A transcriptional response to singlet oxygen, a toxic byproduct of photosynthesis

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The ability of phototrophs to convert light into biological energy is critical for life on Earth. However, there can be deleterious consequences associated with this bioenergetic conversion, including the production of toxic byproducts. For example, singlet oxygen ($^1O_2$) can be formed during photosynthesis by energy transfer from excited triplet-state chlorophyll pigments to O_2. By monitoring gene expression and growth in the presence of $^1O_2$, we show that the phototrophic bacterium *Rhodobacter sphaeroides* mounts a transcriptional response to this reactive oxygen species (ROS) that requires the alternative σ factor, σ^E. An increase in σ^E activity is seen when cells are exposed to $^1O_2$ generated either by photochemistry within the photosynthetic apparatus or the photosensitizer, methylene blue. Wavelengths of light responsible for the generating triplet-state chlorophyll pigments in the photosynthetic apparatus are sufficient for a sustained increase in σ^E activity. Continued exposure to $^1O_2$ is required to maintain this transcriptional response, and other ROS do not cause a similar increase in σ^E-dependent gene expression. When a σ^E mutant produces low levels of carotenoids, $^1O_2$ is bacteriocidal, suggesting that this response is essential for protecting cells from this ROS. In addition, global gene expression analysis identified ≈180 genes (≈60 operons) whose RNA levels increase ≥3-fold in cells with increased σ^E activity. Gene products encoded by four newly identified σ^E-dependent operons are predicted to be involved in stress response, protecting cells from $^1O_2$ damage, or the conservation of energy.

σ factor | reactive oxygen species | *Rhodobacter sphaeroides* | photochemistry | carotenoids

Light energy captured by plants and microbial phototrophs provides O_2 and the reducing power needed to assimilate atmospheric gases (CO_2 and N_2) into compounds used by humans, animals, and other heterotrophs. Although the ability to capture light energy is of great advantage to photosynthetic (PS) organisms, there are risks associated with this bioenergetic lifestyle. For example, the reactive oxygen species (ROS) singlet oxygen ($^1O_2$) is an inadvertent byproduct of energy transfer from excited triplet-state chlorophyll pigments in the PS apparatus to ground-state triplet oxygen (1–5). $^1O_2$ is a strong oxidant that can destroy the integrity of membranes, abolish the function of many biomolecules, and reduce photochemical activity by inactivating enzymes of the PS apparatus (2, 6–12).

Carotenoids within the PS apparatus are known to quench $^1O_2$ (2–4, 12), but the PS growth of cells lacking carotenoids suggests there are other mechanisms to protect cells from $^1O_2$ damage (12–14). In the case of other ROS (superoxide, hydrogen peroxide, or hydroxyl radicals), transcriptional responses are critical in activating the expression of genes needed for survival (15, 16). We show that the facultative phototropic bacterium *Rhodobacter sphaeroides* requires the alternative σ factor, σ^E, to mount a transcriptional response to $^1O_2$. *R. sphaeroides* σ^E is a member of the extracytoplasmic function family (ECF) of alternative σ factors. The basal activity of σ^E is normally low, because it forms an inhibitory complex with a zinc-dependent anti-σ factor, ChrR (17–19).

Previous studies indicate there are transcriptional responses to conditions known or proposed to generate $^1O_2$ in plants (20, 21), algae (22), and bacteria such as *Escherichia coli* (23) and *Myxococcus xanthus* (24). However, information is lacking on the transcription factors and function of genes that protect cells from $^1O_2$. We found a 10- to 20-fold increase in activity from the *R. sphaeroides* ECF σ factor, σ^E, under conditions known to generate $^1O_2$ (1, 2). We showed this σ^E-dependent transcriptional response is required for viability in the presence of $^1O_2$ when cells contain low levels of carotenoids. In addition, we identified members of the σ^E regulon that include a heat-shock σ factor, $RpoH_{II}$; a putative cyclopropane-fatty-acyl-synthetase, CfaS; a potential photolyase, PhrB; and several proteins of unknown function.

Methods

Bacterial Strains and Plasmids. *R. sphaeroides* 2.4.1 (WT), ΔChrR (*chrR*-1::*drf*), or TF18 (*rpoEchrR*-1::*drf*) was grown at 30°C in Sistrom's succinate-based minimal medium A (25). Media used for growth of strains containing low-copy *lacZ* reporter plasmids (17, 18, 26) was supplemented with 25 μg/ml kanamycin.

Growth Conditions. For aerobic respiratory growth, 500 ml of media was bubbled with a mixture of 69% N_2/30% O_2 and 1% CO_2 in the dark. PS cultures were grown by bubbling 500-ml cultures with a mixture of 95% N_2 and 5% CO_2 in front of an incandescent light source (10 W/m^2, as measured with a Yellow–Springs–Kettering model 6.5-A radiometer through a Corning 7-69, 620- to 110-nm filter).

