involved in compaction of bacterial chromatin, along with the other missing regulators Fis and Lrp. This suggests that other proteins play this role in *R. sphaeroides*, or that the strategy for organization of the nucleoid and chromatin compaction in *R. sphaeroides* differs significantly from that in *E. coli*. The principal global regulators present in *R. sphaeroides*, but absent in *E. coli*, are the Prr signal transduction system and the repressor PpsR, four homologues of the histone-like protein Spb (HvrA), and a tryptophan-rich regulatory protein (TspO).

Signal transduction: the process by which cells sense their environment and vary their gene expression patterns to accommodate changes in the environment

E. coli Global Regulators Not Found in *R. sphaeroides*

R. sphaeroides does not appear to have a specific sigma factor involved in general stress or entry into stationary phase, e.g., RpoS. Thus, these functions may be ascribed to non-sigma-factor-type regulator(s), which by definition must have a different strategy for RNA polymerase recruitment onto gene promoters under general stress or entry into stationary-phase conditions. Lrp has also been described to function like a histone-like protein because it has roles in DNA topology (18) and nucleoid organization (50). Whereas some α-Proteobacteria contain Lrp (122), the *Rhodobacterales* are devoid of it, although they have other members of the Lrp-like (AsnC) family of proteins, e.g., PutR, an LRP-like regulator specific for proline utilization, is present in *R. sphaeroides* (RSP2165) and *R. capsulatus* (55). RSP0722 and RSP1867 in *R. sphaeroides* are also members of the AsnC

family. If one were to consider the role of Fis as a histone-like protein, then this function could be ascribed to one or more of the Spb (HvrA in *R. capsulatus*) homologues in *R. sphaeroides* (8, 102).

One- and Two-Component Signal Transduction Systems in *R. sphaeroides*

Prokaryotic signal transduction is effected by both one- and two-component systems (111). One-component systems refer to individual signal transduction proteins in which the input domain is directly fused to the output domain, thus eliminating phosphate transfer (111). Two-component systems evolved later, with the need for an input domain localized to the membrane and an output domain possessing DNA-binding activity. In an analysis of 145 complete and draft prokaryotic genomes it was found that *R. sphaeroides* contains 452 signal transduction domains in a total of 295 proteins. These domains were organized into 15 different categories. The data are shown in **Figure 2** (111). Considering that there are 4369 genes encoding 4242 proteins, nearly 7% of the *R. sphaeroides* proteins are dedicated to signal transduction. Furthermore, there are 116 two-component proteins, as shown in **Table 1**, although only 46 transmitter domains and 64 receiver domains were found.

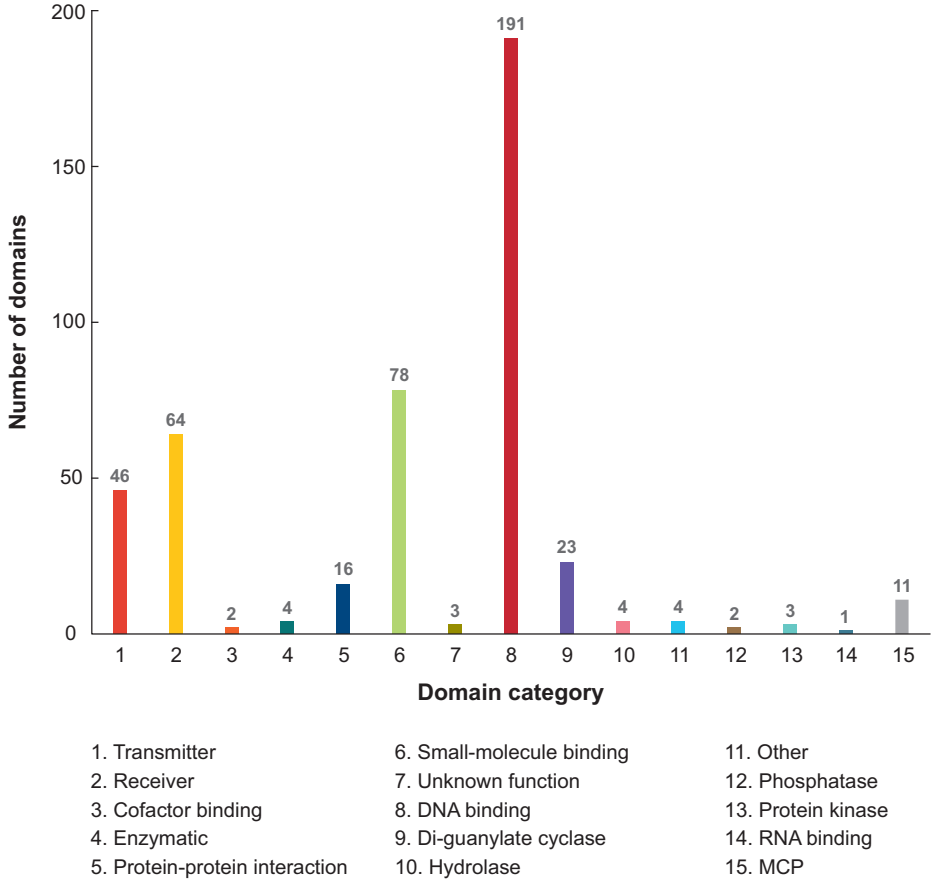


Figure 2

The *R. sphaeroides* 2.4.1 signal transduction profile. There are 452 signal transduction protein domains in *R. sphaeroides* distributed among 15 categories. The graph is based on a computational domain analysis of protein sequences in the *R. sphaeroides* genome (111). Input and output domains for signal transduction proteins are based on the P-famA (4), SMART (60), and COGs (108) resources. This work is reproduced with permission from the authors (111).

