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Interfering with Different Steps of Protein Synthesis Explored by Transcriptional Profiling of *Escherichia coli* K-12†

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Escherichia coli **responses to four inhibitors that interfere with translation were monitored at the transcriptional level. A DNA microarray method provided a comprehensive view of changes in mRNA levels after exposure to these agents. Real-time reverse transcriptase PCR analysis served to verify observations made with microarrays, and a chromosomal *grpE*::*lux* operon fusion was employed to specifically monitor the heat shock response. 4-Azaleucine, a competitive inhibitor of leucyl-tRNA synthetase, surprisingly triggered the heat shock response. Administration of mupirocin, an inhibitor of isoleucyl-tRNA synthetase activity, resulted in changes reminiscent of the stringent response. Treatment with kasugamycin and puromycin (targeting ribosomal subunit association as well as its peptidyl-transferase activity) caused accumulation of mRNAs from ribosomal protein operons. Abundant biosynthetic transcripts were often significantly diminished after treatment with any of these agents. Exposure of a *relA* strain to mupirocin resulted in accumulation of ribosomal protein operon transcripts. However, the *relA* strain's response to the other inhibitors was quite similar to that of the wild-type strain.**

Protein synthesis is a multistep process (Fig. 1). Free amino acids are selected with high specificity by aminoacyl-tRNA synthetases and charged to tRNA. The resulting aminoacyl-tRNAs are essential components in the initiation and elongation steps of protein synthesis that take place on the ribosome. In *Escherichia coli* these processes are integrated through the stringent response (9). When uncharged tRNA occupies the ribosomal A site, the alarmones ppGpp and pppGpp accumulate due to the action of the *relA* gene product, the stringent factor. These alarmones encourage transcription of amino acid biosynthetic genes (41) and discourage initiation at the strong promoters that drive formation of the translational apparatus (3, 4).

Over the years, many herbicides and antibiotics have been developed that target these processes (reviewed in references 11, 19, and 20). Rhodius et al. have recently reviewed evidence that DNA microarray-mediated gene expression profiling sheds light on the consequences of administration of inhibitors to *E. coli* (35). Cellular responses to both the glutamine mimic acivicin (39) and the DNA-damaging agent mitomycin C (53) have been investigated. Such success encouraged us to consider the consequences of inhibiting protein biosynthesis at a variety of steps (Fig. 1).

Inhibitors exist that interfere with the synthesis of branched-chain amino acids and their utilization in translation. Sulfometuron methyl (SM), a slow tight-binding inhibitor of the branched-chain amino acid biosynthetic enzyme acetolactate synthase (21, 38), has been extensively studied from many perspectives. These include an *E. coli* gene expression-profiling study with a set of gene fusions (47) estimated to be about 30% complete (48). SM administration results in starvation for the branched-chain amino acids and pantothenate as well as 2-ketoacid imbalances (22, 24, 25). Moreover, the global transcriptional response to this inhibitor is characterized by a strong *rpoS* regulon (14) signature, as if SM triggers a premature conversion from exponential- to stationary-phase growth (47).

Two inhibitors of branched-chain aminoacyl-tRNA formation have been studied in some detail. One, 4-azaleucine (AZL), is a competitive inhibitor of leucine binding to *E. coli* leucyl-tRNA synthetase (LeuRS) that does not progress to the azaleucyl-adenylate in vitro (40). AZL differs from leucine by having a tertiary, titratable N at the branch point of the R group (42). *E. coli* mutations conferring resistance to AZL have been identified in a variety of genes, including genes encoding amino acid transport and LeuRS (19). The second inhibitor, mupirocin (MUP), a mimic of isoleucyl-adenylate also known as pseudomonic acid (15), is utilized as a topical antibiotic. Resistance to MUP is caused by alterations in IleRS structure (7, 57). Thus, it is expected that SM treatment causes a paucity of branched-chain amino acids and 2-ketoacid imbalance and that AZL treatment saturates LeuRS with an amino acid analog that cannot condense with ATP. In addition it is speculated that MUP binding to IleRS yields an enzyme with an activated amino acid analog incapable of being condensed with tRNA. That is, MUP treatment mimics what happens when a supply of uncharged tRNA is inaccessible.

