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Intracellular Generation of Superoxide as a By-Product of *Vibrio harveyi* Luciferase Expressed in *Escherichia coli*

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Luciferase genes are widely used as reporters of gene expression because of the high sensitivity of chemiluminescence detection and the possibility of monitoring light production in intact cells. We engineered fusions of the *Escherichia coli soxS* promoter to the luciferase structural genes (*luxAB*) from *Vibrio harveyi*. Since *soxS* transcription is positively triggered by the activated SoxR protein in response to agents such as paraquat that generate intracellular superoxide, we hoped to use this construct as a sensitive reporter of redox stress agents. Although a *soxR*$^+$ *soxS'*::*luxAB* fusion exhibited a paraquat-inducible synthesis of luciferase, a smaller increase was consistently observed even in the absence of known *soxRS* inducers. This endogenous induction was *soxR* dependent and was further characterized by introducing a plasmid carrying the luciferase structural genes without the *soxS* promoter into a strain carrying a *soxS'*::*lacZ* fusion in the bacterial chromosome. These cells exhibited increased β-galactosidase expression as they grew into mid-log phase. This increase was ascribed to luciferase activity because β-galactosidase induction was suppressed (but not eliminated) when the substrate *n*-decanal was present in the medium. The *soxS'*::*luxAB* plasmid transformed superoxide dismutase-deficient strains very poorly under aerobic conditions but just as efficiently as a control plasmid under anaerobic conditions. The production of hydrogen peroxide, the dismutation product of superoxide anion, was significantly increased in strains carrying bacterial luciferase and maximal in the absence of *n*-decanal. Taken collectively, these data point to the generation of significant amounts of intracellular superoxide by bacterial luciferase, the possible mechanism of which is discussed. In addition to providing insights into the role of superoxide in the activation of the SoxR protein, these results suggest caution in the interpretation of experiments using luciferase as a reporter of gene expression.

Luciferases have been widely used as reporter genes to determine temporal and tissue-specific regulation of promoters in both prokaryotes (bacterial luciferase) (10, 13, 22) and eukaryotes (firefly luciferase) (1, 18). Bacterial luciferase is also the target gene in a mutagenicity assay (50) and has been used to quantitate long-chain aldehydes (49), protease activity (3), and intracellular concentrations of NADH, NADPH, and flavin mononucleotide (FMN) (46).

Bacterial luciferase catalyzes the $FMNH_2$-dependent oxidation of *n*-decanal (29–31, 35). Both the substrate used experimentally (*n*-decanal) and the measurable product (photons of blue-green light) can cross cell membranes rapidly, which allows luciferase activity to be monitored continuously without damage to the cell. It should be noted that the physiological substrate of luciferase is thought to be *n*-tetradecanal (37), which is not generally used for experimental work because of its poor stability and low solubility (30). The applicability of bacterial luciferase as a reporter enzyme in eukaryotic systems has been enhanced by fusing the *luxA* and *luxB* genes, which encode the two subunits of bacterial luciferase (6, 20, 42, 43). Successful application of this fused bacterial luciferase in yeast (6) and plant (34) cells has been reported.

We have used the *Vibrio harveyi* luciferase genes in an attempt to develop a noninvasive reporter system for the activation of the *soxRS* regulon in *Escherichia coli*. The *soxRS* regulon is positively controlled by a single locus in response to oxidative stress imposed by superoxide-generating viologens and quinones or by nitric oxide (2, 16, 27, 36, 40, 41). This induction increases the levels of various antioxidant defense enzymes such as Mn-containing superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G6PD), endonuclease IV, and fumarase C (2, 27, 36, 47).

The functional *soxRS* locus includes two genes, *soxR* and *soxS*, arranged head to head (2, 16, 52). Activation of the *soxRS* regulon proceeds in two transcriptional stages in which existing SoxR protein, triggered by an intracellular redox signal, first stimulates transcription of the *soxS* gene (40, 53). The elevated level of SoxS protein then activates transcription of the various *soxRS* regulon genes (2, 16). Although numerous experiments connect *soxRS* activation to an increased production of intracellular superoxide (27, 40, 41, 47, 52), it has been also proposed that activation can be controlled by the intracellular NAD(P)H/NAD(P)$^+$ ratio (36).