Table 1 The distribution of genes encoding one- and two-component systems in the *R. sphaeroides* genome

Replicon	Size in Mb	One-component proteins	Two-component proteins
Chromosome I (CI)	3.19	98	88
Chromosome II (CII)	0.94	53	21
Plasmid A	0.11	5	0
Plasmid B	0.11	12	3
Plasmid C	0.11	3	2
Plasmid D	0.10	4	2
Plasmid E	0.04	4	0
Total	4.6	179	116

The Prr Two-Component System of *R. sphaeroides*

The PrrBCA two-component system of *R. sphaeroides* is a master regulator that is involved in expression of approximately 850 genes, >20% of the genome (53; J. Eraso & S. Kaplan, manuscript in preparation), and it acts both as an activator and a repressor. The system comprises the membrane-associated histidine kinase/phosphatase PrrB (24, 82), the response regulator PrrA (23), and the Cu^{2+}-binding, membrane protein PrrC (25, 67). A similar model for redox regulation of gene expression by the RegBA system in *R. capsulatus* has also been described (22, 71, 100, 106). It was recently reported that RegB interacts with the quinone pool at a site located in periplasmic loop 2, between helices 3 and 4, and that it senses the redox state of the quinone pool. A reduced quinone pool would favor kinase activity of the protein, at the expense of phosphatase activity. This is similar to the way the histidine kinase ArcB of the ArcBA system in *E. coli* senses redox conditions in the cell (32).

This cannot explain regulation by PrrB in *R. sphaeroides*, because removal of the cbb_3 oxidase activates PS gene expression despite the fact that more than 70% of the reductant moves through the aa_3 terminal oxidase, thus not substantially affecting the redox state of the quinone pool, yet PS gene expression is induced. Conversely, removal of the aa_3 oxidase would be expected to result in a reduction of the quinone pool, yet PS gene expression is not induced. Thus there are significant differences between these two redox sensing systems.

The cbb_3 oxidase expression in *R. capsulatus* (104) is similar to that in *R. sphaeroides*, with Prr and FnrL being the major regulators. It is possible that PrrB, in addition to interacting directly with the cbb_3 oxidase (80), interacts with a diffusible quinone pool through the cbb_3 oxidase. The amino acid signature GGXXNPF found in RegB, which is involved in the quinone interaction (107), is also present in the second periplasmic loop of PrrB (amino acids 102–108). In addition, a mutation in this loop of PrrB a number of years ago (24, 79) rendered cells unable to grow properly under aerobic conditions, presumably because of high PS gene expression, leading to the conclusion that this region within PrrB is important for interactions involved in redox sensing. When various deletions of the membrane span of PrrB were made (79), the second membrane-spanning region in PrrB was found to be critical to its function as a regulator. Whether this is the site for interaction with the cbb_3 oxidase and/or the quinone pool remains to be determined. Thus, the role of the cbb_3 oxidase and the membrane-spanning region of PrrB and particularly the second periplasmic loop of PrrB have brought together the electron transport chain at the level of the histidine kinase to effect gene regulation.

ECF: extracytoplasmic function family

As mentioned above, the role for PrrA as a master regulator has been extended by the discovery that PrrA controls expression of approximately 850 genes (53; J. Eraso & S. Kaplan, manuscript in preparation). Work in several laboratories (22, 53, 127) has revealed that PrrA (RegA) regulates expression of genes involved in photosynthesis, carbon dioxide fixation, nitrogen fixation, hydrogen uptake, aerotaxis, denitrification, electron transport, aerobic and anaerobic respiration, and heme biosynthesis, among others, thus emphasizing its global role. It is evident, when comparing *prrA*$^{+}$ and *prrA*$^{-}$ strains, that PrrA is responsible for a major metabolic readjustment. On the basis of the number of genes involved and the density of metabolic pathways, there must be considerable overlap between the PrrA regulon and other major regulatory cascades in the cell. The regulator AppA, for which the only known target is PpsR (41, 47), would also be implicated in this complex control system. PrrA (RegA) binds DNA both in a specific and nonspecific manner (48, 58) via the helix-turn-helix (H-T-H) motif located at its carboxy terminus. The fold of this binding domain is a Fis-type fold. In addition, in order to bind DNA in a specific manner, the protein must be phosphorylated (52, 58), which results in its dimerization (57, 58) and subsequent occupation of the two DNA half-sites.

