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Constricted Flux through the Branched-Chain Amino Acid Biosynthetic Enzyme Acetolactate Synthase Triggers Elevated Expression of Genes Regulated by *rpoS* and Internal Acidification

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The first common enzyme of isoleucine and valine biosynthesis, acetolactate synthase (ALS), is specifically inhibited by the herbicide sulfometuron methyl (SM). To further understand the physiological consequences of flux alterations at this point in metabolism, *Escherichia coli* genes whose expression was induced by partial inhibition of ALS were sought. Plasmid-based fusions of random *E. coli* DNA fragments to *Photorhabdus luminescens luxCDABE* were screened for bioluminescent increases in actively growing liquid cultures slowed 25% by the addition of SM. From more than 8,000 transformants, 12 unique SM-inducible promoter-*lux* fusions were identified. The *lux* reporter genes were joined to seven uncharacterized open reading frames, *f253a*, *f415*, *frvX*, *o513*, *o521*, *yciG*, and *yohF*, and five known genes, *inaA*, *ldcC*, *osmY*, *poxB*, and *sohA*. Inactivation of the *rpoS*-encoded sigma factor, σ^S, reduced basal expression levels of six of these fusions 10- to 200-fold. These six genes defined four new members of the σ^S regulon, *f253a*, *ldcC*, *yciG*, and *yohF*, and included two known members, *osmY* and *poxB*. Furthermore, the weak acid salicylate, which causes cytoplasmic acidification, also induced increased bioluminescence from seven SM-inducible promoter-*lux* fusion-containing strains, namely, those with fusions of the σ^S-controlled genes and *inaA*. The pattern of gene expression changes suggested that restricted ALS activity may result in intracellular acidification and induction of the σ^S-dependent stress response.

Sulfometuron methyl (SM) is a potent and specific inhibitor of the first common enzyme of isoleucine and valine biosynthesis (Fig. 1) in bacteria, fungi, and plants (23, 24, 41). Thus, SM is a useful tool for localized constriction of metabolic flux (26). Such inhibition of acetolactate synthase (ALS; EC 4.1.3.18) by SM results in starvation for isoleucine and valine as well as accumulation of its substrates, the α-ketoacids pyruvate and α-ketobutyrate (13, 27). These and other α-ketoacids, and their acyl coenzyme A derivatives, are important central metabolites, as they account for about 70% of carbon flux in *Escherichia coli* (17). Hence, changes in intracellular levels of α-ketoacids resulting from metabolic perturbations such as ALS inhibition may have multiple physiological consequences.

Accumulation of α-ketobutyrate plays an important role in the deleterious biological effects of ALS inhibition in bacteria. This has been demonstrated in studies using an isoleucine feedback-resistant mutant threonine deaminase, which catalyzes conversion of threonine to α-ketobutyrate. Although SM-mediated growth inhibition of wild-type *Salmonella typhimurium* is fully reversed by isoleucine and valine addition, growth inhibition of the mutant is not fully alleviated by this addition (27). Thus, continued synthesis of α-ketobutyrate accounts for the isoleucine- and valine-independent SM-mediated inhibition. Such growth inhibition of the mutant strain is alleviated by the immediate biosynthetic precursor of valine, α-ketoisovalerate, suggesting the deleterious effects of competition between these two α-ketoacids (55). Furthermore, a number of SM-hypersensitive *S. typhimurium* mutants are defective in α-ketobutyrate degradation (27, 56). Lack of these α-ketobutyrate catabolic pathways, such as that mediated by acetate kinase and phosphotransacetylase (54), is suggested to result in higher levels of either this toxic intermediate or a by-product. However, there is not a close correlation at sublethal doses of SM between the degree of growth inhibition of *S. typhimurium* caused by SM and the accumulation of α-ketobutyrate (13). Likewise, the role of α-ketobutyrate accumulation in the phytotoxicity of ALS inhibition in plants is not certain (47). Thus, other approaches that may yield further insights into the physiological ramifications of flux alterations at this key point in intermediary metabolism are needed.

In this study, we analyzed alterations in gene expression induced by sublethal doses of SM that partially constrict flux through ALS. Typically, bacteria regulate transcription in response to conditions that alter cellular physiology. Often the set of proteins induced by a particular stress, a stimulon (37), includes some that eliminate the stress and others that are important for maintenance of cellular homeostasis. Thus, the profile of gene expression changes induced by any agent will reveal the nature of and responses to the stress condition. Such an approach may be particularly useful in understanding the biological consequences of metabolic flux alterations, such as that mediated by SM.

Reporter genes are commonly used to discover and charac-

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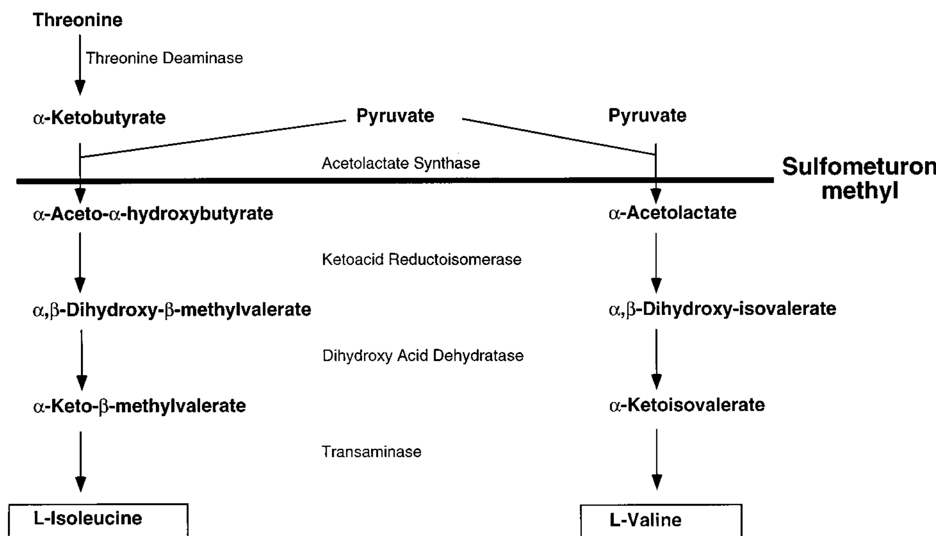