To simplify the analysis of *soxRS* activation, we constructed fusions of the *V. harveyi luxAB* genes to the *soxS* promoter. Unexpectedly, analysis of the expression of these fusions led to the realization that bacterial luciferase itself generates a redox signal capable of activating this oxidative stress response.

MATERIALS AND METHODS

Reagents. Paraquat (PQ), *n*-decanal, antimycin A, ampicillin, luciferase, scopoletin, type VI horseradish peroxidase, FMN, NADH, cytochrome *c*, bovine SOD, and bovine catalase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Other chemical reagents were of analytical grade.

Strains and plasmids. Table 1 describes the strains and plasmids used in these studies. Plasmid pMLS1, which contains

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or characteristics	Reference or source
Strains		
GC4468	K-12 Δ(*lac*) *U169 rspL soxRS*$^+$	11
DJ901	As GC4468, Δ(*soxR-zjc-2205*) *zjc-2204*::Tn*10*Km	27
DF100	*fadL701 relA1 pit-1 spoT1 rrnB2 phoH510*	21
DF2001	As DF100, *zwf*Δ*2*	21
QC909	As GC4468, (*sodA*::MudPR13)*25* (*sodB*::*kan*)Δ*1-2*	11
QC781	As GC4468, (*sodA*::MudPR13)*25*	D. Touati (11) via I. Fridovich
TN520	As GC4468, λφ(*soxR*$^+$ *soxS'*::*lacZ*)	41
Plasmids		
pBR322	Vector, Ap^r, Tc^r	5
pBluescript	Vector, Ap^r	Stratagene
pMLS1	pBluescript derivative carrying *luxAB*	A. T. Schauer (45) via A. O. Summers (13)
pBD100	pBR322 derivative carrying *soxRS*	2
pBGF131	pBluescript derivative carrying *soxR*$^+$ soxS'::*luxAB*	This work
pBGF254	pBluescript derivative carrying Δ*soxR soxS'*::*luxAB*	This work

the *luxAB* genes, was constructed by Alan Schauer (University of Texas, Austin) and provided by Anne Summers (University of Georgia, Athens). Two operon fusions were constructed for this study: pBGF131 (*soxR*$^+$ *soxS'*::*luxAB*) and pBGF254 (Δ*soxR soxS'*::*luxAB*). To construct pBGF131, the 3-kb *Eag*I-*Eco*RV fragment from pBD100 (2) containing the intact *soxR* gene and the 5′ 50% of *soxS* was inserted into the *Eag*I-*Eco*RV digest of pMLS1. Deletion from pBGF131 of the 2-kb *Eco*RI-*Bsm*I fragment containing the 3′ 60% of *soxR*, blunting, and religation resulted in pBGF254.

Growth conditions. The cells carrying pBGF131 or pBGF254 were inoculated into LB broth (38) containing 100 μg of ampicillin per ml and incubated at 37°C for 12 to 16 h with gentle shaking (100 rpm). The saturated cultures were diluted 100-fold into fresh LB-ampicillin and incubated at 37°C for 60 min to reach an optical density at 600 nm (OD_{600}) of about 0.1. For some induction experiments, PQ was added to 2 ml of culture to reach a final concentration of 50 μM, and the culture was shaken vigorously (300 rpm) at 37°C for 90 min.

Luciferase activity. Blue-green light emission (485 nm) (31) by strains harboring *lux* fusion plasmids was measured using a Beckman liquid scintillation counter (model LS 1801; Beckman Instruments, Fullerton, Calif.) set in the out-of-coincidence mode. This counter has photomultipliers responsive in the range of 380 to 620 nm. The dark current value in the absence of sample was 30 ± 2 cpm. At the times indicated, samples were taken, diluted into 3 ml of LB supplemented with 0.1% (vol/vol) *n*-decanal, and placed in low-potassium glass vials, and light emission was measured. Results are reported as counts per minute per 10^6 cells. Cell density was estimated by OD_{600}. Calibration curves were performed with a semipurified enzyme fraction containing both luciferase and FMN reductase (Sigma). The rate of NADH consumption and the light emission by luciferase in vitro were measured in a reaction mixture containing 50 mM phosphate buffer (pH 7.5), 100 μM NADH, and 50 μM FMN (31).