By combining hierarchical clustering of microarray data with sequence scanning, a consensus PrrA-binding sequence was predicted with two nucleotide blocks of six and five nucleotides, respectively, with a high degree of degeneracy, and an imperfect palindrome (64). These blocks are separated by a variable spacer region, which was proposed earlier (57) and which can have anywhere from 0 to 10 nucleotides.

The third member of the Prr signal transduction system, and the least well studied, is PrrC (24) (SenC in *R. capsulatus*) (7, 105). It is a homologue of eukaryotic Sco proteins. A regulatory role for PrrC was revealed when it was found that a *prrC* deletion mutation leads to PS gene expression under aerobic growth conditions (25). The amino-terminal domain of PrrC is located in the cytoplasm, but most of the protein lies within the periplasm (25). Within this periplasmic domain there is a conserved CXXXCP motif, which is involved in copper binding and in intermolecular disulfide bond formation (67).

Global Regulatory Proteins

In *R. sphaeroides* and *R. capsulatus*, genes coding for proteins similar to H-NS and HU have been observed: The *spb* (*Sphaeroides puf*-binding) and *hvrA* genes code for proteins that bind upstream of the *puhA* gene and the *puf* operon, respectively, under light conditions (high or low intensities), thus altering the availability of DNA-binding sequences for other transcription factors (8, 75). In *R. sphaeroides*, the *himAD* genes coding for the two subunits of integration host factor (IHF) (31) have been cloned and the regulatory region upstream of the coding sequence of the *puc* operon houses a consensus binding sequence for IHF. This global regulator plays a critical role on the enhanced expression of *puc* operon transcription by oxygen and light (59).

Recently, the role of the σ^{E} polypeptide encoding an alternative sigma factor belonging to the ECF (extracytoplasmic function family) group was explored in *R. sphaeroides*. This ECF sigma factor is required to mount a transcriptional response to singlet oxygen (1), a reactive oxygen species that could appear during energy transfer from excited triplet-state chlorophyll pigments in the PS apparatus to ground-state triplet oxygen. The ChrR gene coding for a zinc-dependent antisigma factor inhibits the function of the σ^{E} factor by forming a σ^{E}-ChrR complex.

FnrL

The FnrL protein of *R. sphaeroides* is considered the homologue of the FnR (fumarate and nitrate reduction regulatory) protein of *E. coli*

involved in anaerobic regulation processes, increasing the transcription of genes when oxygen tension is reduced (126). An FnrL$^-$ mutant strain (126) of *R. sphaeroides* is unable to grow under anaerobic conditions, i.e., photosynthetically or in the dark with DMSO as the terminal acceptor of electrons (124). Genes of *R. sphaeroides* whose upstream regulatory sequences show the presence of the TTGAT-N_4-ATCAA FNR-binding site were studied, and a role for FnrL was confirmed in each case (76, 126). The following genes belong to the FnrL regulon: the *puc* operon and the *bchE* gene critical for the synthesis of the photosynthetic apparatus (76, 126); the *ccoNOQP* operon encoding the cbb_3-type cytochrome *c* oxidase; the *dorSR* genes encoding the two-component system that stimulates the expression of *dorBAC* genes; the DMSO reductase containing the molybdopterin active site and a DMSO-inducible *c*-type cytochrome (72); and the *hemA* gene coding for 5-aminolevulinic acid (ALA) synthase catalyzing the formation of ALA, the first precursor to all tetrapyrroles synthesized by *R. sphaeroides* (27, 76, 125, 126).

PpsR and the AppA-PpsR Antirepressor/Repressor System

The inactivation of the PpsR protein in *R. sphaeroides* leads to derepression of the expression of PS gene operons (*bch*, *crt*, *puc*) under aerobic conditions (40, 88). In the purple bacteria where *ppsR* was detected, this gene is always located in the PS gene cluster (21). This protein shows 53% identity to the product of the *crtJ* gene of *R. capsulatus* at the amino acid level, whereas the alignment of all known sequences of the PpsR protein shows a lower percentage of identity (21). Nevertheless, a common protein structure is observed in all the PpsR proteins: a H-T-H domain at the C terminus, two PAS (Pern-Arnt-Sim) sites in the central domains, and cysteine residues (C251 and C424 in *R. sphaeroides*) proposed to play a critical role in the transcriptional activity of PpsR (38).

Biochemical studies of the PpsR protein from *R. sphaeroides* and *R. capsulatus* showed the critical role of an intramolecular disulfide bond (65, 66). The function of PpsR is critical to prevent PS gene expression in the presence of oxygen, and a probable role for the cysteine residues is likely (38, 65, 66). In *R. sphaeroides*, the two cysteine residues are reduced and PpsR binds to target DNA promoters under all growth conditions (11). This contradicts the results obtained in vitro that showed the formation of an intramolecular disulfide bridge when exposed to high oxygen tension (66).