Kasugamycin (KAS) inhibits initiation of polypeptide synthesis (32). Resistance mutations map to several loci in *E. coli*

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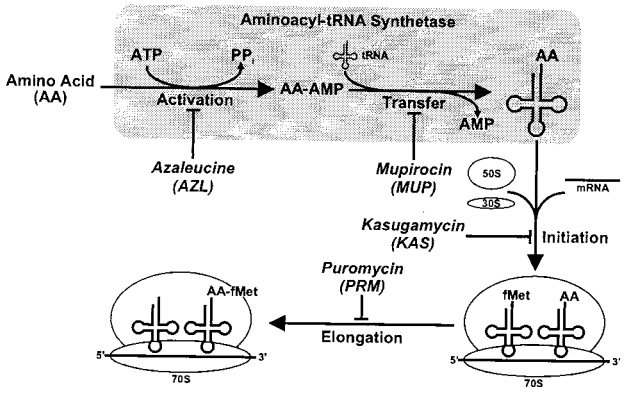

FIG. 1. Scheme of protein synthesis showing the sites of action of the inhibitors used. The indicated sites of action were deduced from references cited in the introduction to this article. Inhibitor abbreviations are indicated.

that encode either structural components or modifying activities of the ribosome (19). Thus, we expect that branched-chain and other aminoacyl-tRNAs accumulate upon KAS treatment since aminoacyl-tRNA synthesis is unrestrained while the major route of aminoacyl-tRNA consumption is blocked. Puromycin (PRM) is an analog of aminoacyl-tRNA that binds to the acceptor site of the ribosome, blocking elongation and causing premature release of the growing polypeptide chain (11). Thus, PRM elevates the titer of unfolded proteins in the cytoplasm, triggering the heat shock response (13).

Here we investigate the action of AZL, MUP, KAS, and PRM through the use of DNA microarray-mediated gene expression profiling of stringent and relaxed derivatives of *E. coli* K-12 and compare this work to recent studies of antibiotic treatment of the pathogen *Streptococcus pneumoniae* (31) and of *E. coli* growth transitions (10).

MATERIALS AND METHODS

Chemicals. Kanamycin, PRM, and 4-aza-D,L-leucine were purchased from Sigma (St. Louis, Mo.). KAS was obtained from Calbiochem (San Diego, Calif.). MUP was a gift from Smith Kline Beecham Pharmaceuticals (West Sussex, United Kingdom).

Strains, growth conditions, and materials. The *E. coli* K-12 derivatives (obtained from M. Cashel [National Institutes of Health]) used were CF1943 (W3110) and CF1944 (W3110 Δ*relA251*::*kan*) (56). Strains used in other microarray experiments were the near-wild-type strain MG1655 (2), the *tolC* mutant DE 112 (49), and the *ilvB* mutant DPD1675 (47). Prototrophic *E. coli* strain DPD3084 harbors a *grpE*::*luxCDABE* fusion at the chromosomal *lac* locus (51).

Aerobic growth was carried out at 37°C in M9 minimal medium (28) supplemented with 0.4% glucose and treated with shaking (250 rpm) unless otherwise specified. The concentration of each inhibitor of translation used to treat cultures prior to RNA isolation was determined by dilution of overnight cultures into fresh M9 glucose medium. At an A_{600} of 0.2, individual cultures were split before treatment with various concentrations of each inhibitor and growth was monitored for 4 h after the inhibitor addition.

Range finding determined that the concentration of each of the inhibitors AZL, MUP, KAS, and PRM needed to decrease the growth rate (increase the doubling time) by a factor of 5 for the *relA*$^+$ strain CF1943 was 750, 8.5, 750, and 818 μg/ml, respectively, while a concentration of each inhibitor of only 375, 4.3, 750, and 546 μg/ml, respectively, was needed to retard growth of the isogenic *relA* strain CF1944 to the same extent. These inhibitor concentrations were later used to determine gene expression changes after a chronic, 30-min administration of the antibacterial agents.

RNA isolation. Total RNA used in microarray procedures and real-time PCR measurements was extracted (following established methods) from cultures. Overnight cultures of each strain were diluted into fresh M9 glucose medium and incubated at 37°C. Once the cultures had reached an A_{600} of 0.4, they were split into two portions. One portion was treated with the inhibitor at the previously determined concentration, while the other was left unchallenged. The cells were pelleted after 30 min of shaking at 37°C, and the total RNA was immediately extracted using RNeasy minicolumns (Qiagen, Inc., Valencia, Calif.) as previously described (54).