Intracellular steady-state concentration of hydrogen peroxide. On the basis of the free diffusion of hydrogen peroxide through cell membranes (14), its intracellular steady-state concentration can be estimated by measuring the hydrogen peroxide concentration in the incubation medium of cells after allowing the diffusional steady state to be reached (the intracellular concentration equals the extracellular concentration) (25, 44). Exponential-phase cultures were pelleted, washed, and resuspended in 120 mM NaCl–30 mM phosphate buffer (pH 7.4) at a density of 1×10^6 to 2×10^6 cells per ml. Samples were taken at 0, 2, 4, 6, and 10 min and divided in two aliquots. One aliquot of each time point was incubated 10 min at 30°C with 1 μM catalase. Samples were incubated in final concentrations of 2 μM horseradish peroxidase and 2 μM scopoletin, and the resultant fluorescence was measured at 350 nm (excitation) and 460 nm (emission) in a Hoefer fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.). Hydrogen peroxide concentration was calculated by subtracting the value of the sample treated with catalase from the value of the untreated sample (25).

Superoxide anion detection. The rate of O_2^- generation during in vitro reactions was measured as the SOD-sensitive rate of cytochrome *c* reduction. Reduction of ferricytochrome *c* was monitored spectrophotometrically at 550 nm (E = 24 $mM^{-1}\ cm^{-1}$) (33) in a 1-ml reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), 20 μM cytochrome *c*, 50 μM FMN, 100 μM NADH, and luciferase, with and without 50 U of SOD.

Enzymatic activities. β-Galactosidase activity in sodium dodecyl sulfate-$CHCl_3$-treated cells was determined as described by Miller (38). Results were expressed as units per OD_{600}. For catalase and fumarase C determinations, exponential cultures were pelleted, resuspended in 120 mM NaCl–50 mM Tris (pH 7.4), and lysed with a mini-bead beater cell disrupter. Catalase activity in bacterial extracts was determined by measuring the decrease in the A_{240} of hydrogen peroxide in a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide (25). Fumarase C activity was measured independently of fumarase AB as described by Liochev and Fridovich (36) by monitoring the increase in the fumarate concentrations as a function of A_{250} in 1-ml reaction mixtures containing 50 mM phosphate buffer (pH 7.4) and 50 mM sodium malate (32). Protein concentration in the extracts was measured by the method of Bradford (7), using bovine serum albumin as the standard. All the measurements were carried out in a Perkin-Elmer UV/Vis spectrophotometer (model Lambda 3A; Perkin-Elmer, Oak Brook, Ill.).

Statistics. Results are indicated as the mean value of four independent experiments ± standard error of the mean. Statistical significance were analyzed by analysis of variance followed by Dunnett's test for other comparisons (51).

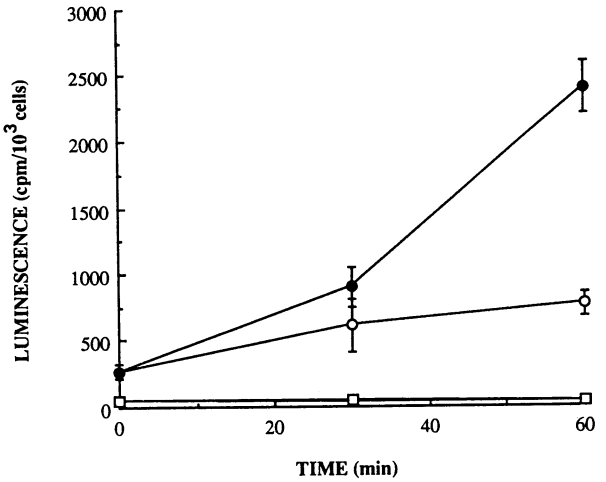

FIG. 1. *soxR*-dependent induction of *soxS'::luxAB* by PQ or luciferase by-products. Cell cultures were incubated at 37°C in the absence (open symbols) or presence (filled symbols) of 50 μM PQ in air with shaking. Samples were taken at 30-min intervals and diluted into LB medium, and *n*-decanal was added (final concentration, 0.1%) to measure chemiluminescence. Plasmids pBGF131 (*soxR*$^+$; circles) and pBGF254 (Δ*soxR*; squares) were present in DJ901 (Δ*soxRS*).