To test the effects of $^1O_2$, PS cultures were exposed to aerobic growth conditions (69% N_2, 30% O_2, and 1% CO_2) in the presence or absence of light (10 W/m^2). Where indicated, light was passed through a 1283 filter (Kopp Glass, Pittsburgh) that impedes >99% of light at wavelengths <770 nm but transmits >45% of light at 830 nm and >80% of light at 900 nm. When using methylene blue (Sigma–Aldrich) to generate $^1O_2$, a final concentration of 1 μM was added to aerobic cultures in the presence or absence of incandescent light (10 W/m^2). To test the effects of other ROS, 0.5 mM H_2O_2, 1 mM diamide, or 1 mM paraquat (Sigma–Aldrich) was added to aerobic cultures (27).

All experiments were initiated when cultures reached ≈2 × 10^8 colony-forming units per ml to minimize light or O_2 limitation to PS and aerobic cells, respectively. To measure cell viability, samples were removed, diluted, and plated in media (25) supplemented with 25 μg/ml kanamycin to select for the *rpoE* P1::*lacZ* reporter plasmid. The whole cell abundance of carotenoids was measured as described (28).

Determining Promoter Activity. Promoter activity was determined by measuring β-galactosidase activity from low-copy *rpoE*

Abbreviations: $^1O_2$, singlet oxygen; ROS, reactive oxygen species; PS, photosynthetic; ECF, extracytoplasmic function family.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus database (accession no. GSE2219).

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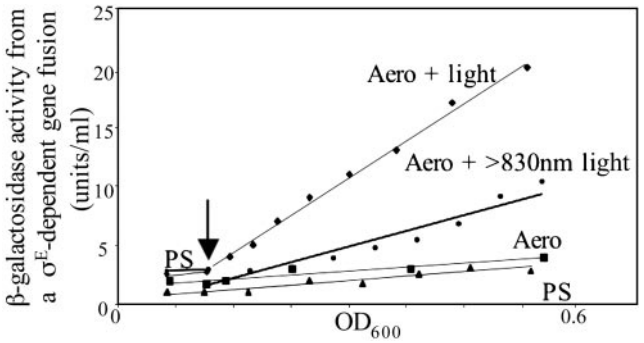

Fig. 1. Conditions that generate $^1O_2$ increase *R. sphaeroides* σ^E activity. Shown is β-galactosidase activity from a σ^E-dependent reporter gene in either steady-state cultures or after shifting cells from PS to aerobic conditions in the presence of light. The arrow indicates the time of shift. Shown are results from experiments where cells were exposed to white unfiltered light (light) or placed behind a filter to remove light ≥830 nm (>830-nm light).

P1::*lacZ* (17, 18) or *trxA*::*lacZ* reporter plasmids. The promoter for the thioredoxin gene [*trxA*, −214 to +27 relative to the known transcription initiation site (27)] was fused to *lacZ* (29) and mobilized into *R. sphaeroides*. β-Galactosidase activity (units/ml of culture) was calculated as follows: $(A_{420} \times 1{,}000)/$ [Cell volume in assay (ml) × time of assay (min)]. Culture density was typically monitored by measuring A_{600} in a BioSpec 1601 spectrophotometer (Schimatzu, Columbia, MD). The density of cultures treated with methylene blue was monitored at 500 nm, because this photosensitizer absorbs light between 609 and 668 nm. The differential rate of β-galactosidase synthesis was determined by calculating the slope from plots of enzyme activity (units/ml of culture) against optical density. All experiments were repeated a minimum of three times with differential rates of β-galactosidase synthesis typically deviating <2-fold between experiments.

Identification of σ^E Target Genes. Triplicate cultures of *R. sphaeroides* 2.4.1 and ΔChrR were grown aerobically to ≈2–3 × 10^8 colony-forming units/ml. RNA was isolated and cDNA was synthesized, labeled, and hybridized to *R. sphaeroides* GeneChip Custom Express microarrays [Affymetrix, Santa Clara, CA (30, 31)]. After data extraction using Affymetrix MAS 5.0 software, data sets were imported into GENESPRING software (Silicon Genetics, Redwood City, CA) for normalization and analysis (Gene Expression Omnibus accession no. GSE2219).

Candidate σ^E promoters (extending ≈200 bp upstream of the predicted start of translation; Table 5, which is published as supporting information on the PNAS web site) were amplified from 20 ng of 2.4.1 chromosomal DNA in EasyStart PCR tubes (Molecular BioProducts, San Diego) with 2.5 units of Pfu Turbo (Stratagene). PCR products were cloned into a plasmid (pRKK96) containing a known transcriptional terminator for *in vitro* assays (32) or into a *lacZ* reporter plasmid (pRKK200) for determining activity *in vivo* (29). *In vitro* transcription assays with reconstituted *R. sphaeroides* Eσ^E were performed with 20 nM of plasmid DNA (19).