FIG. 1. The biosynthetic pathway for synthesis of L-isoleucine and L-valine. ALS catalyzes the decarboxylation of pyruvate and condensation of the resultant hydroxyethyl-thiamine pyrophosphate moiety with α-ketobutyrate in isoleucine biosynthesis and condensation with another molecule of pyruvate in valine biosynthesis. In *E. coli* K-12, two ALS isozymes are normally expressed. In the strain used in this study, ALS I, which is insensitive to SM, was not expressed. Catalysis of this step was by ALS III, which is inhibited by SM.

terize bacterial promoters activated by environmental stresses. Of the various reporter systems available, bacterial bioluminescence has the unique advantage that gene expression can be monitored in real time without cell lysis. Moreover, if a five-gene *luxCDABE* reporter is used, all of the agents required for bioluminescence, the five Lux polypeptides, O_2, ATP, reduced flavin mononucleotide, and NADPH, are present in aerobically grown cells (32). In this work, we used a moderate-copy-number promoter probe vector, pDEW201, that contains a multiple cloning site between transcriptional terminators and a *luxCDABE* reporter gene complex from *Photorhabdus luminescens*. The expressed Lux proteins are stable at temperatures up to 45°C (52). Such a plasmid-based system can identify expression changes in essential genes because the chromosome remained unaltered. In addition, amplification of weak transcriptional signals may be important for detection of transcriptional activity from promoter fusions that would be undetected in single copy. Our experience has been that plasmid-based *lux* genetic fusions respond to the same regulatory controls as do chromosomal genes for one negatively (60) and several positively (8, 12, 50) controlled regulatory circuits.

We describe the use of such plasmid-based *lux* fusions to *E. coli* promoters to characterize gene expression changes following imposition of a metabolic perturbation. Partial inhibition of ALS by SM led to moderate increases in bioluminescence from *E. coli* strains containing SM-induced (*smi*) promoters controlling expression of *luxCDABE*. The majority of the 12 identified *smi-luxCDABE* fusions were induced by weak acid treatment and regulated by σ^S. These results suggested that the physiological consequences of partial inhibition of ALS activity may be intracellular acidification and induction of the σ^S-dependent stress response. This work thus provides an example of the interplay between metabolic flux alterations and global control of gene expression.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** *E. coli* W3110 (14) was used for isolation of chromosomal DNA. *E. coli* DPD1675 [*ilvB2101 ara thi* Δ(*proAB-lac*) *tolC*::miniTn*10*] (58) was used as the host strain for screening for induction of bioluminescence from the chromosomal-*luxCDABE* genetic fusions. Strains isolated from this screening are listed in Table 1. Additional *E. coli* strains used were the otherwise isogenic pair MP180 (HfrH *thi-1*) and UM122 (HfrH *thi-1 rpoS13*::Tn*10*) (30) and an otherwise isogenic set: GC4468 (F⁻ Δ*lac-4169 rpsL*) (3), N7840 [F⁻ Δ*lac4169 rpsL* Δ*(mar sad)1738*] (43), N8452 [F⁻ Δ*lac-4169 rpsL* Δ*(mar sad)1738 rob*::*kan*] (from J. L. Rosner), and DPD2209 [F⁻ Δ*lac-4169 rpsL* Δ*(mar sad)1738 rob*::*kan rpoS13*::Tn*10*]. The latter strain was constructed by generalized transduction using phage P1*clr100* (34) grown on strain UM122 as the donor and strain N8452 as the recipient, selecting for tetracycline resistance.

Plasmid pDEW201 (58) has the origin of replication and *bla* gene conferring ampicillin resistance from pBR322 and four transcription terminators upstream of promoterless *P. luminescens luxCDABE* genes. The multiple cloning site that lies between the terminators and *lux* contains unique *Eco*RI, *Bam*HI, *Kpn*I, and

TABLE 1. Characteristics of *E. coli* strains containing *smi* promoter-*luxCDABE* fusions

Strain	Plasmid	Gene fused to *luxCDABE*	Basal RLU[a]	SM response ratio[b]	No. found[c]
DPD2081	pDEW213	*f415*	0.2	1.83 ± 0.14	3
DPD2084	pDEW215	*yciG*	0.27	1.58 ± 0.22	1
DPD2087	pDEW218	*inaA*	9.8	1.57 ± 0.40	1
DPD2088	pDEW219	*yohF*	0.14	1.71 ± 0.12	2
DPD2089	pDEW220	*o82*/*o521*	1.4	1.54 ± 0.11	1
DPD2090	pDEW221	*osmY*	0.08	1.78 ± 0.10	1
DPD2092	pDEW223	*o513*	5.1	1.35 ± 0.19	1
DPD3501	pDEW301	*frvX*	13.4	3.09 ± 0.96	1
DPD3505	pDEW305	*f253a*	0.9	1.52 ± 0.29	2
DPD3507	pDEW307	*sohA*	62.4	1.20 ± 0.06	1
DPD3509	pDEW309	*poxB*	1.5	1.72 ± 0.09	1
DPD3512	pDEW312	*ldcC*	0.5	2.10 ± 0.22	1
DPD2083	pDEW201	None	0.002		

[a] The RLU reading from the culture grown in the wells of a microplate, untreated with SM, at the initial time point of the primary screen was recorded as the basal light output. This value was expected to roughly correlate to the amount of transcription initiation at promoters upstream of *luxCDABE.*

[b] Calculated by dividing the RLU of the SM-treated culture by the RLU of the untreated culture at about 180 min (except the ratio for strain DPD3507, which was calculated at 120 min) after addition of cells to SM in the second screening test. Values are means and standard deviations of the triplicate cultures.

[c] Number of independent plasmids found with the same fusion of *E. coli* chromosomal DNA to *luxCDABE* as in the representative strain listed. In each case when multiple hits were obtained, the joint of *lux* to the chromosomal segment was identical.