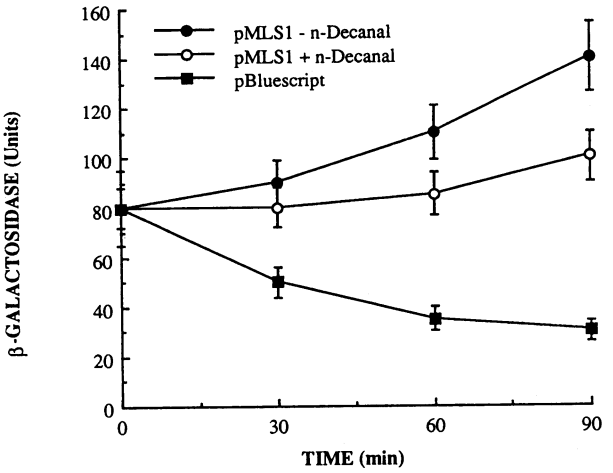

FIG. 2. Induction of *soxS'::lacZ* by luciferase. Cultures of strain TN520 containing plasmid pMLS1 were incubated at 37°C in the absence (filled circles) or presence (open circles) of 0.5 mM *n*-decanal. Samples were taken at 30-min intervals to assay β-galactosidase activity.

RESULTS

PQ-independent induction of *soxS'::luxAB*. Fusion of the *E. coli lacZ* gene to the *soxS* promoter gives rise to β-galactosidase that is inducible by PQ and other superoxide-generating agents in a manner completely dependent on a functional *soxR* gene (40, 41, 53). In a similar fashion, the *soxR*$^+$ *soxS'::luxAB* construct in a multicopy plasmid (pBGF131) directed substantial time-dependent, PQ-inducible luciferase expression (Fig. 1). Unexpectedly, a small increase in luciferase activity (about 2.5-fold per 60 min) was observed as the cells grew to mid-logarithmic phase in the absence of PQ. Neither the PQ-induced nor the spontaneously induced luciferase was observed with the Δ*soxR* plasmid pBGF254 (Fig. 1). The increased expression of *soxS::luxAB* contrasts with the pattern observed for the *soxR*$^+$ *soxS'::lacZ* fusions in the same genetic background, which consistently show a decline with growth into mid- or late logarithmic phase (40).

Induction of *soxS* by luciferase by-products. It seemed possible that the unexpected behavior of the *soxS'::luxAB* fusion could be due to either altered regulation compared with *soxS'::lacZ* or the presence of luciferase in the cells. Oxidation of long-chain aldehydes by luciferase consumes reducing equivalents from NAD(P)H to form the reduced flavoprotein intermediate (29). Reaction of the partially reduced flavins with O_2 yields superoxide anion (8, 39). By alternating these reactions, luciferase might set up a redox cycle that generates O_2^- and depletes NAD(P)H. Long-chain aldehydes would compete with this reaction by consuming reducing equivalents in the light-generating reaction. According to this scheme, superoxide production by luciferase would be maximal in the absence of the aldehyde.

To test this hypothesis and to eliminate the possibility of altered regulation of *soxS'::luxAB* compared with *soxS'::lacZ*, we introduced into a strain (TN520) that carries a chromosomal *soxR*$^+$ *soxS'::lacZ* fusion plasmid pMLS1, which carries the *luxAB* genes lacking their native promoter (45). Nevertheless, a low level of luciferase activity was detected in intact cells of strain GC4468 or DJ901 containing pMLS1 (luminescence values of ~125 cpm/10^3 cells). In contrast to strains carrying pBGF131 (Fig. 1), the luciferase activity expressed by pMLS1 did not increase as a function of cell growth (26).

β-Galactosidase activity was monitored to measure *soxS* transcription in the presence and absence of *n*-decanal (Fig. 2). As with the *soxS'::luxAB* fusion, an increased expression of β-galactosidase occurred during the growth of this strain. However, the induction from the *soxS* promoter was significantly dampened upon addition of *n*-decanal. A similar dampening effect was observed with the natural substrate *n*-tetradecanal (26), although its poor solubility prevented an accurate estimate of its concentration. These results suggest that superoxide production, rather than the consumption of NAD(P)H (which the addition of *n*-decanal does not prevent), is the activating signal generated by luciferase. Control experiments showed no effect of *n*-decanal on the induction of *soxS'::lacZ* by PQ (26).