Results

Conditions That Generate $^1O_2$ Within the PS Apparatus Increase *R. sphaeroides* σ^E Activity. Mutations that inactivate an early enzyme in carotenoid biosynthesis, CrtB, cause a small increase in σ^E activity (data not shown). Because carotenoids play a protective role against $^1O_2$ (2–4, 12), we asked whether this toxic byproduct of photosynthesis directly affected σ^E activity.

To determine whether *R. sphaeroides* σ^E activity responds to $^1O_2$, we monitored the differential rate of β-galactosidase synthesis from a σ^E-dependent *rpoE* P1::*lacZ* reporter fusion (17) after anaerobic PS cells were exposed to O_2 in the presence of light. After this shift, cell growth continues at approximately the same doubling rate, because O_2 is used as a respiratory electron acceptor (33). However, after this shift, the differential rate of β-galactosidase synthesis from the σ^E-dependent promoter increased ≈10-fold (from 6 to 65) when compared with a control culture grown under either steady-state PS (light in the absence of O_2) or respiring (30% O_2) conditions (Fig. 1 and Table 1). This transcriptional response was maintained throughout the experiment, suggesting that σ^E activity was sustained. There was a <2-fold increase in the differential rate of β-galactosidase synthesis from the *rpoE*P1::*lacZ* reporter fusion when PS cells were shifted to aerobic conditions in the dark (Table 1). This was expected, because little $^1O_2$ is made under this condition due to the lack of light needed to produce triplet-state chlorophyll molecules. From these results, we concluded that the combination of light and O_2, conditions known to generate $^1O_2$ within the PS apparatus (1, 2), are required for this transcriptional response.

Table 1. Differential rates of β-galactosidase synthesis from the σ^E-dependent *rpoE*::*lacZ* fusion under conditions that either do (+) or do not (−) generate $^1O_2$

Strain	Growth	$^1O_2$	Rate
WT	PS	−	6
WT	Aero	−	8
WT	PS → Aero + light	+	65
WT	PS → Aero (dark)	−	8
WT	PS (>830 nm)	−	2
WT	PS → Aero (>830 nm)	+	35

PS, cells grown photosynthetically; Aero, cells grown by aerobic respiration (30% O_2).

Control experiments indicated this response depended on σ^E, because the differential rate of a β-galactosidase synthesis from the *rpoE*P1::*lacZ* reporter fusion in a Δσ^E mutant (<1 unit) did not increase upon exposure to $^1O_2$. Cells lacking σ^E grow under these conditions, presumably because the carotenoids within the PS apparatus quench $^1O_2$ (see below). In addition, it appears that $^1O_2$ does not fully induce σ^E activity, because the differential rate of β-galactosidase synthesis from the *rpoE*P1::*lacZ* reporter fusion in WT cells exposed to $^1O_2$ was 10-fold less than that seen in a strain lacking the anti-σ factor, ChrR (65 vs. 650).

Wavelengths of Light That Excite Chlorophyll Pigments Are Sufficient to Increase σ^E Activity. If production of $^1O_2$ by the PS apparatus was responsible for this transcriptional response, then wavelengths of light known to generate triplet-state chlorophyll molecules within the light-harvesting complexes should increase σ^E activity. *R. sphaeroides* contains two light-harvesting complexes, B800-850 and B875, named for their absorption maxima in the near infrared (34–36). To determine whether light absorbed by the light-harvesting complexes could cause this response, we looked at the action spectrum of this transcriptional response. Under PS conditions with light that was filtered to remove wavelengths <830 nm, the differential rate of β-galactosidase synthesis from the σ^E-dependent promoter was ≈4-fold lower than that observed with cells grown in white light (Table 1), presumably because the cells grow slower when light <830 nm is removed. However, there was an ≈17-fold increase in the differential rate of β-galactosidase synthesis when cultures illuminated with >830-nm light were exposed to O_2 (Table 1). The magnitude of this response was similar to that observed when PS cells were exposed to O_2 and white light (≈17- vs. ≈10-fold,

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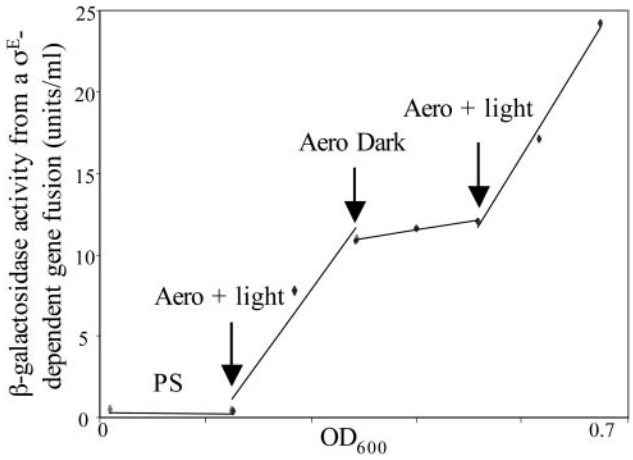

Fig. 2. Continued exposure to $^1O_2$ is required for increased σ^E activity. Shown is β-galactosidase activity from the σ^E-dependent reporter gene when PS (PS) cells are shifted to aerobic conditions (Aero) in the presence or absence of light. Arrows indicate the time of each shift.