*Sac*I sites. A plasmid, pDEW207, that contained the *grpE* heat shock promoter driving expression of *luxCDABE* was constructed by ligating the 0.6-kb *Bam*HI fragment from plasmid pGrpELux5 (57) into *Bam*HI-digested and calf intestinal alkaline phosphatase-treated pDEW201. The correct orientation of the *grpE* promoter was confirmed by *Hin*dIII digestion of the resultant plasmid. *E. coli* DPD2077 is a transformant of DPD1675 that contains pDEW207.

Growth media and chemicals. The defined growth medium was Vogel-Bonner medium (11), with glucose as a carbon source, supplemented with thiamine, uracil, and proline. Ampicillin was added at either 25 or 10 μg/ml to this medium. The rich medium was LB (34) to which ampicillin was added at 150 or 50 μg/ml. SM was obtained from the Agricultural Products Department of the DuPont Company. A 2-mg/ml solution of SM in 0.01 N NaOH was prepared and stored at −20°C. Dilution of this SM stock to 32 μg/ml or less into the Vogel-Bonner medium did not affect the resultant pH. A 1 M stock solution of sodium salicylate, purchased from EM Science, in water was stored at −20°C.

Turbidity measurements and growth rate determinations in microplates. Culture turbidity was routinely measured with a Klett-Summerson colorimeter by using the red filter. For measurement of growth rate inhibition by SM and ethanol, *E. coli* DPD1675 was grown at 37°C in the defined medium in a flask to early exponential phase (8 to 20 Klett units). Then 50 μl of this culture was placed in the wells of a sterile, clear microplate (Falcon Microtest III 96-well, flat-bottom tissue culture plate with low-evaporation lid) containing 50 μl of medium with various concentrations of the chemicals. The covered plate was incubated at 37°C. At various times after inoculation, the plate was shaken and the optical densities at 650 nm of the cultures in the wells of uncovered microplates were measured with a Molecular Devices 96 well plate reader. The background optical density at 650 nm from wells containing 100 μl of medium only was subtracted from all readings prior to plotting and calculation of growth rates.

Gene fusion library generation. Chromosomal DNA isolated from *E. coli* W3110 was partially digested with *Sau*3A1 and size fractionated by agarose gel electrophoresis. A fraction with an average size of approximately 1.8 kb was ligated to pDEW201 that had previously been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase. The ligation products were used to transform ultracompetent *E. coli* XL2Blue cells (Stratagene) to ampicillin resistance, using the protocol provided by Stratagene. Preliminary characterization of individual random XL2Blue transformants indicated that all (16 of 16) contained insert DNA with sizes ranging from 0.9 to 3.0 kb. Approximately 24,000 of these transformants were pooled and used as a source of heterogeneous plasmid DNA isolated by using Qiagen tip20 columns. This plasmid DNA pool was used to transform (38) *E. coli* DPD1675, selecting for ampicillin resistance and using a 30-min phenotypic expression time to minimize the presence of siblings. Individual transformants were used to inoculate the 96-well sterile Falcon Microtest III tissue culture plates containing 190 μl of the defined medium with 25 μg of ampicillin per ml. These plates were covered and incubated overnight at 37°C.

Bioluminescence analysis. The overnight cultures in 96-well plates were used for both permanent cryogenic storage (33) and dilution and regrowth to exponential phase in the defined medium containing 10 μg of ampicillin per ml. A 15-μl aliquot of the overnight culture was added to 150 μl of prewarmed medium in microplates and incubated at 37°C without shaking for 3 h. In the primary screen, these actively growing cultures were divided into SM-treated and untreated wells of sterile white microplates (Microlite; Dynex). Addition of 50 μl of the culture to 50 μl of fresh prewarmed medium lacking ampicillin but containing 4 μg of SM per ml yielded a final SM concentration of 2 μg/ml. For each culture, the untreated control was in the same microplate. Light production was measured in a Dynatech (now Dynex) ML3000 luminometer at 0, 90, and 180 min of incubation at 37°C after addition of cells to chemical. The dimensionless units of light production, relative light units (RLU), are obtained by comparison with the light reading from an internal light-emitting diode. The levels of light production of the SM-treated and untreated wells were compared for each culture. A ranged set of criteria was used to identify putative SM-inducible fusions. These criteria, which considered the increase in expression as calculated by both a difference in light production (ΔRLU = RLU [SM treated] − RLU [control]) and the ratio of light production (ratio = RLU [SM treated]/RLU [control]), were as follows: for ΔRLU between 0.02 and 0.1, the ratio was required to be ≥1.5; for ΔRLU between 0.1 and 1.0, the ratio was required to be ≥1.35; for ΔRLU between 1.0 and 10.0, the ratio was required to be ≥1.25; and for ΔRLU of >10.0, the ratio was required to be ≥1.20. Due to variabilities inherent in growing cells in microplates and the narrow range of SM resulting in induction of bioluminescence, it was likely that the number of SM-inducible genetic fusions identified represents an underestimate of the actual proportion of *smi* promoters in the *E. coli* chromosome.

Putative SM-inducible transformants were reisolated from the appropriate wells of the duplicate cultures stored at −80°C and retested in triplicate under the same conditions as above except that data were semicontinuously collected by using the cycle mode of the ML3000 luminometer, similar to previous descriptions (57). Those that showed SM-induced bioluminescence increases in the secondary screen were grown to exponential phase in a flask and then tested a third time at a variety of SM concentrations, using the cycle mode of the ML3000 luminometer. For all experiments, the actively growing culture was divided at the time of SM addition to ensure identical populations when the stress was imposed. Response ratios were calculated by dividing the RLU of the SM-treated culture by the RLU of the untreated control culture at each time point. Because SM reduced the growth rate, the increase of bioluminescence was an underestimate of the fold increase in light production per cell, as there were fewer cells in the SM-treated cultures than the untreated cultures at the end of the experiments.