The generation of superoxide by luciferase might constitute a substantial burden for cells lacking normal antioxidant defenses such as SOD. To test this possibility, we examined the transformation efficiency of luciferase-expressing plasmids into SOD-deficient strains, which lacked either one (QC781) or both (QC909) of the bacterial SOD enzymes. Low relative efficiencies of transformation were observed for pMLS1 and especially for pBGF131 when introduced into QC781 and QC909 compared with the SOD-proficient strains GC4468 and DJ901 (Table 2). Although DJ901 lacks the *soxRS*-dependent induction of SOD, this strain has normal basal SOD activity (27). Under conditions of low oxygen tension, in which the intracellular generation of superoxide should be strongly diminished, the transformation efficiency of the SOD-deficient strains by the *lux* plasmids was similar to that of SOD-containing cells under aerobiosis (Table 2).

Deficiency in G6PD sensitizes *E. coli* to *soxRS* activation by PQ (36). This was also true for the luciferase-dependent activation, as measured by using the *soxS'::luxAB* fusion (Fig. 3A), or following the induction of the *soxS*-regulated enzyme fumarase C (Fig. 3B). After 16 h of growth, 56- and 20-fold increases in luciferase and fumarase C expression, respectively, were measured for the G6PD-deficient strain, 10- and 5-fold increases were measured in the *zwf*$^+$ strain (26).

TABLE 2. Transformation efficiencies for luciferase-containing plasmids in different genetic backgrounds

E. coli strain	Plasmid	Transformation efficiency (%)	
		Aerobic	Anaerobic[a]
GC4468	pBluescript	100 (7×10^3)[b]	ND
	pMLS1	100	ND
	pBGF131	100	ND
DJ901	pBluescript	100	ND
	pMLS1	100	ND
	pBGF131	100	ND
QC781	pBluescript	100	100 (3×10^3)
	pMLS1	30	90
	pBGF131	1	90
QC909	pBluescript	100	100
	pMLS1	40	100
	pBGF131	0	90

[a] A partial anaerobiosis during the growth of the transformants was achieved by placing the plates in an Atmos Bag (Aldrich) that was flushed and filled with argon gas. ND, not determined.

[b] Numbers in parentheses indicate the total number of transformants obtained for the control plasmid.

Oxidative stress and redox changes associated with luciferase activity. Generation of superoxide anion by autoxidation of flavoproteins is well documented: flavoproteins acting as electron transferases and dehydrogenases appear likely to act in one-electron steps by formation of a stable semiquinone, which reacts with oxygen to yield superoxide anion (8, 39, 48). We sought biochemical evidence that luciferase generates superoxide. Purified luciferase, incubated in vitro with FMN and NADH, formed reduced cytochrome *c* (Fig. 4A). This cytochrome *c* reduction was inhibited by superoxide dismutase (Fig. 4A) and was linearly correlated with the luciferase concentration (Fig. 4B). These data indicate that superoxide anion is a significant by-product of the luciferase reaction in vitro. In addition, the rate of superoxide anion production by luciferase in vitro was significantly diminished by both *n*-decanal (Fig. 4B) and *n*-tetradecanal (26), which is consistent with the hypothesis that superoxide production and *n*-decanal oxidation are alternative pathways competing for a common intermediate.

Because superoxide anion is unable to cross cell membranes, the intracellular concentration of O_2^- cannot be measured directly in an intact cell. In SOD-containing cells, O_2^- is rapidly converted by SOD to H_2O_2 ($k = 10^9\ M^{-1}\ s^{-1}$ [14, 23]), which does cross membranes (14). Increased generation of intracellular O_2^- can therefore be observed as an increase in intracellular and extracellular concentrations of hydrogen peroxide. This noninvasive approach has been applied to mammalian tissues (25) and cultured cells (9) as well as to the protozoan *Trypanosoma cruzi* (24).

We applied this approach to confirm the luciferase-dependent generation of superoxide anion in intact *E. coli*. After a steady state is reached in which the rate of H_2O_2 production equals the rate of H_2O_2 utilization (mainly by catalase [28]), the rate of H_2O_2 generation can be estimated from the H_2O_2 concentration and the cellular content of catalase, using the following relation: $d[H_2O_2]/dt = k \cdot [\text{catalase}] \cdot [H_2O_2]$, where k is the rate constant for the decomposition of H_2O_2 by bacterial catalase ($k = 10^6\ M^{-1}\ s^{-1}$, calculated from reference 28), and the catalase and steady-state H_2O_2 concentrations are the experimental values.