DNA microarray experiments. The procedures used to generate fluorescently labeled cDNA for microarray experiments, as well as those used in hybridization, data acquisition, and analysis, have been extensively described (55) and reviewed (34) previously. A total of 4,290 distinct open reading frames (ORFs) were spotted in duplicate on each glass slide (55).

Measurement of relative transcript levels using real-time PCR. Bulk RNA isolated as described above was used as a template for cDNA production using random primers (Invitrogen, Carlsbad, Calif.) and Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen). These cDNA samples were diluted 20-fold and used as the template for real-time PCRs. Primer pairs specific to several genes of interest (Table 1) were designed using software on the Primer3 website (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (36). The PCR was carried out using QuantiTect SYBR Green PCR Master Mix (Qiagen), and the thermal cycling and real-time monitoring of SYBR green fluorescence were performed on an iCycler (Bio-Rad, Hercules, Calif.) according to the protocol supplied with the master mix. Data were collected in duplicate (using cDNA prepared from two independently isolated bulk RNA samples) for each gene in each data set. The severalfold changes in expression levels were calculated using a ΔCt method described elsewhere (54).

Confirmation of *rel* phenotypes. Upon amino acid starvation, stringent (*relA*$^+$) strains greatly reduce incorporation of precursor into RNA while the bulk incorporation of UTP into macromolecules proceeds at an unaffected pace in a relaxed (*relA*) strain (9). The *rel* phenotype of each strain tested was determined by monitoring the incorporation of [^{14}C]uracil into macromolecules during isoleucine starvation induced by addition of L-valine to the culture medium (27). Overnight cultures were diluted into M9 glucose medium and incubated at 30°C. Upon reaching an A_{600} of 0.4, the culture was split into two portions and [^{14}C]uracil (0.55 mCi/mmol) was added to achieve a final concentration of 20 μg/ml. L-valine was added to 0.2 mg/ml to one sample, while the other remained untreated. At various times before and after the addition of valine, 0.5 ml was taken from each culture and added to 2.5 ml of cold 6% trichloroacetic acid. Samples were incubated on ice for 20 min before being collected on Whatman GF/A filters. Each sample was washed three times with 5% trichloroacetic acid and twice with 70% ethanol and allowed to dry. The extent of incorporation of [^{14}C]uracil into macromolecules was measured by scintillation counting.

Bioluminescence of *grpE*::*lux* operon fusion. A 10-ml culture of strain DPD3084 was incubated with shaking (250 rpm) in a 50-ml Erlenmeyer flask at 37°C in minimal medium E (12) supplemented with 0.4% glucose and thiamine until early exponential-phase growth was achieved. At that point, 50-μl samples of the culture were exposed in microtiter plates to twofold dilution series with inhibitors in the same medium (49). Light production was monitored as a function of time as described previously (49), with updated instrumentation (Labsystems Lunminoskan Ascent). The response ratio and maximal-response ratio for such a time course study have been defined previously (5); the minimal-response ratio is the smallest fraction of light emitted by a treated culture relative to that emitted by an untreated control over the duration of an experiment. Experiments were performed at 37°C because the gene products of the *luxCDABE* operon utilized (47) (derived from *Photorhabdus luminescens* [50]) are thermotolerant.

RESULTS

Expression profile of wild-type *E. coli*. Preliminary characterization determined the inhibitor concentrations used to treat each *E. coli* strain (see Materials and Methods). The stringent and relaxed phenotypes of the wild-type (CF1943) and *relA* (CF1944) strains were verified by monitoring the bulk incorporation of uracil into RNA (data not shown). *E. coli* strain CF1943 was then treated with each of the four protein synthesis inhibitors at the concentrations specified above.