Another mechanism proposed to reflect the redox-sensing capacity of PpsR in *R. sphaeroides* is inherent in the AppA-PpsR system. AppA is a flavoprotein that contains an N-terminal FAD-binding domain and a Cys-rich motif at the C-terminal domain and plays an essential role in the development of the photosynthetic apparatus as an antirepressor of the PpsR protein in vivo (39, 41, 42). The release of the repressor effect of PpsR by AppA is proposed to occur by reduction of the disulfide bond in PpsR (65) and by the formation of an AppA-$PpsR_2$ antirepressor-repressor complex. Two signals modulate the antirepressor role of AppA: (*a*) the change of oxygen availability and (*b*) the presence of (blue) light (42, 78). Thus AppA is the first example of a protein able to integrate redox and light signals.

In the case of the second signal integrated by AppA, the N-terminal domain that noncovalently binds FAD functions as a new type of photoreceptor named BLUF that is not involved in oxygen control (5, 43). AppA forms an AppA-$PpsR_2$ complex that dissociates under blue-light exposure, making PpsR available for DNA binding and PS gene repression (5, 43, 65).

Recently, several transcriptome studies allowed a better characterization of the *ppsR* regulon, and it was shown that the genes *hemC* and *hemE*, involved in the tetrapyrrole biosynthesis, as well as the *puf* and *pubA* operons encoding photosystem core proteins, were

repressed by PpsR (70). Also, a study of the transcriptome profile of *R. sphaeroides* in response to hydrogen peroxide shows a modification of the expression of the *appA* gene coding for the PpsR antirepressor after 7 min of exposure, leading to a decrease of gene expression involved in the synthesis of the photosynthetic apparatus. These data suggest the existence of another regulatory system that controls PS gene expression by modulating the expression of the *appA* gene (128).

The *tspO* gene of *R. sphaeroides* codes for an outer-membrane-localized protein involved in the efflux/control of porphyrin intermediates that negatively modulates the expression of PS genes, *puc*, *crtA* and *crtI*, also under the control of the AppA-PpsR antirepressor/repressor system. (119–121, 129). Moreover, the presence in multicopy of the *hemN* gene that catalyzes the formation of protoporphyrinogen IX produces a TspO$^-$-like phenotype in the wild type (120).

The *ppaA* gene encodes a new regulatory protein. Its presence in extra copy activates photopigment production and *puc* gene expression under aerobic conditions, and an interrupted *ppaA* strain of *R. sphaeroides* exhibits a lower quantity of photopigment and decreased expression of the *puc* genes under aerobic conditions (37). However, no distinct phenotype related to the formation of the photosynthetic apparatus was observed under anaerobic conditions in a PpaA mutant strain of *R. sphaeroides* (37). The PpaA protein contains a corrinoid-binding domain, thus suggesting that the activity of PpaA could depend on the availability/structure or redox status of a bound corrinoid cofactor.

The *ppsr*$^-$*prrA*$^-$ double-mutant-containing strain of *R. sphaeroides* was observed to grow again under photosynthetic conditions (70; J.M. Eraso, unpublished data). Studying the effect of *ppsR* inactivation in a *prrA*$^-$ background using postgenomic tools will provide significant, new information. In fact, pairwise comparison of transcriptome profiles (one strain to another) and cross-referencing these pairwise comparisons has led to (*a*) a better characterization of the *ppsR* regulon, (*b*) the determination of genes strictly necessary for PS growth, and (*c*) the discovery of branch points between the AppA-PpsR and PrrBA regulatory pathways (P. Bruscella & S. Kaplan, manuscript in preparation).

GENOME ARCHITECTURE

Complex Genome Organization of *R. sphaeroides*

The genome of *R. sphaeroides* 2.4.1 as well as that of other related organisms (63) consists of two circular chromosomes (CI and CII) and five endogenous circular plasmids (103). The origin and evolution of a second chromosome implicate either the dissolution of a large ancestral chromosome, the insertion of essential gene(s) into preexisting plasmids, or horizontal replicon transfer. Genome analysis of *R. sphaeroides* revealed an ancient association of the two chromosomes possibly prior to the formation of the species (13) and significant dispersal of essential genes between the two chromosomes (61, 62). This supports the hypothesis that CII of *R. sphaeroides* possibly originated by division of the principal chromosome. However, the low coding density of CII and the possession of *repABC* suggest a derivation from a plasmid ancestor.

Reviewing the Process of Bacterial Cell Division: Multiple Chromosomes

Multipartite chromosomes in bacteria contain different types of origins of replication (*ori*), which is consistent with the idea of different replication factors being required for replication. The principal chromosome of several species of α-Proteobacteria possesses *Cori* (origin of replication as in *Caulobacter crescentus*), which depends on DnaA initiation. In contrast, the second chromosome contains *RepABC* origins that are characteristic of plasmids whose replications are initiated by *RepC*

independently of DnaA. A different type of origin of replication on CII of *Vibrio cholerae* (*oriII*) has recently been discovered and it requires two novel genes (*rctA* and *rctB*) to regulate its replication (19). Cellular localizations of two chromosomes in *V. cholerae* were detected by cloning the binding sites for fluorescent proteins adjacent to each origin of replication. CI and CII of *V. cholerae* localize at the poles and at the center of the cell, respectively (30).