Introduction of luciferase structural genes into GC4468 and DJ901 caused 2.5-fold increases in both strains in the hydrogen peroxide steady-state concentration in the absence of *n*-decanal (Table 3). This H_2O_2 generation in luciferase-expressing cells was lowered when *n*-decanal was present but was still significantly higher than that measured for cells not expressing luciferase (Table 3). The basal rate of H_2O_2 production in cells expressing luciferase was nearly doubled in the presence of *n*-decanal (15 ± nmol/min/10^6 cells) and nearly tripled without this substrate (26 ± 3 nmol/min/10^6 cells [$P < 0.001$ compared with the value in the presence of *n*-decanal]) compared with control cells (9 ± 1 nmol/min/10^6 cells [no difference was found in the endogenous rate of hydrogen peroxide production between GC4468 and DJ901]).

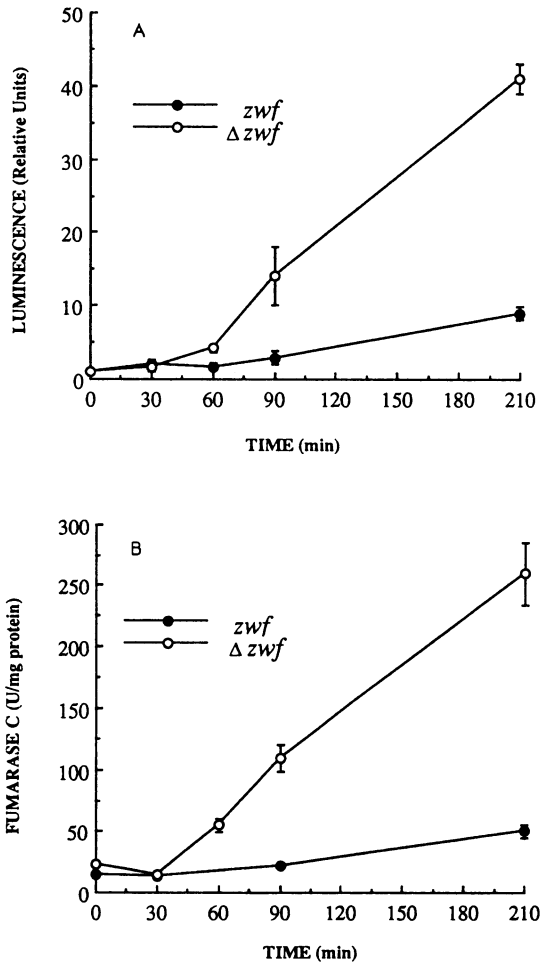

FIG. 3. Luciferase-induced expression of *soxS* in a G6PD-deficient strain. Overnight cultures of DF2001 (Δ*zwf*; open circles) and DF100 (*zwf*$^+$; filled circles) carrying pBGF131 (*soxS'::luxAB*) were diluted 1/100 and grown for about 1 h to reach an OD_{600} of ≈0.1 to 0.2 (time 0). Cultures were incubated in the absence of any exogenous inducer. Samples were taken at 30-min intervals and diluted, and *n*-decanal was added to measure luminescence (A) or fumarase C activity was assayed (B).

DISCUSSION

Bacterial luciferase genes are frequently used as reporters to monitor promoter activity. We were therefore surprised when fusion of the *V. harveyi lux* genes to the *E. coli soxS* promoter revealed induction independent of the usual inducing treatments. The results presented here show that this *lux*-dependent