The effects of other chemicals on bioluminescence were tested by using cultures that were grown to exponential phase at 37°C and divided at the time of chemical addition. Bioluminescence was quantitated in the cycle mode by using the ML3000 luminometer, and response ratios were calculated.

Plasmid isolation and insert analysis. Plasmid DNA was isolated by using Qiagen tip100 columns and the protocol provided by the manufacturer. The size of the insert DNA of individual clones was estimated by digesting with *Eco*RI and *Sac*I, followed by agarose gel electrophoresis and comparison with markers. DNA sequence data were obtained by using ABI Prism dye terminator cycle sequencing kits with AmpliTaq DNA polymerase and oligonucleotide primers specific for the regions of pDEW201 flanking the multiple cloning site in the transcription terminator region (5′-GGATCGGAATTCCCGGGGAT-3′) and in the *luxC* region (5′-CTGGCCGTTAATAATGAATG-3′). The sequence reactions were run on ABI 373A and 377 sequencers. DNA homologies with the entire *E. coli* genome sequence (9) were determined with the program BLAST (1) on the NCBI database. The ECDC database (20, 61) was used to determine the genetic map positions of the genes fused to *lux.*

RESULTS

Identification of *smi* promoters. The ideal *E. coli* strain for these studies contains both *ilvB* and *tolC* mutations. An *ilvB* mutation eliminates the SM-resistant ALS isozyme I (25), and a *tolC* mutation results in lack of an outer membrane channel for efflux pumps (15, 19), making the cells sensitive to growth inhibition at reduced chemical concentrations (46). The growth of strain DPD1675, which contains both of these key mutations, was inhibited by addition of 1, 2, 4, or 8 μg of SM per ml. The treated cultures maintained exponential growth but at decreasing rates with increasing doses of SM (data not shown). For subsequent screening, 2 μg/ml (5.5 μM), which resulted in 25% growth rate inhibition, was used. This SM concentration also resulted in a partial decrease in the bioluminescence from strain DPD2077, which carries a plasmid with the *E. coli* heat shock promoter, *grpE*, driving *P. luminescens luxCDABE* (data not shown).

A screening protocol was used to identify isolates of strain DPD1675 containing rare *E. coli* promoter-*luxCDABE* fusions that, in contrast to the *grpE-luxCDABE* fusion and about 99% of the fusions in the library, yielded an increase in bioluminescence upon treatment with the sublethal dose of SM. Individual transformants of *E. coli* DPD1675 containing fusions of random *E. coli* chromosomal DNA to the *P. luminescens luxCDABE* were challenged with SM at 2 μg/ml while actively growing in microplates. Of 8,066 individual cultures screened, the bioluminescence from 19 strains was reproducibly SM inducible. Thus, the chromosomal DNA upstream of the *luxCDABE* reporter in these 19 fusion plasmids was presumed to contain an *smi* promoter.

The identity of the *E. coli* chromosomal DNA in each of the 19 plasmids containing *smi* promoters was determined by DNA sequencing of each end of the inserted DNA followed by comparison to the complete *E. coli* genome sequence (9). Of these 19 plasmids, 3 contained regions of DNA from differing distal portions of the *E. coli* chromosome, most likely due to insertion of two or more independent *Sau*3A1 fragments into one plasmid. These were not further considered. Of the remaining 16, there were 12 unique chromosomal regions represented. Figure 2 shows the structures of these regions and their fusion point to the *lux* operon. In 11 of the cases, the *lux* operon was inserted within the coding sequences of genes or open reading frames (ORFs). In each of these cases, the direction of transcription of that gene or ORF was the same as that of the *lux* operon. Our assumption was that the promoter that drives the expression of the gene into which *lux* is inserted also controls *lux* operon expression. One exception was found in plasmid pDEW220, where the *lux* operon was inserted in an intergenic

