BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 305–309 (1997)
ARTICLE NO. RC977469

RpoS Dependent Overexpression of Carotenoids from *Erwinia herbicola* in *OXYR* Deficient *Escherichia coli*

Michelle Becker-Hapak,*[,1] Erik Troxtel,† James Hoerter,† and Abraham Eisenstark*[,‡,2]

**Cancer Research Center, 3501 Berrywood Drive, Columbia, Missouri 65201; †Ferris State University, Department of Biology, Big Rapids, Michigan 49307; and ‡University of Missouri, Division of Biological Sciences, and Department of Molecular Microbiology, Columbia, Missouri 65211*

Received August 25, 1997

Carotenoid synthesis in *Escherichia coli,* when transformed with plasmid containing a carotenoid gene cluster from *Erwinia herbicola* (pPL376), is regulated by RpoS. When the plasmid was transformed into *E. coli* mutants that were *oxyR* minus, the intracellular carotenoid concentration dramatically increased from that observed in an *oxyR* plus allele. The higher carotenoid concentration in these mutants correlated with an increase in *rpoS* transcription as indicated by *β*-galactosidase activity from a *rpoS::lacZ* promoter fusion. This indication of a higher concentration of carotenoids correlated with an increased resistance to hydrogen peroxide and near-ultraviolet radiation (310–400 nm; near-UV).

Bacteria have evolved complex mechanism by which they respond to a myriad of stresses that they may encounter. In *Escherichia coli,* oxidative stress caused by metabolic processes, near-UV, exogenous toxins or mutagens is dealt with both at transcriptional and post-transcriptional levels. Many studies have shown the importance of OxyR as a sensor of oxidative stress and its role as a transcriptional activator of stress response genes such as *katG, ahp, gor* and *dps* (1). The protein is active under oxidizing conditions as a tetramer that binds to four ATAGxt motifs in the major groove of the DNA helix in log phase upon OxyR sensing of oxidative stress (2). Specific genes of rapid transcription proceed via sigma-70 form of RNA polymerase holoenzyme through conformational changes in the promoter (3).

RpoS is known as a stress response protein in various enteric organisms, primarily in stationary phase. Because of the wide diversity of phenotypes noted in sigma-38 minus cells, it has been given many names (*nur, katF* and *rpoS*). When the gene was eventually identified, cloned and expressed, the *rpoS* protein product was given the name sigma-38 for its size as a sigma factor. RpoS is responsible for effecting a multitude of cellular proteins (4). Its expression and half life are highly regulated at the transcriptional level and by protein stabilization factors (reviewed in ref. 4, 5). In addition, Sandmann et al (6) showed that *E. coli* RpoS can regulate foreign genes as well. They noted that when the carotenoid genes in plasmid pPL376, derived from *Erwinia herbicola* (a closely related plant pathogen) was transformed into *E. coli* devoid of RpoS, the expression of the carotenoids decreased. The identity of the carotenoid genes on the pPL376 have been established (7) and their affect on cells exposed to UV radiation has also been discussed (6, 8, 9).

We have used the pPL376 plasmid to identify a key change in the expression of wildtype *E. coli* in which *oxyR* is absent. The lack of *oxyR* resulted changes in *rpoS* transcription and, presumably, protein levels as indicated by higher concentrations of carotenoids and greater resistance to near-UV.

MATERIALS AND METHODS

Strains and plasmids are noted in Table 1. Cells were grown in LB broth supplemented with the appropriate antibiotics at 30°C for *Erwinia* cultures and 37°C for *E. coli* cultures. Media were purchased from Difco Laboratories (Detroit, MI), and all reagents from Sigma Chemicals (St. Louis, MO).

The *β*-galactosidase assays were performed as described by Ivanova et al (10).