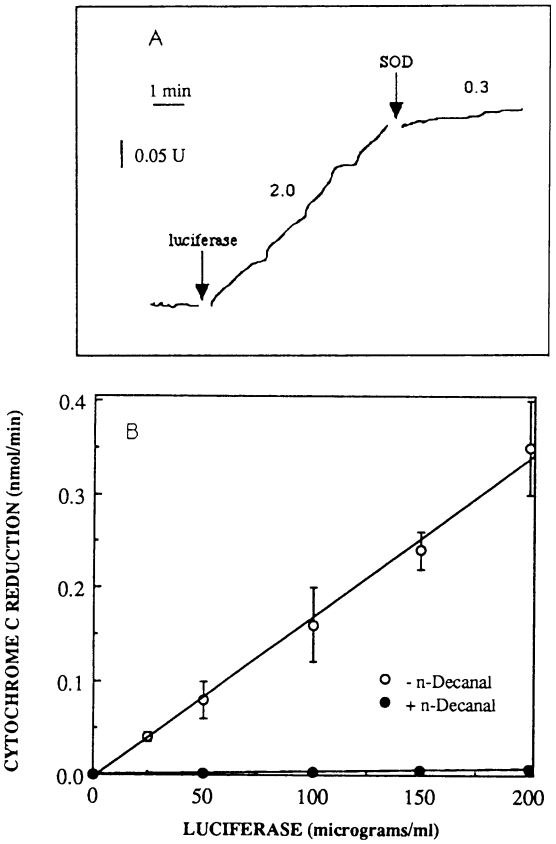

FIG. 4. Superoxide anion production by luciferase. (A) Time course of generation of superoxide anion by luciferase (0.2 mg/ml) in vitro; (B) relationship between the amount of luciferase and the rate of cytochrome *c* reduction. Luciferase extracts from *V. harveyi* were assayed for superoxide anion production as described in Materials and Methods. The numbers near the traces indicate nanomoles of O_2^- produced per minute per milligram of protein.

induction of *soxS* is apparently due to side products of the luciferase reaction. This conclusion prompts a reappraisal of the luciferase mechanism and suggests caution in the use and interpretation of luciferase as a reporter for gene activity.

The molecular aspects of the reaction catalyzed by bacterial luciferase have been studied extensively since 1965 (29–31). Briefly, the reaction involves the reduction of FMN by a NAD(P)H-dependent oxidoreductase (Fig. 5, step 1). The reduced forms of FMN react with molecular oxygen to yield a stable hydroperoxide in a reaction catalyzed by luciferase (steps 2 and 3). The hydroperoxide intermediate oxidizes a long-chain aldehyde (step 4), forming an excited intermediate that is responsible for the blue-green emission associated with the reaction (step 5). Finally, the oxidized form of FMN is dissociated from luciferase (step 6), starting a new catalytic cycle.

TABLE 3. Intracellular steady-state concentration of hydrogen peroxide and catalase in *E. coli* carrying luciferase

Strain/plasmid	[Catalase] (pmol/10^6 cells)	$[H_2O_2]$ (μM)	
		−*n*-Decanal	+*n*-Decanal
GC4468	0.17 ± 0.03	0.20 ± 0.03	0.25 ± 0.05
GC4468/pMLS1	0.17 ± 0.03	0.50 ± 0.04[a]	0.40 ± 0.06[a,b]
DJ901	0.21 ± 0.03	0.20 ± 0.03	0.23 ± 0.04
DJ901/pMLS1	0.23 ± 0.01	0.50 ± 0.07[a]	0.30 ± 0.04[a,b]

[a] $P < 0.001$ compared with the value in the absence of pMLS1.
[b] $P < 0.05$ compared with the value in the absence of *n*-decanal.

Reactive oxygen species as by-products of luciferase were proposed by Kurfust et al., who suggested that H_2O_2 they measured is generated by cleavage of the hydroperoxide intermediate (35). However, a significant body of evidence shows that the FMN semiquinone can transfer single electrons to oxygen to yield superoxide anion rather than hydrogen peroxide (8, 39, 48). We therefore postulate the reaction described in step 3′ for the production of superoxide anion by luciferase. It is noteworthy that after dissociation from oxidized FMN, luciferase can again bind $FMNH^-$ or $FMNH_2$ (step 2) to set up a redox cycle unconnected to aldehyde oxidation. An interesting feature of this model is the alternative fate for electrons between two-step (aldehyde oxidation cycle, steps 3 and 4) and one-step reactions (futile redox cycle, step 3′). The partition between these alternative pathways would depend on the relative availabilities of the oxidizable aldehyde and of molecular oxygen. The effects of *n*-decanal and hypoxia presented here are both consistent with this model.

These observations shed some light on the intracellular signal(s) that activates SoxR protein, the primary sensor/regulator of the *soxRS* regulon. Although much evidence implicates intracellular superoxide as the key signal generated by redox-cycling agents (40), the NAD(P)H/$NAD(P)^+$ ratio has also been suggested to regulate SoxR activity (36). In the situation described here, the consumption of NAD(P)H is expected to proceed both in the presence of *n*-decanal (to support oxidation of the aldehyde) and in its absence (to support the superoxide-generating redox cycle). The lower level of *soxRS* activation in the presence of *n*-decanal paralleled the reduced generation of H_2O_2, which indicates that the superoxide-generating pathway operates in *E. coli* even when the light-generating reaction is allowed. We know of no observations suggesting that luciferase activity causes oxidative stress in *V. harveyi* or other luminescent bacteria. Perhaps the coordinated activity of specific FMN reductase(s) and the synthetic machinery to produce the correct aldehyde substrate intracellularly avoids this problem in such cells.