Table 1). Thus, wavelengths of light that excite the light-harvesting complexes are sufficient to increase σ^E activity.

Continued Exposure to Conditions That Generate $^1O_2$ in the PS Apparatus Are Needed to Sustain This Response. The half-life of $^1O_2$ in cells is <100 ns (37). We took advantage of the relatively short half-life of this ROS to further test whether σ^E activity was responding to $^1O_2$. For example, if increased σ^E activity required $^1O_2$, then placing PS cultures that had previously been exposed to O_2 in the dark might terminate this transcriptional response. When PS cells were shifted to aerobic conditions in the presence of light, we saw the expected increase in the differential rate of β-galactosidase synthesis from the σ^E-dependent promoter (≈10-fold; Fig. 2 and Table 2). However, when this culture was placed in the dark (conditions that allow growth via respiration but prevent $^1O_2$ formation), the differential rate of β-galactosidase synthesis decreased ≈9-fold (Fig. 2 and Table 2). In addition, placing the same culture back into the light to restore $^1O_2$ formation caused an ≈8-fold increase in the differential rate of β-galactosidase synthesis from the σ^E-dependent promoter (Fig. 2 and Table 2), suggesting this transcriptional response to $^1O_2$ is reversible, and that increased σ^E activity requires continued exposure to $^1O_2$.

***R. sphaeroides* σ^E Activity Is Increased by Formation of $^1O_2$ in the Absence of the PS Apparatus.** If $^1O_2$ was responsible for the observed σ^E transcriptional response, then other conditions that generate this ROS should also increase σ^E activity. To test this hypothesis, we asked whether generating $^1O_2$ by illumination of methylene blue in the presence of O_2 produced a similar response (1). When aerobically grown WT cells were exposed to 1 μM methylene blue in the presence of light and O_2, cell growth continued (see below), and the differential rate of β-galactosidase synthesis from the *rpoE* P1::*lacZ* reporter fusion increased ≈20-fold compared with aerobic cells grown in the absence of methylene blue (Table 3). Control experiments indicated there was a <2-fold increase in the rate of β-galactosidase synthesis when aerobic cultures were exposed to methylene blue in the dark (Table 3). The lack of a comparable increase in σ^E activity in aerobic cells exposed to methylene blue in the dark is expected, because both light and O_2 are required for this compound to generate $^1O_2$ (38). For these experiments, cells were grown in the presence of 30% O_2, a condition where pigment–protein complexes of the PS apparatus are not detectable (34). Therefore, we conclude that this transcriptional response to $^1O_2$ can occur in cells that either contain or lack the PS apparatus.

Other ROS Do Not Produce a Similar Increase in σ^E Activity. We recognize that the damaging effects of $^1O_2$ on many biomolecules (1, 6, 38) could stimulate the formation of other ROS. To test whether other ROS could produce an increase in σ^E activity, we monitored the differential rate of β-galactosidase synthesis from a *rpoE* P1::*lacZ* reporter fusion in aerobic cells treated with concentrations of H_2O_2, paraquat (to stimulate superoxide formation), or diamide (to alter the oxidation-reduction state of the cytoplasmic thiol pool) previously shown to generate an oxidative stress response in *R. sphaeroides* (27). For these experiments, we also monitored the differential rate of β-galactosidase synthesis from a control *trxA*::*lacZ* reporter fusion, because the *trx* promoter has previously been shown to respond to oxidative stress in *R. sphaeroides* (27).

We found that addition of paraquat or H_2O_2 to aerobic cells produced increases in the differential rate of β-galactosidase synthesis from the *trxA*::*lacZ* reporter gene that are consistent with changes in abundance of *trxA* transcripts produced by these compounds in previous studies (Table 4) (27). However, the differential rate of β-galactosidase synthesis from the σ^E-dependent reporter fusion either decreased (paraquat) or increased no more than 1.2-fold (H_2O_2) when compared with untreated cells (Table 4). Any observed increase in σ^E activity in the presence of these ROS was below the 10-fold increase in σ^E activity seen when cells are exposed to $^1O_2$. We did not monitor σ^E activity in the presence of diamide, because previous work has shown that σ^E activity does not increase upon exposure to this compound (39). Based on these results, we concluded that the transcriptional response observed when $^1O_2$ is generated does not occur in the presence of other ROS.