AZL. Expression profiling of transcripts corresponding to the 4,290 *E. coli* ORFs indicated that the response to AZL was quite broad. Expression of 134 genes was elevated >2-fold,

TABLE 1. Primer pairs used in real-time PCR

Gene name	B number	Forward primer (5′-3′)	Reverse primer (5′-3′)
argA	b2818	GCCAAAACACTGGAACTGGT	ATCGACAGGCGAGCAGTAAT
argB	b3959	CGGCAAATAAAACCCTGTTG	CGTCACCGAGAAACAAACCT
aroF	b2601	CATTGAGCCTGCAACAAGAA	CCCGGCGATAATATCTGAAA
cysK	b2414	GGTATTGGCGCTGGTTTTAT	TTGGTGATGCCAATGACTT
dnaK	b0014	TCGTATGCCAATGGTTCAGA	TCCGGGTTAACGTCTTTACG
glnA	b3870	TCCGCTGAACACGTACTGA	TGAAGCGCAAATCAACAAA
ilvC[a]	b3774	TGTACGAAATGAACGTGGTTATC	CACCAAAGCGTAAGAGAACAGAT
ilvC[b]	b3774	CCGCAGTATGAAGGCAAAAT	TCACCATCGCAATCATCAGT
lacZ	b0344	CACCCGAGTGTGATCATCTG	GATACAGCGCGTCGTGATTA
leuS	b0642	AAACCGACACTTTCGACACC	TGTACTGCGGGCAAGTGTAG
livJ	b3460	GAAAGCGAACTCCGTGGATA	AAGCCTTTCAGATCGCCTTT
metE	b3829	GTACATAATCCGGCGGTAGAA	CAGCACGCACTTCATAGACAT
mopA	b4143	AAAGATGGTGTTTCCGTTGC	ATCTGCGCACCCATATTTTC
ompA	b0957	ACGGTGCATACAAAGCTCAG	GTCCAGGTCGTCAGTGATTG
rplV	b3315	CATGCTCGTTCTTCTGCTCA	GCCTGCGACACTTTCTTACC
rpmD	b3302	CTGCGTCGTATTGGTCACAC	TGATCATACCGCGAATAGCA
rpsL	b3342	TGCGTAAAGTATGCCGTGTT	TGACCTTCACCACCGATGTA
sfsA	b0146	GTTAGCGGAGAACGAACAGG	TCAACTCCCGAAGGTGTTTC
soxS	b4062	ACCAGCCGCTTAACATTGAT	CGGAACATTCGTTGCAAGT
trmD	b2607	CCGAAATTGACGAAGAATGG	GCGTCATTGCTGGTAACTCA
trpC	b1262	TATCGATACGCTGCGTGAAG	GGTTTCACCGACGCTTAATG
trxA	b3781	AACTGACCGTTGCAAAACTG	TACCACGGATGCCATATTTC
wrbA	b1004	AAAACTGGCGAGCGTCTTTA	CAGGTGGATGTGATGGTTTG

[a] Used in measures of RNA decay.
[b] Used in microarray verification.

while expression of 33 genes was elevated >4-fold and transcript levels of 16 ORFs were elevated >8-fold in response to the challenge. Examination of the 58 genes encoding transcripts whose levels were elevated >3-fold was revealing (Table 2 and supplementary material [Table 1A; http://trna.chem.yale.edu/supdata/sup001/]). Of the 58 genes, 12 were heat shock genes (*dnaK*, *dnaJ*, *htrA*, *htpG*, *htpX*, *clpB*, *grpE*, *ibpA*, *hslU*, *hslV*, *mopB*, and *mopA*), another 4 were genes induced by other stresses (*suhB*, *mazE*, *soxS*, and *nrdG*), and 12 specified proteins of uncharacterized function. Elevated expression of a limited subset of amino acid biosynthesis genes was noted; it was striking that of the 11 genes in this class whose expression was elevated, 9 were *arg* and 2 were *met*. Similarly, unanticipated elevation of *arg* and *met* transcripts has been noted in a microarray study of the *E. coli* response to the histidine biosynthesis inhibitor acivicin (39).

Down-regulation was also observed. Of the 34 genes whose transcripts were decreased >4-fold (Table 3 and supplementary material [Table 2A; http://trna.chem.yale.edu/supdata/sup001/]), functions cannot yet be assigned to 22. Similarities among members of this group of down-regulated genes were not discerned.

MUP. The response to MUP was quite different from that caused by AZL (for up-regulation, see Table 2 and supplementary material [Table 1B; http://trna.chem.yale.edu/supdata/sup001/]; for down-regulation, see Table 3 and supplementary material [Table 2B; http://trna.chem.yale.edu/supdata/sup001/]). Only 20 mRNA levels were modestly elevated (between 2.5- and 7.5-fold), and 10 of the corresponding genes have no assigned function. It was comforting that *ilv*, *leu*, and *thr* transcription was elevated by MUP treatment, since the *ilvGMEDA*, *thr*, and *leu* operons are thought to be responsive to isoleucyl-tRNA limitation due to the presence of isoleucine codons in each operon's attenuation leader genes (18, 33, 45).