Carotenoid content was determined by growing 100 mL of cells containing plasmid for 24 hours in LB broth with 20 μg/ml ampicillin at 30°C with constant agitation. The growth flasks were placed in an ice water bath for 15 min. and then the cells were collected by centrifugation at 4,000 X g for 20 min. Pellet fraction was weighed, suspended in 2:1 (v/v) chloroform:methanol (8) and vortexed for two min. The suspension was separated by centrifugation at 16,000 × g

[1] Present address: Howard Hughes Medical Research Institute, Washington University, Department of Pathology/Molecular Oncology, 660 S. Euclid, Box 8022, St. Louis, MO 63110.

[2] To whom correspondence should be addressed. (Fax: 573-443-1202; E-mail: abe@biosci.mbp.missouri.edu).

0006-291X/97 $25.00

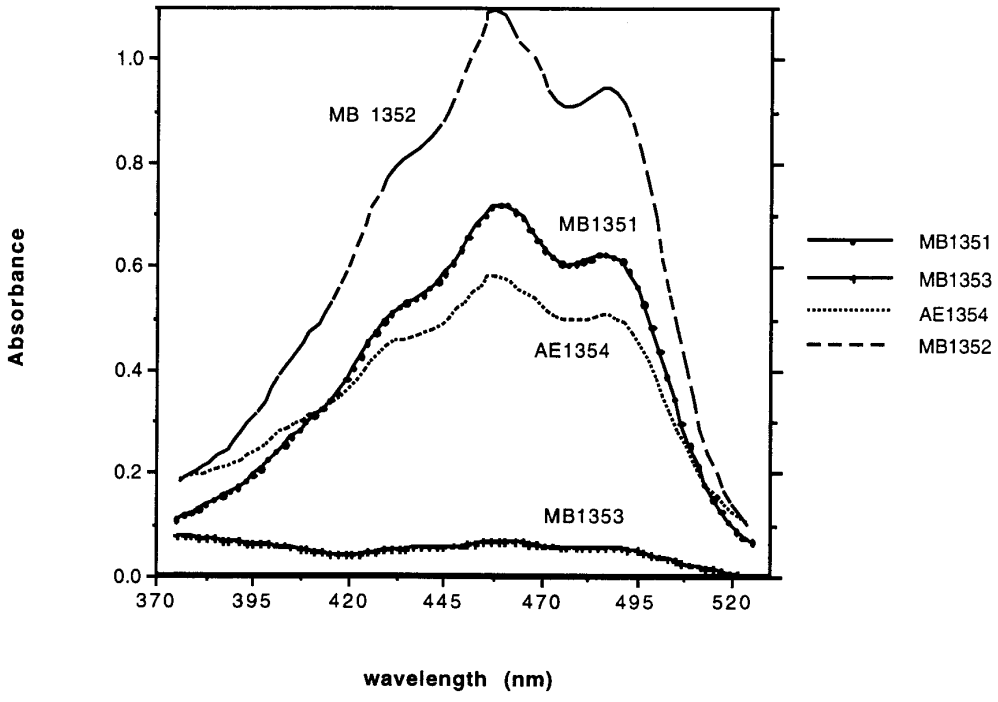

FIG. 1. The carotenoid spectra of wild type *E. coli* with pPL376 diluted 5 fold (MB1351), and the *katF*::tn10 mutant, undiluted, with pPL376 (MB1353). The *oxyR* mutant (MB1352) and parent (MB1354) with pPL376 were diluted three fold. The spectra were completed in 2:1 (v/v) chloroform:methanol. The absorbance maxima for the crude carotenoids was 460 nm.

for 10 min. The organic layer containing the carotenoids was diluted in the chloroform:methanol solvent and compared to a β-carotene standard. The wavelength maximum for β-carotene in the chloroform:methanol solvent was 461 nm and the extinction coefficient was 0.0616 $(mg/ml)^{-1}$ $(cm)^{-1}$.

The hydrogen peroxide disk sensitivity assay was performed as described by Greenberg and Demple (11).

Near-UV radiation studies were completed as described previously (12).