Two master regulators of the cell cycle, CtrA and GcrA, were discovered and thoroughly investigated in *C. crescentus* (49), also a member of α-Proteobacteria. The CtrA/GcrA regulatory circuit in *C. crescentus* resembles the cyclin/cyclin-dependent kinase (CDK) system driving cell cycle transitions in eukaryotes. Cell cycle regulatory genes, *ctrA* (RSP 2621), *gcrA* (RSP 2007), and *cckA* (RSP 0454), that correspond to respective gene homologues of *C. crescentus* have recently been identified in *R. sphaeroides*. These regulatory genes appear to be essential at least for photosynthetic growth. In addition, all genes involved in DNA replication, chromosome segregation, and cell division, which are also controlled by *ctrA/gcrA* master regulators, have been identified in *R. sphaeroides*. Except for *bupA* (RSP 3589), all cell cycle-regulated genes, including *ctrA/gcrA*, are located on CI.

Multiple Chromosomes Facilitate Genetic Diversity

Recently, multiple genome alignment of three strains of *R. sphaeroides* demonstrated that of the three *R. sphaeroides* strains, 2.4.1, ATCC 17025 (accession number AAME00000000), and ATCC 17029 (accession number AAMF00000000), 2.4.1 and ATCC 17029 share the most extensive DNA homology. The frequency and the amount of total intergenomic DNA duplications between these three strains revealed that the genome of strain ATCC 17025 had diverged more from the other two strains and possibly separated before the split of 2.4.1 and ATCC 17029 (14). Thus, these two independent analyses demonstrate a close phylogenetic relationship between the genomes of *R. sphaeroides* 2.4.1 and ATCC 17029 and a distant relationship between these two strains and ATCC 17025.

The rapid nucleotide divergence of CII-specific sequences as well as the existence of more unaligned regions between CII from different strains reveals its faster evolution, which could be attributed to different evolutionary pressures brought on this replicon. CII may also be susceptible to rapid chromosomal changes because it has a relatively low coding capacity with long intergenic sequences, which together make CII more prone to the accumulation of genetic variants.

THE EVOLUTION OF *R. sphaeroides*?

R. sphaeroides and the Tree of Life

The recent deluge of finished and unfinished bacterial genomes into the public domain has forced us to reassess our perceptions of where *R. sphaeroides* lies in the Tree of Life and what its closest relatives are. When we sequenced the *R. sphaeroides* genome and compared its genes to those in the NCBI database using BLAST, the top gene match, if one existed, was generally found in one of the following three organisms: *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, and *Brucella melitensis*. Occasionally genes of *Paracoccus denitrificans* would also be found.

One reason that *R. sphaeroides* genes may be similar to genes from these organisms may have been purely artifactual, i.e., there were simply more genes of *A. tumefaciens*, *S. meliloti*, and *B. melitensis* in the databases than those of other closely related purple nonsulfur photosynthetic organisms such as *Rhodopseudomonas palustris* or *Rhodospirillum rubrum*, which for biochemical reasons (photosynthesis) had been considered close relatives.

Clade: a taxonomic group of organisms that are descended from a single common ancestor

CDS: coding sequences

However, once draft genomes of *R. palustris* and *R. rubrum* were deposited, probing the database still revealed best matches to the earlier common trio.

From the 16S ribosomal Wosean Tree of Life, *A. tumefaciens*, *S. meliloti*, *B. melitensis*, and *P. denitrificans* were known to be closely related to *R. sphaeroides*. In addition, they all had a common feature, i.e., a similar genome architecture with multiple chromosomes and/or megaplasmids that buttressed the 16S RNA tree perspective. As an *R. sphaeroides*-like organism had been proposed to be the ancestor of the eukaryotic mitochondrion (45), then we could envisage a unique cluster of bacteria with interesting genome architecture that may have facilitated a mechanism to establish symbiotic relationships with other organisms. Indeed it had previously been hypothesized that the agrobacteria and rhizobia may have evolved through the initial infection of water plants by purple nonsulfur photosynthetic bacteria (34). Therefore our BLAST results returned matches to the organisms that we would have predicted in the 16S Tree of Life.

It therefore came as a surprise when a recent release of the NCBI database gave, almost exclusively, top matches to gene translations of *Silicibacter pomeroyi* DSS-3, a member of the marine *Roseobacter* clade (69). Also of interest was that *S. pomeroyi* has genes advantageous for associations with plankton and suspended particles, including genes for uptake of algal-derived compounds, use of metabolites from reducing microzones, rapid growth, and cell density-dependent regulation (69), many features that are broadly analogous to those found in nodulating or tumor-forming bacteria. *S. pomeroyi* also has a circular chromosome of 4.1 Mb and a 0.49 Mb megaplasmid, i.e., its genome architecture is similar to that of *R. sphaeroides* and its near relatives.