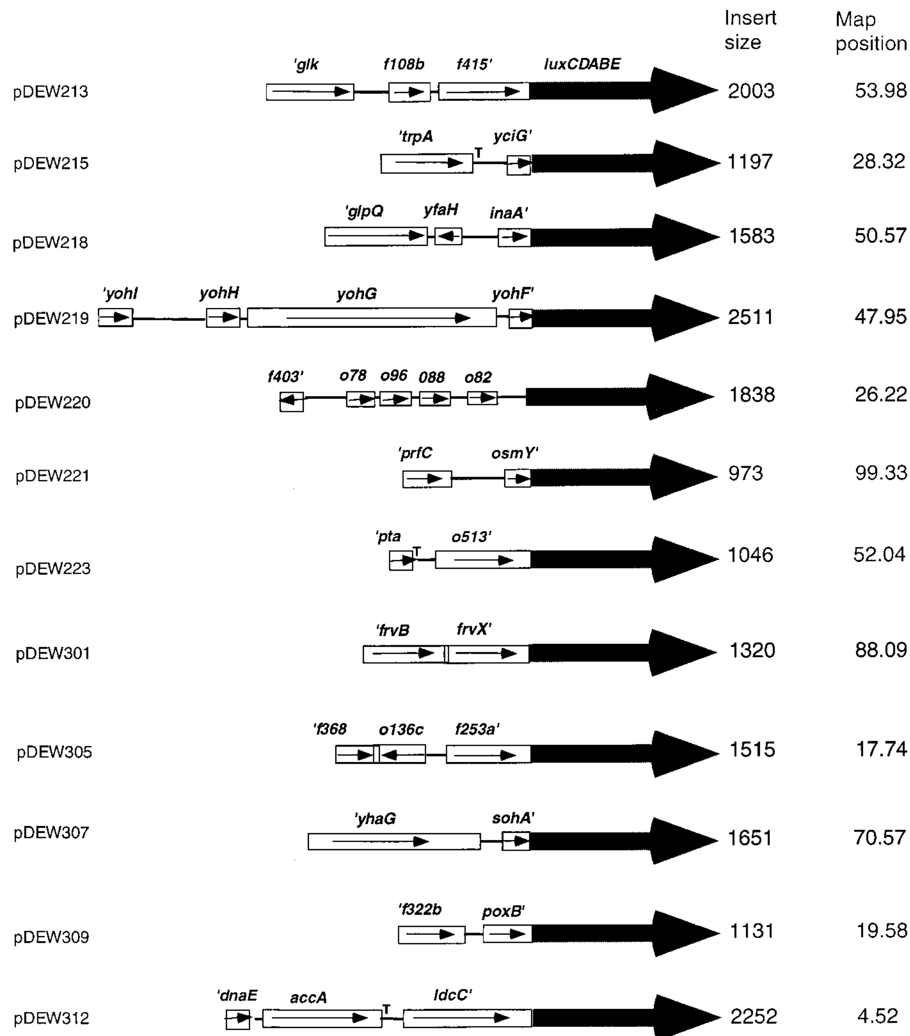

FIG. 2. Structure of *smi-luxCDABE* gene fusions (drawn approximately to scale). Known or proposed terminators (T) are shown. The designations for ORFs are from ECDC, release 28 (20, 61), as are the map positions in minutes for the gene proximal to *luxC*.

space. The direction of transcription of the nearest upstream ORF, *o82*, and the nearest downstream ORF, *o521*, was the same as for the *lux* operon. Since 201 of the 290 bp of the intergenic space separating *o82* and *o521* were present in this plasmid, it was not clear whether the promoter that drives *o82* expression or that driving *o521* expression was responsible for the *lux* operon expression.

Characterization of 12 *smi* promoter-*lux* fusions. Table 1 summarizes the basal, uninduced bioluminescence and the SM-induced response ratio of strain DPD1675 containing each of the 12 unique *smi* fusions. Also shown in Table 1 is the number of times each chromosomal segment was found in these screens. Saturation of the genome was clearly not reached because most *smi* fusions were found only once. Although this survey was not exhaustive, the genes found should be representative of the types of genes that are induced by SM-mediated inhibition of ALS. The basal level of light production from strain DPD1675 containing each fusion was substantially greater than that of strain DPD1675 containing plasmid pDEW201. This was consistent with the presence of promoter sequences in each of the DNA inserts. Furthermore, the range of promoter strengths among these 12 *smi* promoters was large; the uninduced bioluminescent activities differed by a factor of more than 500. Upon treatment with the sublethal SM dose of 2 μg/ml, the induction responses observed were modest, ranging from 20% increases to threefold increases.

The time course of bioluminescence induction of one such *smi* fusion is shown in Fig. 3. These kinetics represent a typical response in that there was lag time with little change in bioluminescence, followed by an increase in bioluminescence relative to the control untreated sample. The lag time presumably represents the time required for the stress response to be initiated and for transcription and translation of the *luxCDABE* reporter complex.

Dose-response curves for SM effects on bioluminescence revealed that a narrow range of SM concentrations yielded an induction response. Figure 4 shows a representative example of the strain containing pDEW213. At SM concentrations that resulted in growth rate inhibition of 50% or more, the light production was less than that of the parallel, untreated culture. This "lights-off" response at higher SM concentrations was likely due to an insufficiency of isoleucine and valine that limited formation of the Lux proteins. As expected, the SM effects of increased bioluminescence at lower concentrations and the lights-off response at higher concentrations upon each of the 12 *smi* fusions were overcome by isoleucine and valine

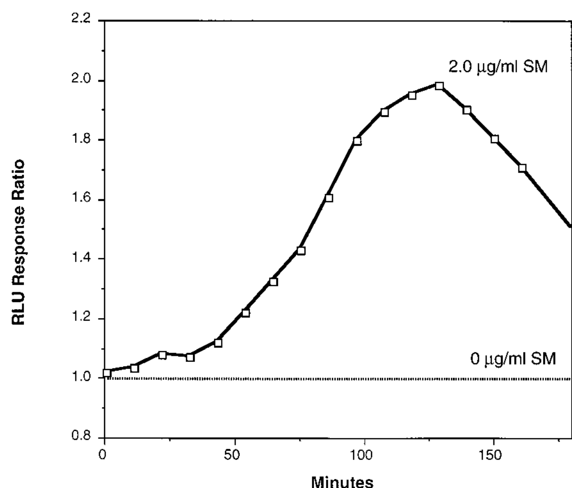

FIG. 3. Kinetics of bioluminescent induction by SM treatment of *E. coli* DPD2088 containing plasmid pDEW219. The actively growing culture in defined medium was treated with 2 μg of SM per ml at time zero. The response ratio is the RLU of the SM-treated culture divided by the RLU of the untreated culture at each time point. The average of duplicates for both treated and untreated cultures was plotted.

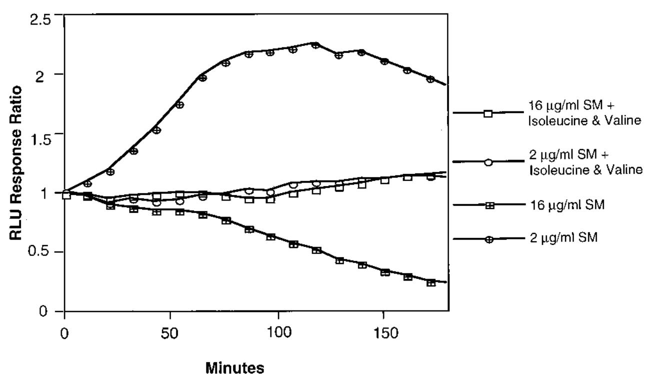

FIG. 5. Prevention of toxic and inducing effects of SM by addition of isoleucine and valine in strain DPD3505 containing plasmid pDEW305. A culture was grown to exponential phase in defined medium without isoleucine or valine. Addition of these two amino acids (each at 42 μg/ml) was made at the same time as addition of SM to the divided culture. The response ratios were calculated at each time point by dividing the RLU from the SM-treated culture without isoleucine and valine by the RLU from the otherwise identical culture lacking SM. Likewise, the response ratio for the SM-treated culture with isoleucine and valine was calculated from the control with isoleucine and valine addition but lacking SM. The average of duplicates was used for each data point.

supplementation. An example is shown in Fig. 5. Light production in the presence of 16 μg of SM per ml from the strain containing pDEW305 was less than that from the untreated control but was restored by isoleucine and valine addition. Likewise, the induction of bioluminescence by SM at 2 μg/ml observed in the absence of isoleucine and valine was prevented by their addition. Thus, adequate SM needed to be added for a response to be induced, but higher concentrations precluded a bioluminescent report of the response.