RESULTS AND DISCUSSION

Elevated carotenoid levels in oxyR mutant E. coli. During the course of our investigation of oxidative stress mechanisms, we noted that when *E. coli* cells that were *oxyR* deficient and transformed with the carotenoid genes on plasmid pPL376, colonies were a much deeper orange than the allelic *oxyR*$^+$ parent strain. Visible spectrographic analysis showed that the absorbance profile of the carotenoids in the *oxyR* mutant did not change. However, quantitative changes in expression levels were obvious (Fig. 1). These changes were assayed by comparison to a β-carotene standard which produced similar spectra to those expressed from the pPL376 plasmid (Table 1). When an *rpoS*::Tn10 was transduced into this strain, 98% of the carotenoid

TABLE 1

Bacteria and Plasmids Used in This Study

Strain or plasmid	Description	Source
AE 908	MC4100 (F$^-$ *araD*139 Δ(*lacIOPZYA*)U169 *rpsL thi A*	Our stock
AE1393	RK4936 (*araD*139, (*arg-lac*)205, *flb*5301, non-9 *gyrA*219, *relA*, *rpsL*150, *metE*70, *btuB*::tn10	
AE1195	*Erwinia herbicola* (EH106W)	A. Chatterjee
CM1276	AE908 *katF*::tn10	Our stock
MB1353	AE908 *katF*::tn10 with pPL376	This study
MB1351	AE908 with pPL376	This study
MB1354	AE1393 with pPL376	This study
AE1262	TA4112(*oxyRΔ3*)	G. Storz
MB1352	AE1262 with pPL376	This study
MB1390	AE1393 with pR*SkatF*5	This study
MB1391	AE1262 with pR*SkatF*5	This study
pPL376	carotenoid cluster from *E. herbicola* in pHC79	R. Tuveson
pRSkatF5	*rpoS::lacZ* promotor	P. Loewen

TABLE 2

Carotenoid Content of *E. coli* Strains with and without the Carotenoid Plasmid

Strain	Relative Genotype	Carotenoid content (μg per gram) wet cell paste
AE908	MC4100 (wild type)	not detected
MB1351	AE908 + pPL376	230 ± 4 229.20 ± 3.90
MB1353	AE908 *katF*::Tn10 + pPL376	4.15 ± 0.06
MB1354	RK4936 (WT) + pPL376	160 ± 20 160.10 ± 16.10
MB1352	RK4936 Δ *oxyR* + pPL376	420 40 415.50 ± 44.10

Note. The carotenoid contents are the averages of six independent determinations.

content was lost. The enhancement of color noted in the *oxyR* mutant strain corresponded to a 2.6 fold increase in total carotenoid content over its allelic parent. There was also a two fold change in the amount of carotenoids being expressed between the two parent strains of *E. coli*. At this point in our study, it was unclear why this occurred (Table 2).

The low level of carotenoid expression that remained in *rpoS* mutants could be due to weak recognition of the promoter by sigma-70 or selective involvement of sigma-38 in a portion of carotenoid biosynthetic pathway leading to the accumulation of one of the major carotenoids synthesized in *Erwinia*, or perhaps the stability of any of these products. The only known regulatory requirement for the synthesis of the carotenoids from the pPL376 plasmid is cAMP (7). The implications of this requirement are not clear but it is known that cAMP is a negative regulator of sigma-38 synthesis (4, 5, 13). These data pointed to the possibility that other carotenoid expressing organisms could have a sigma-38 homologue.

The gram positive organism *Staphylococcus aureus* which also produces carotenoids (14) was examined in a similar fashion. No decrease in *S. aureus* carotenoid expression occurred in when carotenoid genes were transformed into various *rpoS E. coli* mutants (data not shown). To date, no *rpoS* homologues have been reported in other carotenoid expressing organisms.