A BLAST database of the *R. sphaeroides* coding sequences (CDS) has been constructed. The CDS from each of the following *Roseobacter* genomes, *Jannaschia* sp. CCS1, *Oceanicola granulosus* HTCC2516, *Oceanicola batsensis* HTCC2597, *Roseobacter denitrificans* OCh 114, *Roseobacter* sp. MED193, *Roseovarius* sp. 217, *S. pomeroyi* DSS-3, *Silicibacter* sp. TM1040 and other α–proteobacterial genomes; *A. tumefaciens* C58, *Bradyrhizobium japonicum* USDA 110, *B. melitensis* 16M, *P. denitrificans* PD1222, *R. palustris* CGA009, and *S. meliloti* 102, were used to query the database using WU-BLAST 2.0 (35) in BLASTP mode. The *E. coli* K12 genome was also used as a comparative outlier. The top-hit of each CDS BLASTP output was then extracted and all CDS in the genome were sorted according to P value. The percentage of the genes in each genome that fell within the following P-value ranges of $<10^{-200}$, $10^{-200-150}$, $10^{-150-100}$, $10^{-100-75}$, 10^{-75-50}, 10^{-50-35}, 10^{-35-20}, and 10^{-20-10} was then determined. P values of $>10^{-10}$ were not included in the analysis.

From our preliminary result (not shown) three things were apparent. Compared with other α–Proteobacteria, members of the *Roseobacter* clade had the greatest proportion of genes with high-quality matches to the *R. sphaeroides* database, i.e., their BLAST returns had small P values. Of the *Roseobacter* species, *Roseobacter denitrificans* OCh 114 had the greatest percentage of genes (5.6% = 221/3944 genes in its genome) with the highest-quality matches (P value of $<10^{-200}$) to genes in the *R. sphaeroides* database, and just slightly behind by ∼0.2% were the results for *O. granulosus* HTCC2516 (205/3792 genes), ∼0.3% for *S. pomeroyi* DSS-3 (229/4250 genes), and ∼0.4% for *Silicibacter* sp. TM1040 (204/3863 genes). Finally, about 5% of the genes of the *P. denitrificans* PD1222 genome (248/5101 genes) had matches (P value of $<10^{-200}$) to the *R. sphaeroides* genome. The level of similarity was comparable to members of the *Roseobacter* clade, i.e., in terms of P values *P. denitrificans* clustered with the *Roseobacter* clade. For comparison, the genome of *B. melitensis*, the best-matching genome in the non-*Roseobacter* α–Proteobacteria group, has approximately half the number of high-quality matches, i.e., ∼3% of the genes

(89/3194) in its genome have P values of $<10^{-200}$. This last finding confirms a long-standing suspicion held by members of the photosynthetic bacteria community: *P. denitrificans* is a nonphotosynthetic *R. sphaeroides*, i.e., it may be an *R. sphaeroides* that some time ago lost its ability to photosynthesize. This notion of the closeness of *R. sphaeroides* and *P. denitrificans* to the *Roseobacter* clade was reinforced further by 16S ribosomal phylogenies (see **Figure 3**), which placed *P. denitrificans* on the same side branch of the tree as *R. sphaeroides*.

An overview of the BLASTP results from the database searches described above was compiled by plotting the mean number of genes that fell into different P-value ranges from the *Roseobacter* species alongside the numbers of genes from the other α–Proteobacteria. This data is summarized in