Specificity of *smi* promoter induction. To test if the induction of any of these promoters was due simply to partial growth rate inhibition, the responses to an unrelated growth inhibitory chemical were tested. Cultures growing in defined medium in microplates were stressed by ethanol additions at the sublethal concentrations of 2 and 4% (44). These concentrations of ethanol reduced the growth rate of strain DPD1675 in microplates by 8 and 12%, respectively. The bioluminescence from strain DPD2077 with the *E. coli* heat shock *grpE* promoter driving *P. luminescens luxCDABE* was increased 1.3-fold at 50 min after addition of either 2 or 4% ethanol. These concentrations of ethanol, however, did not induce increased bioluminescence in 11 of the 12 distinct *smi* fusions. The one exception was strain DPD2081 containing plasmid pDEW213. While the addition of 4% ethanol did not result in bioluminescence of DPD2081 greater than that of the untreated control, the bioluminescence was increased 1.4-fold at 60 min after addition of 2% ethanol. The bioluminescence of DPD2081 was also noted to be induced by a wide variety of growth-inhibiting chemicals (data not shown). Thus, activation of the promoter driving expression of the *f415′-luxCDABE* fusion in DPD2081 may be tied to growth rate reduction. SM induction of the other 11 *smi* promoters, however, was not simply due to reduction in growth rate.

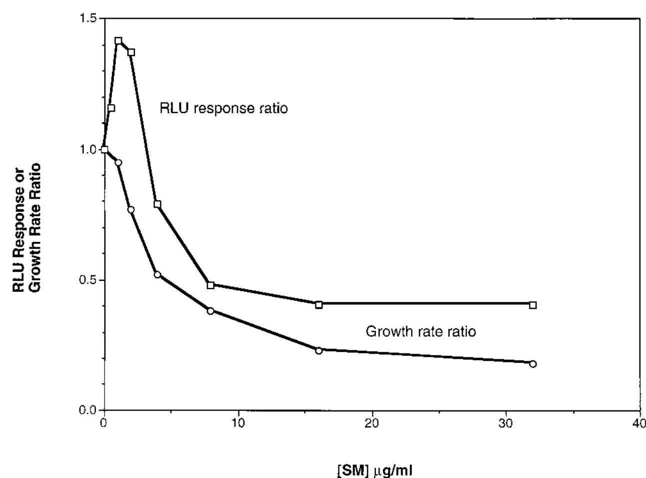

FIG. 4. Dose response to SM of strain DPD2081 containing plasmid pDEW213. The bioluminescence response ratio was calculated by dividing the RLU of each SM-treated culture by the RLU of the untreated culture at 165 min after SM addition, using the average of duplicate cultures. The growth rate of DPD2081 in defined medium growing at 37°C in microplates was determined from the slope of the exponential curve fit of optical density versus time. The growth rate reduction ratio was calculated by dividing the growth rate of each SM-treated culture by the growth rate of the control untreated culture.

Promoters regulated by σ^S. What are the physiological consequences of partial ALS inhibition? Two possibilities were suggested by the identified *smi* promoter-*luxCDABE* fusions. One was that treatment with SM may result in induction of the σ^S-dependent stress response. This regulatory circuit is induced by numerous stresses, including entry into stationary phase. At least two genes, *poxB* (10) in pDEW309 and *osmY* (21, 63) in pDEW221, regulated by σ^S were among the 12 *smi* fusions. Furthermore, the bioluminescence of several of the *smi* fusion strains, including the *poxB* and *osmY* fusions, appeared to increase dramatically as the culture in the microplates approached stationary phase (data not shown), suggesting that others among the *smi* fusions may be controlled by σ^S. This was tested by placing each of the 12 plasmids in a pair of *E. coli* strains, containing either an $rpoS^+$ or nonfunctional *rpoS* allele but otherwise isogenic. As shown in Table 2, five plasmids (pDEW213, pDEW218, pDEW223, pDEW301, and pDEW307) expressed bioluminescence which was not substantially altered by the two *rpoS* alleles. In contrast, the basal bioluminescence expressed from another group of six plasmids was dramatically (11- to 188-fold) depressed in the *rpoS* mutant. Such a result was expected for loss of an element required

TABLE 2. Effect of *rpoS* mutation on *smi* promoter-*luxCDABE* fusions[a]

Plasmid	Gene fused to *luxCDABE*	RLU in[b]:		*rpoS* dependence ratio[c]
		E. coli MP180 (*rpoS*$^+$)	*E. coli* UM122 (*rpoS*)	
pDEW213	*f415*	0.0033 ± 0.0014	0.0079 ± 0.0070	0.42
pDEW215	*yciG*	1.11 ± 0.09	0.0059 ± 0.0020	188
pDEW218	*inaA*	2.44 ± 0.43	2.60 ± 0.55	0.94
pDEW219	*yohF*	0.455 ± 0.040	0.00888 ± 0.00114	51
pDEW220	*o82/0521*	3.82 ± 0.86	0.642 ± 0.040	6.0
pDEW221	*osmY*	0.362 ± 0.073	0.0081 ± 0.0018	45
pDEW223	*o513*	7.09 ± 1.77	9.61 ± 0.74	0.74
pDEW301	*frvX*	0.161 ± 0.034	0.146 ± 0.013	1.1
pDEW305	*f253a*	0.947 ± 0.077	0.0187 ± 0.005	51
pDEW307	*sohA*	30.0 ± 3.0	24.1 ± 2.5	1.2
pDEW309	*poxB*	2.38 ± 0.30	0.0241 ± 0.0121	99
pDEW312	*ldcC*	1.69 ± 0.20	0.148 ± 0.014	11

[a] Visual inspection in a dark room of the bioluminescence of hundreds of transformants on petri plates confirmed the quantitative results presented. Either there was a dramatic loss of bioluminescence in the *rpoS* host or there was no visually discernible difference between two host strains.