rpoS promoter activity increases in oxyR mutant cells. Because of the known *rpoS* dependent expression of the carotenoid genes in *E. coli* pPL376, we examined whether the increase could be due to an increase in *rpoS* transcription. To determine this, a *rpoS::lacZ* operon fusion was transformed into the *oxyR* mutant (AE1262) and its parent (AE1393). Fig. 2 shows a consistently lower promoter activity in early (4 hr), mid (6 hr) and early stationary (8 hr) log phases, but a 1.5 fold increase in promoter activity in late stationary phase, of the *oxyR* mutant. Subsequent to this observation of increased carotenoid synthesis, in similar experiments in our laboratory, Ivanova et al (10) also noted increased *rpoS* transcription in stationary phase cells that lacked OxyR. The reason for low *rpoS* transcriptional activity in log phase is not clear. However, the result obtained in stationary phase cells implied that the increased transcription of *rpoS* probably led to higher concentrations of RpoS protein in the cell, and was the reason for the higher concentration of carotenoids in this cell type. Although the increase was not as profound as would be expected by the carotenoid quantitation data, it is known that RpoS quantities in E. coli are regulated by two post-transcriptional mechanisms involving *clpX* (15) and *rssB* (16). The approxi-

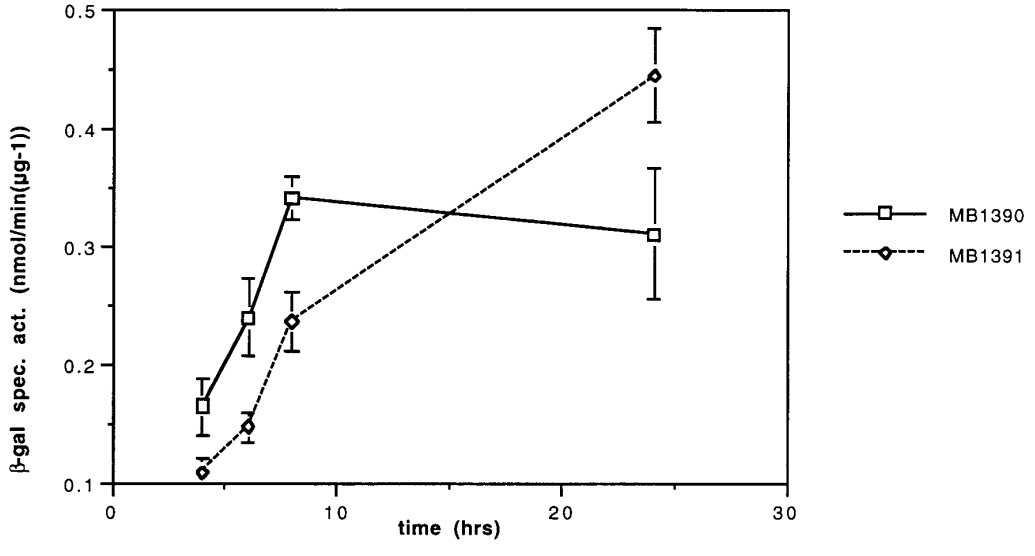

FIG. 2. β-galactosidase activity assay in *E. coli* with pRS*katF*5 (MB1390) and *E. coli* Δ*oxyR* with pRS*katF*5 (MB1391). The results shown are the average of three independent determinations completed in duplicate.