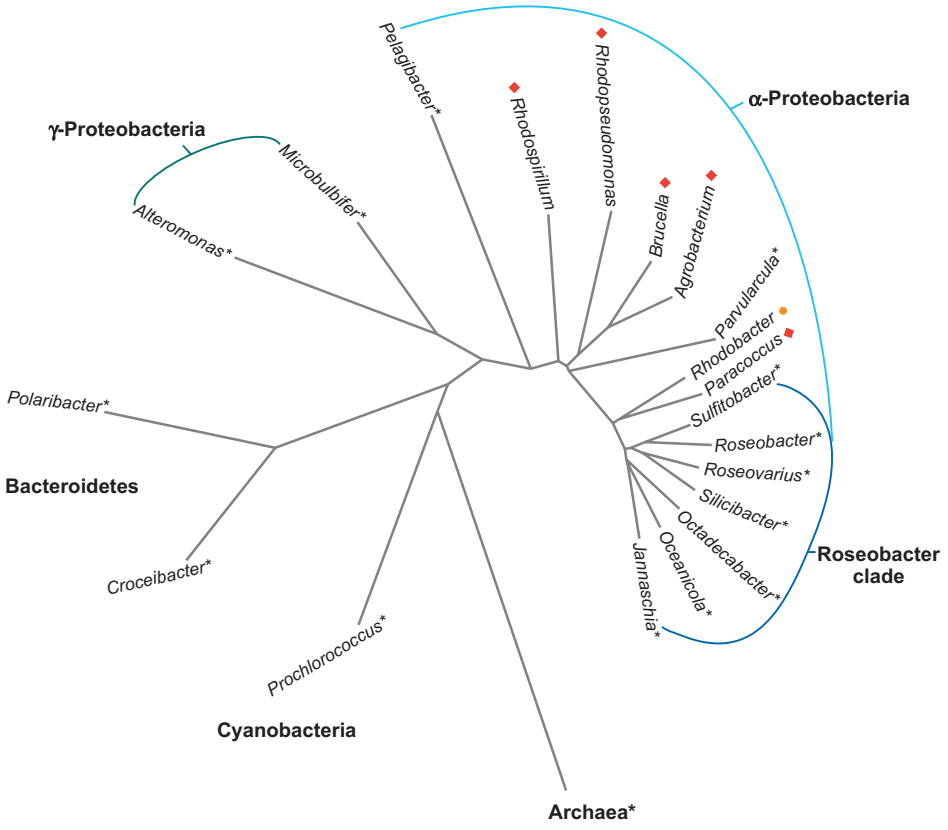


Figure 3

An unrooted phylogenetic tree of 16S rRNA gene sequences. Shown are *R. sphaeroides* (*circle*), historically close relatives (*diamonds*), the *Roseobacter* clade, and other major marine taxa. All marine organisms are marked with an asterisk (*). The neighbor-joining method was used in the construction of this tree, with a member of the marine Archaea as the outlier. The following organisms, represented by their genus names on the tree, were used for its construction: *Agrobacterium tumefaciens*, *Alteromonas macleodii*, *Brucella melitensis*, *Croceibacter atlanticus*, *Jannaschia helgolandensis*, *Microbulbifer hydrolyticus*, *Oceanicola batsensis*, *Octadecabacter antarcticus*, *Paracoccus denitrificans*, *Parvularcula bermudensis*, *Pelagibacter ubique*, *Polaribacter filamentus*, *Prochlorococcus marinus*, *Rhodobacter sphaeroides* 2.4.1, *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Roseobacter denitrificans* OCh 114, *Roseovarius nubinhibens*, *Silicibacter pomeroyi* DSS-3, and *Sulfitobacter pontiacus*. The uncultured archaeon 'KTK 32' (accession number: AJ133617) is the outlier. *P. denitrificans* and *R. sphaeroides* share a distinct branch adjacent to the marine *Roseobacter* clade.

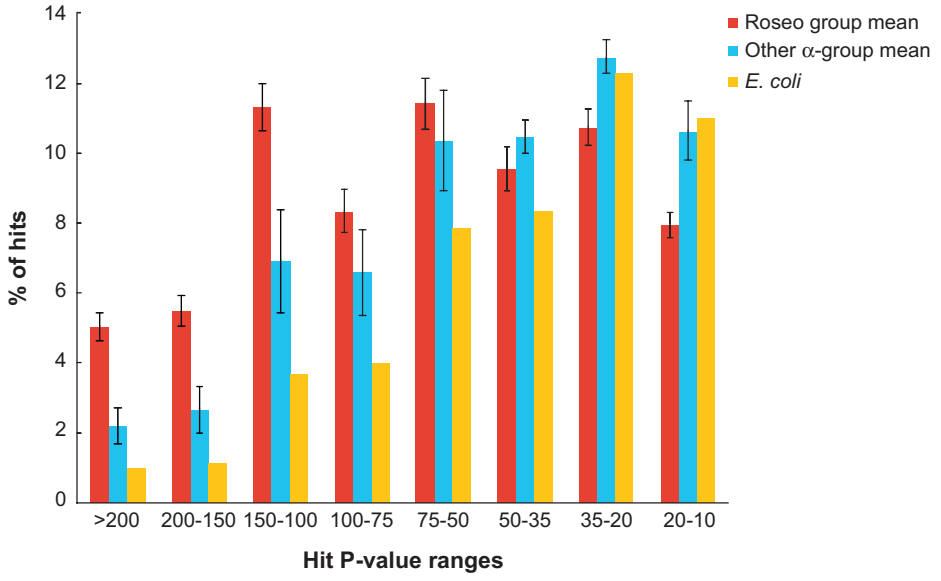


Figure 4

The CDS from the genomes *Agrobacterium tumefaciens* C58, *Bradyrhizobium japonicum* USDA 110, *Brucella melitensis* 16M, *Escherichia coli* K12 (outlier), *Jannaschia* sp. CCS1, *Oceanicola granulosus* HTCC2516, *Oceanicola batsensis* HTCC2597, *Paracoccus denitrificans* PD1222, *Rhodopseudomonas palustris* CGA009, *Roseobacter denitrificans* OCh 114, *Roseobacter* sp. MED193, *Roseovarius* sp. 217, *Silicibacter pomeroyi* DSS-3, *Silicibacter* sp. TM1040, and *Sinorhizobium meliloti* 1021 were run in a WU-BLAST 2.0 search using BLASTP mode against a *R. sphaeroides* 2.4.1 CDS database. The output of each BLASTP search was extracted to give the top hit found in the *R. sphaeroides* genome. All these top hits were then combined into a list and then sorted on the basis of their P value. The percentage of genes in each genome falling within the P-value ranges $<10^{-200}$, $10^{-200-150}$, $10^{-150-100}$, $10^{-100-75}$, 10^{-75-50}, 10^{-50-35}, 10^{-35-20}, and 10^{-20-10} was then determined (x-axis). The percentage of genes (% of hits) within the Roseobacter genomes (Roseo group mean) and genes within non-Roseobacter genomes (other α–group mean) were plotted against their P-value ranges. *E. coli* was used as a recognizable outlier.