[b] Cultures of two transformants were grown in LB medium containing 150 μg of ampicillin per ml overnight at 37°C, diluted into LB medium containing 50 μg of ampicillin per ml, and grown to log phase (20 to 24 Klett units) at 37°C. The bioluminescence from two 100-μl aliquots of each of the duplicate cultures was measured. Values are means and standard deviations of the four measurements.

[c] Calculated as follows: RLU [transformants of MP180 (*rpoS*$^+$)]/RLU [transformants of UM122 (*rpoS*)].

for transcription. Thus, these results suggested a strong σ^S dependence of the promoters driving transcription of the *luxCDABE* fusions to *poxB* (pDEW309), *osmY* (pDEW221), *yciG* (pDEW215), *yohF* (pDEW219), *f253a* (pDEW305), and *ldcC* (pDEW312). For one plasmid, pDEW220, there was an intermediate (sixfold) reduction attributed to the *rpoS* mutation. This plasmid may have more than one promoter in the cloned region. Thus, of 12 *smi* fusions, the expression of 6 was clearly controlled by σ^S.

Weak acid-inducible promoters. Another possible stress sustained by the cell when ALS is partially inhibited is cytoplasmic acidification. Included in the set of *smi* fusions was *inaA*, a known acid-inducible gene (49, 62), and *ldcC*, encoding a lysine decarboxylase (18). The function of *inaA* is not known (43). In contrast, the activity of lysine decarboxylase in converting lysine to cadaverine, an alkaline molecule, could neutralize acids. A prediction of the SM-mediated acidification hypothesis was that other promoters responsive to cytoplasmic acidification may be among the set of *smi* fusions. This was explored by testing for induction by salicylate, a membrane-permeant weak acid that results in cytoplasmic acidification and potent induction of *inaA* expression (49). As expected, the bioluminescence from the strain containing the *inaAluxCDABE* fusion was induced 17-fold after treatment with 5 mM sodium salicylate and 21-fold after treatment with 10 mM sodium salicylate (Table 3). There was also a strong 13-fold induction of bioluminescence upon treatment with 10 mM sodium salicylate of the strain containing the *poxB-luxCDABE* fusion (Table 3). In addition, moderate (two- to fivefold) increases in bioluminescence were induced by 10 mM salicylate for the strains containing the *yciG*, *yohF*, *osmY*, *f253a*, and *ldcC* fusions (Table 3). Yet addition of salicylate can be ruled out as having a general enhancing effect on bioluminescence because there were several fusions that were not induced by salicylate addition (Table 3). The genes represented by these fusions, *o513*, *frvX*, and *sohA*, are probably not involved in a response to internal acidification.

The salicylate-mediated induction of *inaA* is known to be partially regulated by the multiple antibiotic resistance (*mar*) stress response system (43) through binding of salicylate to the repressor of the *mar* operon, MarR (31). As in published results (43), placement of pDEW218 containing the *inaA-luxCDABE* fusion into a Δ*mar* strain greatly decreased (to 2.4-fold) but did not eliminate induction of bioluminescence upon addition of 5 mM salicylate. However, placement of this plasmid in a strain lacking both *mar* and *rob*, which encodes a DNA binding protein (48) that can also activate transcription of *inaA* (3), had a more substantial effect on bioluminescence induction. There was no increase upon addition of 10 mM salicylate (Table 3), and the induction by 5 mM salicylate was reduced to 1.2-fold. Similar results on the effects of a strain carrying both *mar* and *rob* mutations have also been obtained for a chromosomal *inaA-lacZ* transcriptional fusion (42). Interestingly, the effect of the double-mutant host strain differed for the other *smi* fusions. The salicylate-mediated induction of the group of moderately induced *smi* fusions remained in the two- to five-fold range in the *mar rob* double mutant (Table 3). In contrast to both *inaA-luxCDABE* and the moderately induced fusions, the salicylate-mediated induction of the *poxB-luxCDABE* fusion was substantially decreased but not eliminated in the double mutant (Table 3). The residual degree of salicylate induction of this fusion was similar to that of the moderately induced fusions, all of which were controlled by σ^S. In a triple mutant host strain lacking function of *mar*, *rob*, and *rpoS*, the salicylate induction of *poxB-luxCDABE* was eliminated; the response ratio to 10 mM salicylate was 0.45, and that to 5 mM salicylate was 0.65.

DISCUSSION

Specific chemical inhibitors of metabolic enzymes provide a useful mechanism for flux alteration, allowing analysis of actively growing cultures abruptly stressed by constricted flux at a precise point. We used sublethal doses of SM and found unexpected changes in gene expression induced by partial in-

TABLE 3. Effects of 10 mM salicylate on bioluminescence induction responses in *mar*$^+$ *rob*$^+$ and Δ*mar rob* hosts

Plasmid	Gene fused to *luxCDABE*	Salicylate response ratio[a] in:	
		GC4468 (*mar*$^+$ *rob*$^+$)	N8452 (Δ*mar rob*)
pDEW213	*f415*	ND[b]	ND
pDEW215	*yciG*	3.4	5.3
pDEW218	*inaA*	20.6	0.92
pDEW219	*yohF*	5.4	5.2
pDEW220	*o82/o521*	1.8	4.0
pDEW221	*osmY*	2.7	3.9
pDEW223	*o513*	0.05	0.21
pDEW301	*frvX*	0.62	0.45
pDEW305	*f253a*	2.6	1.8
pDEW307	*sohA*	1.1	1.0
pDEW309	*poxB*	12.8	4.3
pDEW312	*ldcC*	3.8	3.2

[a] The cultures were grown to log phase (30 to 42 Klett units) in LB medium containing 50 μg of ampicillin per ml. The culture was then split, and 50 μl was added to 50 μl of LB medium containing or lacking 20 mM sodium salicylate, yielding a final concentration of 10 or 0 mM. The response ratio, which was the RLU of the salicylate-treated culture divided by the RLU of the untreated culture, was calculated from the mean of duplicate cultures at 30 min after combining the cells and salicylate.