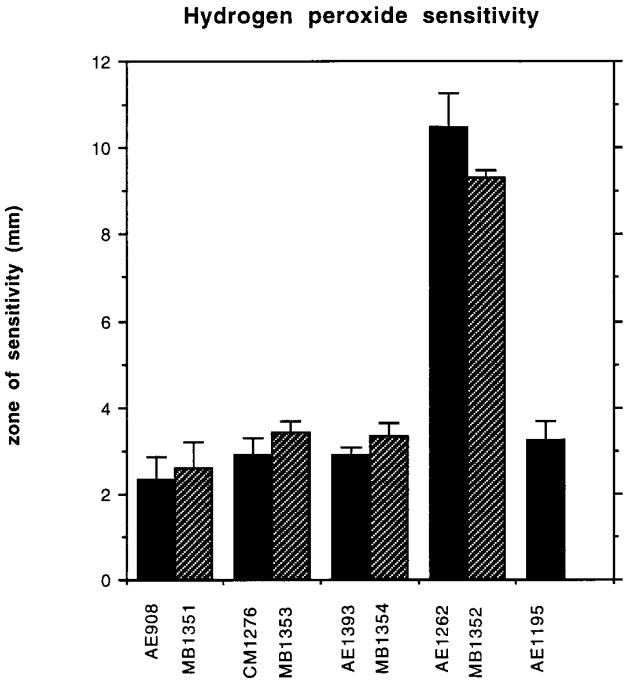

FIG. 3. Hydrogen peroxide sensitivity of log phase cells with or without the carotenoid plasmid pPL376. *E. coli*-MC4100 (AE908), *E. coli katF*::tn10 (CM1276), *E. coli*-RK4936 (AE1393), *E. coli* Δ*oxyR* (AE1262) and *E. herbicola* (AE1195). Assays were completed as described by Greenberg and Demple (11) except the zone of sensitivity was measured and the disk width was subtracted from the measured zone. The results shown are the average of two independent determinations each completed in triplicate.

mate three fold increase in carotenoid content may be due to the lack proteolysis of RpoS by these factors.

Carotenoids protection against H_2O_2 and near-UV. The challenge of *E. coli* mutants with H_2O_2 showed that the carotenoids did protect against this oxidative insult in all cases except the *oxyR* mutant (Fig. 3). The protection was small but approximately five percent consistently. The *E. herbicola* carotenoid positive control also showed approximately the same sensitivity to H_2O_2 as was observed in the strains containing pPL376, demonstrating that the carotenoids do have a protective effect. Because the disk diffusion assay performed is one that challenges cells primarily in log phase of growth, the protection would be expected to be low since it is known that maximal carotenoid expression occurs in stationary phase. The *oxyR* mutant strain was most sensitive to H_2O_2, but contrary to the above results, the carotenoids did not protect against H_2O_2 to the extent that might have been anticipated. This was consistent with the lower level of transcription than the parent in log phase of growth of the *oxyR* mutant.

When the various mutants were stressed with near-UV, all strains carrying the pPL376 plasmid (Fig. 4) were more resistant to near-UV lethality than those without the plasmid. The *rpoS* mutant was highly sensitive to near-UV and was partially protected by the low level of carotenoid being expressed from the plasmid. As expected, wildtype *E. coli* containing the plasmid showed no sensitivity during the exposure.

This study provides additional evidence of the close association of RpoS and OxyR in dealing with oxidative stress insults. Past reports have shown these sigma factors regulate *dps* (3), *katG* (10) and *gor* (17) dually. When *oxyR* is missing, RpoS is probably recruited to take its place in inducing the critical genes required to defend against oxidative stress and to prolong the organism's ability to survive under adverse conditions.

OxyS is a small, stable RNA induced by oxidative stress; its gene is linked to *oxyR*. As described in a recent report (18), its role in regulation of *rpoS* gives us a clearer understanding of the mechanism whereby OxyR-OxyS alters transcription upon environmental