σ^E Is Required to Respond to $^1O_2$ When Carotenoids Are Low. Although cells lacking σ^E are unable to mount this transcriptional response to $^1O_2$ (Fig. 1, Table 1), exponential growth of a $\Delta\sigma^E$ strain continues when a PS culture is shifted to aerobic conditions in the presence of light (data not shown). This occurs presumably because carotenoids within the PS apparatus quench $^1O_2$ (2–4, 12). To assess the relative importance of carotenoids and σ^E in the presence of $^1O_2$, we monitored the growth of cells

Table 2. Continued exposure to $^1O_2$ is required for increased σ^E activity

Growth	$^1O_2$	Rate
PS	−	7
Aero + light	+	73
Aero dark	−	8
Aero + light	+	63

PS, cells grown photosynthetically; Aero, cells grown by aerobic respiration (30% O_2).

Table 3. Light plus methylene blue increases σ^E activity

Strain	Growth	$^1O_2$	Rate
WT	Aero	–	5
WT	Aero + light	–	8
WT	Aero + methylene blue + light	+	151
WT	Aero + methylene blue (dark)	–	8

Differential rates of β-galactosidase synthesis from the σ^E-dependent *rpoE*::*lacZ* fusion when WT cells are grown aerobically under conditions that either do or do not generate $^1O_2$.

Table 4. Other ROS do not increase σ^E activity

Addition	ROS	*rpo*EP1::*lacZ* fusion	*trxA*::*lacZ* fusion
None	–	11	185
Paraquat	Superoxide	6	450
H_2O_2	Peroxide	13	220
Diamide	Oxidizes cysteine thiols	3	ND

Differential rates of β-galactosidase synthesis from the indicated promoters when WT cells are grown aerobically under conditions that either do or do not generate indicated ROS. ND, not determined.

that contain low levels of carotenoids in the presence and absence of σ^E. For this analysis, we grew cells by aerobic respiration (30% O_2), because they have 20-fold less total carotenoids than PS cells grown at 10 W/m^2 ($\approx$10 μg of carotenoid/2 $\times$ 10^{10} cells compared with $\approx$200 μg of carotenoid/2 $\times$ 10^{10} cells, respectively). The use of aerobically grown cells is preferable to studying a carotenoid-minus $\Delta\sigma^E$ mutant, because the lack of carotenoids lowers PS growth rates (12–14).

Exponential growth of aerobically grown WT cells continued after exposure to $^1O_2$ (Fig. 3*A*). In contrast, the number of colony-forming units per ml of the $\Delta\sigma^E$ mutant culture decreased $\approx$10-fold after 8 h of exposure to $^1O_2$ (Fig. 3*B*). The bacteriocidal effect of $^1O_2$ on the σ^E mutant when carotenoid levels are low shows that both σ factor activity and carotenoids are critical to viability in the presence of this ROS.

Additional Members of the σ^E Regulon. To identify genes that are part of this transcriptional response to $^1O_2$, we compared RNA levels from aerobically grown (30% O_2) WT cells with a ΔChrR mutant. ChrR inhibits σ^E activity (17–19), so we looked for RNA that was more abundant in the ΔChrR mutant. As expected, global gene expression analysis showed an increase ($\approx$12-fold) in

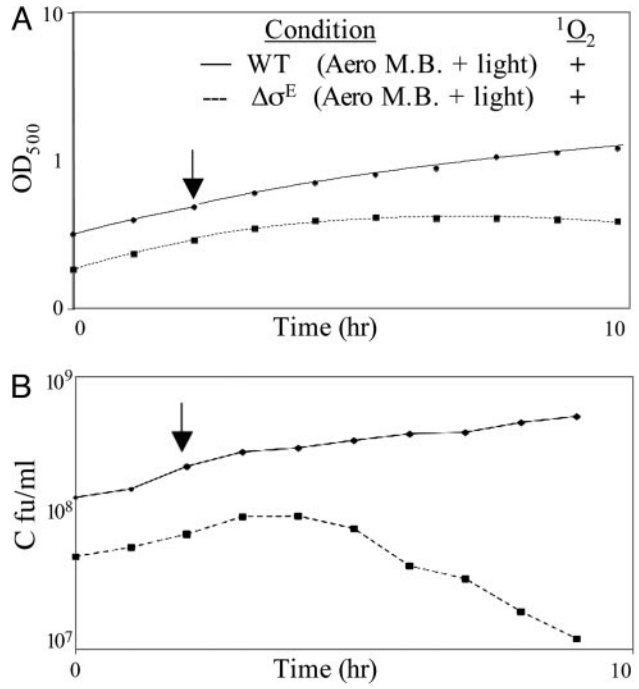

Fig. 3. $^1O_2$ is bacteriocidal to a $\Delta\sigma^E$ mutant when carotenoids are low. (*A*) Optical density measurements ($OD_{500\ nm}$) and (*B*) viable plate counts (colony-forming units/ml) when aerobically grown WT cells or cells lacking σ^E ($\Delta\sigma^E$) were treated with methylene blue in the presence of light. The arrow indicates the time when methylene blue and light were added.