Transcription reduced by MUP treatment was mostly associated with known genes; only 7 of the 64 genes with reduced expression had an unassigned role. Moreover, many of these genes were previously noted to be among the 50 most abundantly expressed ORFs when *E. coli* was cultured in minimal medium to either the exponential (22 of the 64 genes) or transitional (21 of the 64) phase of a typical growth curve (55). Of the 64 genes displaying reduced transcript abundance, 27 were in the list of 73 genes most highly expressed during the exponential or transition phase of growth in minimal medium. Moreover, 33 of these 64 genes showed at least twofold-reduced expression levels after challenge with acivicin, a glutamine analog (39).

KAS. Compared to interference with tRNA aminoacylation, inhibition of translation initiation led to accumulation of more than 200 transcripts (Table 2 and supplementary material [Table 1C; http://trna.chem.yale.edu/supdata/sup001/]). Among those were mRNAs derived from ribosomal protein genes organized into operons and loci encoding transporters for diverse polyvalent molecules such as citrate, taurine, and carnitine. Many of the 150 down-regulated genes (Table 3 and supplementary material [Table 2C; http://trna.chem.yale.edu/supdata/sup001/]) were also familiar. Among them were genes (previously identified to be highly expressed when cells are grown in minimal medium [55]) specifying the glyoxylate bypass (*aceABK*), glycolysis (*ptsHI*, *pgi*, *pgk*, *fba*, *tpiA*, *eno*, and *gapA*), the pentose shunt (*talA* and *tktB*), the TCA cycle (*gltA* and *icdA*), and other central carbon pathways (*zwf*, *pflB*, and *adhE*) as well as biosynthesis (*aroF*, *cysK*, *folE*, *ilvC*, and *metE*). Levels of transcripts normally associated with growth cessation either through membership in the *rpoS* regulon (*cbpA*, *dps*, *hdeA*, *hdeB*, *osmY*, *otsA*, *otsB*, *poxB*, *wrbA*, and *yeaG*) (14) or by empirical study (*rmf*, *gadA*, and *gadB*) (55) were counterintuitively found not to be elevated after the KAS challenge. Tak-

TABLE 2. Transcripts accumulated after inhibitor challenge of a *relA*$^+$ strain[a]

Inhibitor and gene	b no.	Expression ratio
AZL		
ibpA	b3687	260
argA	b2818	39
b1455	b1455	35
argI	b4254	20
mgtA	b4242	14
dnaK	b0014	13
argF	b0273	12
celA	b1738	12
clpB	b2592	12
argC	b3958	11
b0235	b0235	10
htpG	b0473	9.7
argB	b3959	9.2
argG	b3172	9.1
celB	b1737	8.9
sdaA	b1814	8.9
mopA	b4143	7.9
htpX	b1829	7.1
ybdO	b0603.	6.9
dnaJ	b0015	6.7
mopB	b4142	6.7
chaA	b1216	6.2
pstS	b3728	6.0
grpE	b2614	5.9
ycjW	b1320	5.6
hisJ	b2309	5.2
htrA	b0161	4.4
ybdQ	b0607	4.4
dadX	b1190	4.3
dadA	b1189	4.2
MUP		
ycjF	b1322	7.6
b1976	b1976	6.2
cpsG	b2048	6.1
leuC	b0072	5.9
b4103	b4103	4.5
yagU	b0287	4.3
yaiM	b0355	4.3
ilvG	b3767	3.8
b1433	b1433	3.6
mukF	b0922	3.4
rnc	b2567	3.4
yagR	b0284	3.1
thrA	b0002	3.0
hemN	b3867	2.9
ycjI	b1326	2.9
b1762	b1762	2.8
molR	b2115	2.8
xseB	b0422	2.8
mhpT	b0353	2.6
b1513	b1513	2.5
KAS		
yjfI	b4181	52
b1629	b1629	25
b1644	b1644	21
ycjX	b1321	19
glvG	b3681	16
mviM	b1068	15
yceO	b1058	15
yjcB	b4060	15
ydgO	b1630	14
yhaL	b3107	14
ttdB	b3062	13
yfiK	b2578	13
ycjG	b1325	12
ylcB	b0572	12
sgbE	b3583	11
nikA	b3476	10
rpsF	b4200	9.7
caiT	b0040	9.4
yeeT	b2003	9.3
b2460	b2460	9.0
ybgG	b0732	8.9
rpsR	b4202	8.7
priB	b4201	8.1
sgaB	b4194	7.6
ycjT	b1316	7.5
rplI	b4203	7.1
elaC	b2268	7.0
hyfH	b2488	6.4
yjfJ	b4182	6.2
yraJ	b3144	6.2
PRM		
metA	b4013	82
ycdU	b1029	77
chpR	b2783	59
ybeK	b0651	28
b1832	b1832	27
phoQ	b1129	24
uhpT	b3666	24
b1444	b1444	20
b1601	b1601	19
kdpC	b0696	17
arp	b4017	16
mesJ	b0188	15
yfiK	b2578	13
xthA	b1749	12
yhcR	b3242	12
yhaI	b3104	11
celC	b1736	9.6
rpmJ	b3299	9.4
b0011	b0011	8.3
b1447	b1447	8.1
rpmD	b3302	7.9
b1451	b1451	7.8
menC	b2261	7.5
yagS	b0285	7.1
b1600	b1600	6.9
yaeS	b0174	6.9
b2534	b2534	6.5
hemL	b0154	6.5
rpmC	b3312	6.2
b1400	b1400	6.1