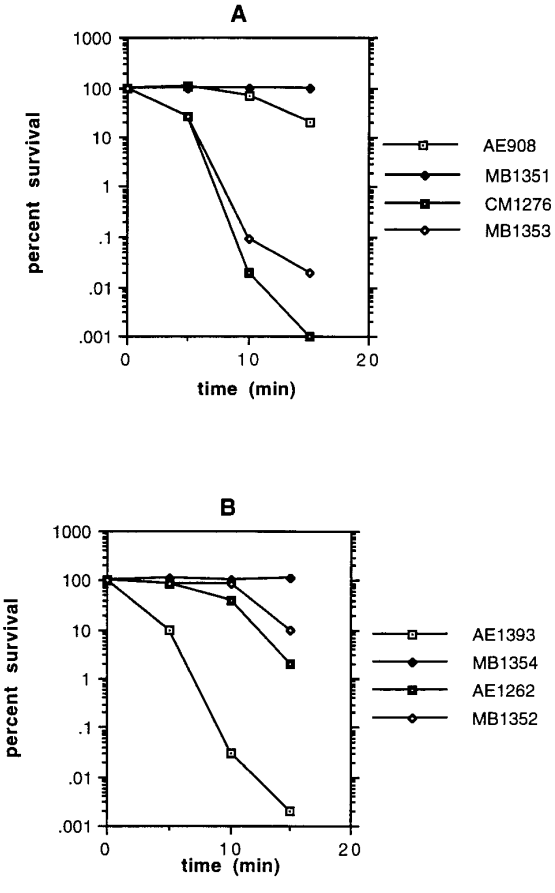

FIG. 4. The effect of near-UV radiation on the survival of strains with and without the carotenoid plasmid: Panel A. MC4100-WT (AE908), MC4100+pPL376 (MB1351), MC4100 *katF*::tn10 (CM1276), MC4100 *katF*::tn10+pPL376 (MB1353). Panel B. RK4936-WT (AE1393), RK4936+pPL376 (MB1354), RK4936 Δ *oxyR* (AE1262), RK4936 Δ *oxyR*+pPL376 (MB1352). The assays were completed as described by Knowles and Eisenstark (12). Each minute of exposure was equivalent to 1.04 kJ/m^2. The relative standard deviation of the data is below 15%.

shifts. This new information does not alter the overall observations of this study.

ACKNOWLEDGMENTS

We thank A. Chatterjee, G. Storz, R. Tuveson, and P. Loewen for kindly providing bacterial strains. We also thank M. Calcutt, A. Ivanova and J. Bullock for their helpful discussion, as well as Dana Carroll and Marnie Tutt for assistance in the preparation of this manuscript. This research was made possible by grant number ESO4889 (to A.E.) and ES06399 (to J.H.) from the National Institute of Environmental Health Science, NIH.

REFERENCES

1. Altuvia, S., Almiron, M., Huisman, G., Kolter, R., and Storz, G. (1994) *Molec. Microbiol.* **13,** 265–272.
2. Storz, G., and Altuvia, S. (1994) *Methods Enzymol.* **234,** 217–223.
3. Kullik, I., Stevens, J., Toledano, M. B., and Storz, G. (1995) *J. Bacteriol.* **177,** 1285–1291.
4. Loewen, P. C., and Hengge-Aronis, R. (1994) *Ann. Rev. Microbiol.* **48,** 53–80.
5. Eisenstark, A., Calcutt, M. C., Becker-Hapak, M., and Ivanova, A. (1996) *Free Radical Biol. Med.* **21,** 975–993.
6. Sandmann, G., Woods, W. S., and Tuveson, R. W. (1990) *FEMS Microbiol.* **59,** 77–82.
7. Perry, K. L., Simonitch, T. A., Harrison-Lavoie, K. J., Liu, S. T. (1986) *J. Bacteriol.* **168,** 607–612.
8. Hundle, B. S., Beyer, P., Kleinig, H., Englert, G., and Hearst, J. E. (1991) *Photochem. Photobiol.* **54,** 89–93.
9. Schnurr, G., Schmidt, A., and Sandmann, G. (1991) *FEMS Microbiol.* **62,** 157–162.
10. Ivanova, A., Glinsky, G., and Eisenstark, A. (1997) *Free Radical Biol. Med.* **23,** 627–636.
11. Greenberg, J. T., and Demple, B. (1988) *EMBO J.* **7,** 2611–2617.
12. Knowles, R., and Eisenstark, A. (1994) *Environ. Health Perspectives* **102,** 88–94.
13. Lange, R., and Hengge-Aronis, R. (1994) *Genes & Development* **8,** 1600–1612.
14. Wieland, B., Feil, C., Gloria-Maercher, E., Thumm, G., Lechner, M., Bravo, J. M., Poralla, K., and Gotz, F. (1994) *J. Bacteriol.* **176,** 7719–7726.
15. Schweder, T., Lee, K., Lomovskaya, O., and Matin, A. (1996) *J. Bacteriol.* **178,** 470–476.
16. Muffler, A., Fischer, D., Altuvia, S., Storz, G., and Hengge-Aronis, R. (1996) *EMBO J.* **15,** 1333–1339.
17. Becker-Hapak, M., and Eisenstark, A. (1995) *FEMS Microbiol. Letters* **134,** 39–44.
18. Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L., and Storz, G. (1997) *Cell* **90,** 43–53.