rpoE-specific RNA from cells lacking ChrR. It also showed that RNA from $\approx$180 genes ($\approx$60 operons) was $\geq$3-fold more abundant in cells that contained increased σ^E activity (Table 5). In contrast, the $\approx$35-fold increase in *cycA* P3 activity that occurs in ΔChrR cells *in vivo* (17) causes only an $\approx$1.6-fold increase in total *cycA*-specific RNA (Table 5). The smaller increase in *cycA*-specific RNA levels reflects the fact that *cycA* contains additional strong promoters that are recognized by other σ factors (40, 41). This suggests that a global gene expression microarray approach might miss other σ^E-dependent genes that also contain multiple promoters.

To test whether any of these candidate operons contained a σ^E-dependent promoter, DNA upstream of the first gene in each of 28 potential operons was tested for transcription by reconstituted Eσ^E (Table 6, which is published as supporting information on the PNAS web site) (17, 42). These operons were chosen either based on their increased levels of expression in cells with elevated σ^E activity or because of a potential role of their gene products in the PS apparatus (a source of $^1O_2$). We found that $rpoH_{II}$, which encodes one of two *R. sphaeroides* heat-shock σ factors (Rsp0601), is transcribed by Eσ^E. Production of the $rpoH_{II}$ transcript is inhibited by addition of ChrR, as is the case with other σ^E-dependent promoters like *rpoE* P1 and *cycA* P3 (Fig. 4*A*). By these criteria, σ^E-dependent promoters are also located upstream of Rsp1087 (which may contain two promoters, because different-sized Eσ^E transcripts are seen), Rsp1409, and Rsp2143 (Fig. 4*A*). Each gene is predicted to be part of a polycistronic operon that encodes uncharacterized proteins (see *Discussion*). The level of transcripts produced from the $rpoH_{II}$, Rsp1087, and Rsp2143 promoters is comparable to that of *rpoE* P1 (within 1.1-fold), suggesting these four promoters are of similar strength. In contrast, the abundance of the σ^E-dependent transcript produced by Rsp1409 *in vitro* is comparable to the σ^E-dependent promoter, *cycA* P3, which has $\approx$80-fold less activity than *rpoE* P1 (17).

The same putative $rpoH_{II}$ and Rsp1087 promoter regions were fused to *lacZ* to test for σ^E-dependent activity *in vivo*. Expression was not detectable from these reporter fusions in WT *R. sphaeroides* cells, but it was comparable to that of *rpoE* P1 in cells lacking the anti-σ factor, ChrR (Fig. 4*B*). In addition, activity from the $rpoH_{II}$ and Rsp1087 promoters was not detectable in a σ^E mutant strain (Fig. 4*B*). This suggests that transcription from this promoter region depends solely on σ^E, as is the case for *rpoE* P1 (17).

The other 24 potential promoters tested (Table 6) produced no detectable σ^E-dependent transcripts *in vitro*. These results suggest that either no σ^E-dependent promoter is located within this region, or that another factor is required to produce a transcript at levels comparable to weak σ^E-dependent promoters like *cycA* P3 or the one upstream of Rsp1409. Reasons why the abundance of RNA from many potential σ^E-dependent genes was increased in cells lacking the anti-σ factor, ChrR, are presented in *Discussion*.

Discussion

The ability of plant and microbial phototrophs to convert light into biological energy is fundamental to life on Earth. However, the photochemical reactions that allow these organisms to conserve the energy in sunlight are also the source of $^1O_2$. $^1O_2$ is a strong oxidant that can cleave peptide and phosphodiester bonds, damage amino acids and nucleosides, oxidize unsaturated fatty acids, and damage other cellular components (2, 6–12). Although changes in expression of individual genes in response to $^1O_2$ have been reported in plants (20, 21), algae (22), and bacteria, including *E. coli* (23) and *M. xanthus* (24), details on the targets or features of these transcriptional responses are lacking. In this work, we show that the PS bacterium *R. sphaeroides* mounts a transcriptional response to $^1O_2$; that the alternative