[b] ND, not determined. The bioluminescence from *E. coli* GC4468 or N8452 containing pDEW213 was very low and was reduced below the detection limit by addition of 10 mM salicylate.

hibition of ALS, the first common step of branched-chain amino acid biosynthesis. Promoters associated with the amino acid starvation response were not found in this survey, which likely indicates that the level of starvation for isoleucine and valine was not severe when SM inhibited the growth rate by 25%. Likewise, this level of ALS inhibition did not induce the heat shock-controlled *grpE* promoter, indicating that the level of amino acid limitation was not severe enough to cause substantial amounts of nonnative proteins to accumulate. Thus, the promoters activated by this flux constriction represent responses to other, perhaps more subtle, physiological perturbations. That these perturbations were not severe may be reflected in the relatively modest degree of inductions observed. Yet these were clearly due to SM-mediated inhibition of branched-chain amino acid biosynthesis rather than an unknown effect of SM because the presence of isoleucine and valine prevented SM-mediated induction of all the identified *smi-lux* fusions.

An interesting and coherent picture emerged from the pattern of promoters found to be activated by partial ALS inhibition. The majority of the *smi* promoters were controlled by σ^S and also induced by weak acid treatment. The latter observation suggested the possibility that inhibition of ALS in *E. coli* results in cytoplasmic acidification. Although such acidification may not have been previously considered as an immediate consequence of partial ALS inhibition, it is plausible because of the considerable flux through the branched-chain amino acid biosynthetic pathway. For example, a maximal synthesis rate of α-ketobutyrate in *S. typhimurium* is estimated at 6 nmol/min/10^9 cells from the rates of its accumulation and degradation (27). Furthermore, in accordance with inhibition of ALS leading to acidification, overexpression of ALS in *E. coli* has been used to direct metabolism away from production of acidic by-products (2). Induction of acid-responsive gene products may allow the cell to combat this acidification stress by neutralization or other strategies.

The majority of the acid-responsive *smi* promoters found were members of the σ^S regulon. This included the known σ^S regulon genes, *osmY* and *poxB*, and several newly identified members of the σ^S regulon, *f253a*, *ldcC*, *yciG*, and *yohF*. The sublethal SM treatment may have resulted in increased cellular levels of ppGpp, a positive effector of σ^S levels (16, 22). Alternatively, acidification stress may be the trigger that initiates the σ^S-dependent stress response. A connection between σ^S and acid stress responses has been characterized in *E. coli* and *S. typhimurium* (6). Weak acid treatment, which results in cytoplasmic acidification, induces expression of *rpoS* in *E. coli* (45). In *S. typhimurium*, *rpoS* mutants do not have the acid-inducible resistance to weak acids characteristic of the wild type (5). It has also been shown that σ^S is acid inducible and controls expression of at least eight other acid-inducible proteins (29). These σ^S-dependent acid-inducible proteins include the *S. typhimurium* homolog of OsmY (6), which was found in this study to have an *smi* promoter. Furthermore, another *S. typhimurium* σ^S-dependent acid-inducible protein is encoded by gene *orf3* of the *tonB-trpA* region or *yciE* (6) that is nearby and possibly cotranscribed with *yciG* (51), another of the *E. coli smi* promoters. The acid induction of five proteins is negatively regulated by *mviA* in *S. typhimurium* (7). The product of *rssB* (or *sprE*), the *E. coli mviA* homolog, plays a similar role in regulating RpoS stability (36, 40). Whether cytoplasmic acidification is one signal that the cell uses to induce the σ^S-dependent stress response generally or whether there are specific σ^S-regulated genes that also respond to acidification remains to be clarified.

The global transcriptional regulator MarA controls responses to some weak acids such as salicylate (35). Here we showed Mar regulation of salicylate induction of two *smi* promoters: *inaA*, a known Mar regulon gene, and *poxB*, not previously known to be a member of the Mar regulon. These two promoters differed in that *inaA* was unaffected by an *rpoS* mutation, while *poxB* was strongly affected by loss of *rpoS* function. Our results are consistent with the salicylate induction of *poxB* expression being under dual regulation by *mar* and *rpoS*. We have also found that expression of the *poxB-luxCDABE* fusion is induced by methyl viologen treatment under the control of *soxRS* (4). Furthermore, expression of *poxB* is affected by mutations in the regulatory genes, *lrp* and *hns* (28).

The multiple regulation of *poxB* suggests a key cellular role for its product, pyruvate oxidase. However, the phenotypic consequences of *poxB* mutations are difficult to ascertain (10). Likewise, pyruvate oxidase induction by SM treatment and the reaction that it catalyzes, the conversion of pyruvate to acetate and CO_2, suggest a role for it in response to ALS inhibition. However, a *poxB* mutant of *E. coli* is not altered in sensitivity to SM (59). Either the induction of pyruvate oxidase plays a minor adaptive role in SM stress or this induction may not be a specific defense response. Possibly the cell responds to certain stresses by inducing a gene expression pattern similar to that found in the stationary phase. Notably, the degree of growth rate reduction by SM was much less severe than that found to be necessary in chemostats for induction of σ^S-controlled genes (39). The induction of the σ^S-mediated stress response may reflect an alternative survival strategy to that afforded from induction of proteins specific for combating the adverse effects of a stress.

The use of an easily assayed transcriptional reporter was critical for our random screening using cells growing in liquid medium. Thus, the five-gene *luxCDABE* operon that does not require breaking cells or substrate addition was chosen. The sensitivity of the *lux* reporter was important in the identification the modestly activated promoters described here. Such threefold or less SM-induced increases in reporter gene activity may not have been readily uncovered in assays using standard reporters with petri plate-based methods or by newer approaches based on cell sorting (53). Furthermore, the advantage of the large dynamic range of the *luxCDABE* reporter was demonstrated by the identification of promoters of widely disparate strengths under identical screening conditions.

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