Figure 4. The results show that the marine *Roseobacter* clade returned nearly a twofold-greater percentage of high-quality matches (ranges with smaller P values) compared with other α–Proteobacteria. In addition, CI of *R. sphaeroides* and the chromosome of *S. pomeroyi* show significant gene synteny over large regions of their chromosomes (data not shown). These regions often include genes of unknown function, suggesting an evolutionary pressure against rearrangement in these regions.

An Evolutionary Mechanism?

The finding that *R. sphaeroides* and the marine *Roseobacter* clade are close relatives led us to consider schemes that would account for how speciation may have occurred in the ancient past. We find the following scenario plausible. Imagine a time when vast areas of the current landmasses were covered by ocean. As the land rose and the sea withdrew saline lakes would have been left behind. It is likely that marine *Roseobacter*-type of organisms would have become stranded in these lakes. With long periods of rainfall, these lakes would have gradually been reduced in their salinity, giving rise to many of the freshwater bodies of water that are seen around the globe today. Given that freshwater nonsulfur photosynthetic bacteria are global in distribution, it seems likely that the ability to photosynthesize preceded the fall in sea level. Such a mechanism would

explain the global distribution of similar freshwater nonsulfur photosynthetic bacteria and the relative constancy of their photosynthetic gene clusters and the synteny of many of their other genes. Indeed such a scenario may go a long way to also explain the possession of two chromosomes in *R. sphaeroides* strains collected from different environments around the globe (74).

If we hold that our thesis is correct, then going forward it can be seen that genomic cross-comparisons between the *Roseobacter* clade, *P. denitrificans* and *R. sphaeroides* will give us insights into the different capacities that are required to live in saline versus freshwater environments and also what capacities beyond the photosynthetic apparatus (RCs, antenna complexes and membranes) are required to live a photosynthetic lifestyle.

EPILOGUE

As described above, the content of this review reflects the bias of the authors, but more importantly, the topics described have depended heavily on knowledge, availability, and use of genomic and postgenomic information to engage the study of *R. sphaeroides* 2.4.1 in an understanding of physiological diversity and microbial evolution.

SUMMARY POINTS

1. *R. sphaeroides* is a highly versatile and metabolically complex organism that can thrive under a wide range of growth conditions.
2. As the oxygen tension in the environment decreases *R. sphaeroides* develops intracytoplasmic membranes that house all the components of the photosynthetic machinery.
3. Proteomic approaches combined with genome data have allowed us to fractionate and localize subcellular components of *R. sphaeroides.*
4. In *R. sphaeroides* motility and sensing are significantly more complex than the *E. coli* paradigm.
5. The *R. sphaeroides* GeneChip has provided a global perspective of broad transcriptional variations that occur under seven different growth conditions.
6. *R. sphaeroides* differs significantly from *E. coli* in terms of its regulators, with PrrA acting as a significant global regulator.
7. *R. sphaeroides* has a complex genome composed of two chromosomes that provides an architecture with increased possibilities for gene rearrangement and exchange.
8. Surprisingly, the closest relatives of *R. sphaeroides* do not appear to be aquatic photosynthetic bacteria, but rather marine members of the *Roseobacter* clade.

FUTURE ISSUES

1. The *R. sphaeroides* community would like to define all the regulatory circuitry involving the Prr system and its interaction with other regulatory circuits.
2. The community would like to obtain a thorough understanding of the global effect of redox regulation and the mechanisms which are involved in its function, with a definition of the movement of cellular redox as a primary source of gene control.

3. Currently we lack a full definition of the proteomic content of the photosynthetic membrane and the physical and chemical interactions defining the process of PS membrane invagination.
4. A complete understanding of the relationships and evolutionary significance of the complex genome of *R. sphaeroides* and the specific role of chromosome II would allow us to grasp the evolutionary basis for this style of genome architecture.
5. As with most bacterial genomes, we still lack, but really need, a complete physiological and structural definition of the proteins encoded by the *R. sphaeroides* genome.
6. The evolutionary relationships between different strains of *R. sphaeroides* remain poorly defined and the functional and genomic role of chromosome II in these relationships remains obscure.
7. We envisage a future study of the evolution of *R. sphaeroides* as it relates to members of the *Roseobacter* clade, particularly to genes that permit each group of organisms to specialize for life within their own environmental niches.
8. Currently 25%–35% of the genome appears nonfunctional under all laboratory growth conditions, suggesting that we have not fully established all the growth conditions that *R. sphaeroides* is capable of exploiting in the wild.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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