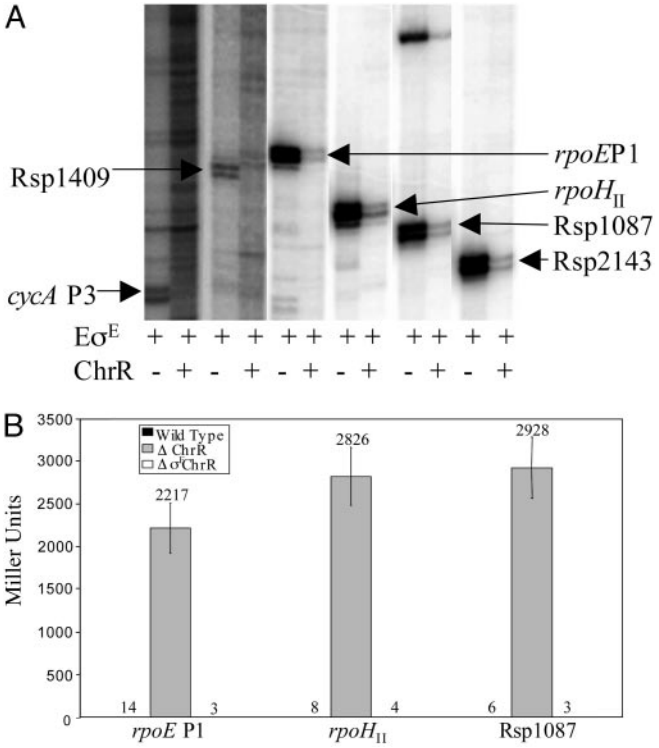

Fig. 4. Identification of additional σ^E-dependent promoters. (*A*) Products of *in vitro* transcription reactions using reconstituted *R. sphaeroides* Eσ^E (17) and the indicated potential promoter. As an additional control to demonstrate the σ^E dependence of these transcripts, ChrR was added to indicated reactions (17–19). Note that the first four lanes were exposed to a phosphoscreen twice as long as the remainder of the gel to detect low-abundance transcripts from the *cycA* P3 and Rsp1409 promoters. Experiments were repeated at least three times, with a representative gel shown. The σ^E-dependent transcripts appear as two products due to termination at different bases within the SpoT 40 transcriptional terminator on the template used (32). (*B*) Activity of selected σ^E-dependent promoters in *R. sphaeroides*. Shown are β-galactosidase levels [Miller units (58)] from the indicated promoter fused to *lacZ* in WT cells (■), ΔChrR cells (increased σ^E activity) (▨), or cells lacking both σ^E and ChrR (□). All assays were performed in triplicate, with bars denoting the standard deviation from the mean.

ECF σ factor, σ^E, is required for this response; and that σ^E is essential for viability in the presence of $^1O_2$ when carotenoid levels are low.

One of the most common sources of $^1O_2$ in biological systems is photochemistry within the PS apparatus (20). However, the formation of $^1O_2$ by photosensitizers in organelles has been reported to alter nuclear gene expression (43), suggesting that cells have a signaling pathway to sense and respond to this ROS. We have shown that wavelengths of light sufficient to excite bacteriochlorophyll molecules within the light-harvesting complexes in the presence of O_2, or the combination of light and a photosensitizer (methylene blue), can cause a sustained increase in σ^E activity. These results implicate *R. sphaeroides* σ^E as a member of a signal transduction pathway that responds to $^1O_2$.

We have shown that σ^E activity increases under two conditions known to generate $^1O_2$. However, under each of these conditions, the amount of σ^E activity is a fraction of that seen in cells lacking the anti-σ factor, ChrR. One possible explanation for this difference in σ^E activity is that the amount of $^1O_2$ generated is insufficient to cause dissociation of all σ^E–ChrR complexes. Alternatively, the destruction of free σ^E by $^1O_2$ could explain the difference in target gene expression between cells lacking ChrR and those exposed to this ROS.

During the preparation of this paper, it was reported that illumination of low-oxygen *R. sphaeroides* cells with blue light produces a transient increase in the abundance of many RNA species (42). Some of these genes were predicted to contain either heat shock or σ^E-dependent promoters by a bioinformatic analysis of intergenic regions (42). The authors show that exposure of low-oxygen cells to blue light can cause significant sustained decreases in RNA levels from many genes. However, the sustained decreases in RNA levels contrast with the transient increases seen in the expression of genes predicted to contain heat shock and σ^E-dependent promoters (42). Thus, illumination of low-oxygen cells with blue light stimulates a short-lived response that differs from the sustained increase we find in the presence of $^1O_2$.

Because anaerobic phototrophs like *R. sphaeroides* do not produce O_2 as a byproduct of photochemical activity and use carotenoids to quench $^1O_2$ (2, 4), it may seem unnecessary for them to mount a transcriptional response to $^1O_2$. However, *R. sphaeroides* is often found in low O_2 environments (44), conditions where significant $^1O_2$ could be generated by photochemical activity. Depending on light availability, dissolved O_2 tension, and carotenoid content, the transcriptional response we discovered could play an important role in mitigating damage from $^1O_2$ in nature (14, 45, 46). The finding that σ^E is essential when cells contain low levels of carotenoids predicts that one or more of its target genes is necessary for viability.