The phenomenon described here would not be restricted to *V. harveyi* luciferase, since luciferases from different bacterial species catalyze the same reaction, although these enzymes differ in their physical, kinetic, and purification properties (29). In contrast, firefly luciferase catalyzes the ATP-dependent oxidation of luciferin without electron transfer, which would not be expected to cause oxidative stress by the mechanism described here.

The increased rate of O_2^- and H_2O_2 generation was not cytotoxic for SOD-containing strains. This contrasts with mammalian cells, for which two- to fourfold increases in the intracellular generation of H_2O_2 were reported to cause irreversible cell damage (25). Peroxide concentrations higher than 100 μM, given in single pulses, can be bacteriostatic or lethal for *E. coli* under some conditions (17), while pretreatment of bacteria with 5 to 50 μM pulses of H_2O_2 induces resistance to H_2O_2 concentrations up to 25 mM (16, 17). Since no increase in catalase concentration was detected, the twofold increase in the H_2O_2 steady state is not enough to activate the *oxyR* regulon significantly (15). Conversely, the corresponding fourfold net increase in the flux of O_2^- may be sufficient to begin triggering the *soxRS* regulon. It remains possible that the effect of luciferase on O_2^- production is even greater than estimated here, with some superoxide consumed in reactions that do not

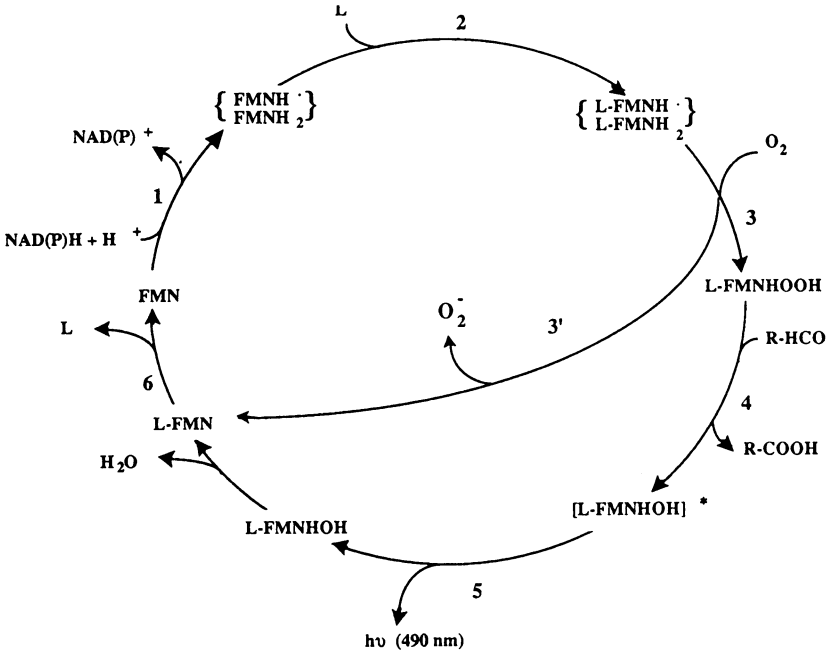

FIG. 5. Proposed mechanism for the generation of superoxide by *V. harveyi* luciferase in *E. coli*. $FMNH^{\cdot}$ and $FMNH_2$, partially and fully reduced forms of FMN; L, luciferase; R-HCO, long-chain aldehyde; RCOOH, fatty acid: O_2^-, superoxide anion.

generate H_2O_2. Alternatively, the simultaneous depletion of NAD(P)H and generation of O_2^- may exert a kind of double signal for *soxRS*, consistent with the effect of *zwf* mutations shown in this work and by others (36).

Apart from revising our understanding of the mechanism of bacterial luciferase operating in *E. coli*, our results also suggest that new caution should be applied in the use of *luxAB* as reporter genes. The generation of superoxide by luciferase in an organism that does not normally express it leaves open the possibility that some inductions might result from the oxidative stress caused by the luciferase itself. Additionally, we have now provided values for the intracellular concentration of hydrogen peroxide and a preliminary approach to the quantitative characterization of the thresholds needed to trigger oxidative stress responses in *E. coli*.

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