From this work and previous studies (17, 42), it appears that members of the σ^E regulon function to protect against and repair $^1O_2$ damage in the cell (6, 8–11, 47–49). Rsp0296, cytochrome c_2, is an essential part of the *R. sphaeroides* PS electron transport chain (17, 50). The P3 promoter for the cytochrome c_2 gene (*cycA*) was previously shown to be σ^E-dependent (17). During photosynthesis, a fraction of the cytochrome c_2 is likely to be in the immediate vicinity of $^1O_2$, because this protein reduces the reaction center complexes that are oxidized after energy transfer from triplet-state chlorophyll molecules (17, 50). $^1O_2$ inactivates the mitochondrial homolog of cytochrome c_2 (51), so increased synthesis of cytochrome c_2 may help maintain function of the PS apparatus when $^1O_2$ is generated.

From our studies, four additional operons have been identified as members of the σ^E regulon. The predicted Rsp1087–1091 operon contains a σ^E-dependent promoter and is located directly upstream of the *rpoEchrR* operon (Rsp1092–1093) in *R. sphaeroides* and other bacteria that contain homologs of σ^E. Although Rsp1087–1091 have no known functions, Rsp1091 shows homology to the flavin-containing amine oxidoreductase family, and Rsp1087 is predicted to be a member of the short chain dehydrogenase/reductase family. Hence, it is possible that a product of the Rsp1087–1091 operon helps cells generate energy when $^1O_2$ is produced. Rsp1409, another member of the σ^E regulon, shows 54% identity to the *tspO*-like regulator from *Sinorhizobium meliloti* (52). In *S. meliloti*, this *tspO*-like protein regulates the *ndi* (nutrient deprivation-induced) locus that is activated in an unknown manner by O_2, N_2, or C deprivation; by osmotic stress; or by entry into stationary phase (52). Therefore, Rsp1409 could aid in the response to damage generated by the formation of a ROS-like $^1O_2$. Another σ^E-dependent operon identified in this study, Rsp2143–2146, encodes a DNA photolyase (Rsp2143-PhrB) that repairs pyrimidine dimers (53), and a cyclopropane-fatty-acyl-phospholipid synthetase (Rsp2144-CfaS) that uses *S*-adenosylmethionine to generate a methylene bridge across the double bonds in unsaturated fatty acids (54). $^1O_2$ is known to modify unsaturated fatty acids, causing a loss of bilayer integrity and an increase in membrane permeability (37, 54). Thus, some combination of Rsp2143 and Rsp2144 could protect membranes and other cellular components from damage by $^1O_2$ (53, 54). We also found that the Rsp0601 gene (*rpoH*$_{II}$) contains a σ^E-dependent promoter. RpoH$_{II}$ is one of two *R.*

sphaeroides heat-shock σ factors, so this response could help cells repair and replace components of the PS apparatus that were damaged by $^1O_2$. Activation of $RpoH_{II}$ probably explains why many transcripts present at increased levels in ΔChrR cells do not appear to contain Eσ^E-dependent promoters *in vitro*. It is possible that there are still unidentified members of the σ^E regulon, especially if these genes contain additional σ^E-independent promoters, like *cycA*, that could mask increases seen from σ^E-dependent promoters.

Among the operons tested that did not contain a detectable σ^E-dependent promoter were several that encode enzymes for carotenoid biosynthesis (Table 6). Thus, *R. sphaeroides* σ^E does not appear to directly control the synthesis of carotenoids that can quench $^1O_2$. This is unlike *M. xanthus*, which uses an ECF σ factor and an anti-σ factor that lacks significant amino acid sequence similarity to ChrR to increase carotenoid synthesis under conditions that are proposed to generate $^1O_2$ (24).

Conclusion

Our data indicate that $^1O_2$, a ROS that can be generated within the PS apparatus, increases the activity of *R. sphaeroides* σ^E. σ^E is a member of the ECF family of alternative σ factors, which control gene expression in response to stress or signals generated beyond the cytoplasm (55, 56). Given the ability of all phototrophs to generate $^1O_2$, it is not surprising to find homologs of *R. sphaeroides* σ^E and its anti-σ factor, ChrR, in the genomes of many PS bacteria (18). It appears likely that non-PS bacteria also mount a transcriptional response to $^1O_2$, because σ^E and ChrR homologs are predicted to exist in proteobacteria that interact with humans, animals, or plants (*Vibrio*, *Pseudomonas*, and *Salmonella*) (18). Animal and plant cells contain peroxidases and other enzymes that are proposed to produce $^1O_2$ to ward off microbial pathogens (57). Thus, further analysis of this transcriptional response to $^1O_2$ is likely to provide insight into a signal transduction pathway found in bacteria with important agricultural, medical, and environmental activities.

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