[a] Additional data can be found at http://trna.chem.yale.edu/supdata/sup001.

ing these data together, it appears that *E. coli* was responding to KAS by attempting to preserve or even enhance its protein biosynthetic capacity while jettisoning its ability to produce energy.

PRM. The expression of 119 genes was enhanced by treatment with the inhibitor PRM (Table 2 and supplementary material [Table 1D; http://trna.chem.yale.edu/supdata/sup001/]), including that of 34 genes that encode ribosomal proteins as well as that of 6 translation-related genes embedded in ribosomal protein operons (*infB*, *nusA*, *prlA*, *rimM* [*yfjA*], *rnpA*, and *trmD*) (16). Transcript levels of 383 genes were found to be decreased after this challenge (Table 3 and supplementary

TABLE 3. Transcripts diminished after inhibitor challenge of a *relA*$^+$ strain[a]

Inhibitor and gene	b no.	Expression ratio	Inhibitor and gene	b no.	Expression ratio
AZL			KAS		
b2880	b2880	0.04	*gadA*	b3517	0.04
b2879	b2879	0.07	*aceA*	b4015	0.05
yhaM	b3108	0.08	*gadB*	b1493	0.05
b2635	b2635	0.09	*gapA*	b1779	0.05
b1445	b1445	0.10	*aceK*	b4016	0.06
b2274	b2274	0.10	*livJ*	b3460	0.06
b2450	b2450	0.10	*hdeB*	b3509	0.08
farR	b0730	0.10	*aroF*	b2601	0.09
b1310	b1310	0.11	*dps*	b0812	0.09
hmpA	b2552	0.12	*b1513*	b1513	0.11
yehT	b2125	0.13	*ilvC*	b3774	0.11
kdpD	b0695	0.14	*osmE*	b1739	0.11
ypfH	b2473	0.15	*aroG*	b0754	0.12
caiC	b0037	0.16	*otsB*	b1897	0.12
yiaB	b3563	0.16	*serA*	b2913	0.12
yjfK	b4183	0.17	*yeaG*	b1783	0.12
yjfL	b4184	0.17	*asd*	b3433	0.13
b2460	b2460	0.19	*pflB*	b0903	0.13
nuoK	b2279	0.19	*icdA*	b1136	0.14
yhaB	b3120	0.20	*ompC*	b2215	0.14
yibG	b3596	0.20	*serC*	b0907	0.14
b2666	b2666	0.21	*pgi*	b4025	0.15
b0359	b0359	0.22	*ppc*	b3956	0.15
gapC_2	b1416	0.22	*aceB*	b4014	0.16
wrbA	b1004	0.22	*gatY*	b2096	0.16
apbA	b0425	0.23	*wrbA*	b1004	0.16
wcaI	b2050	0.23	*eno*	b2779	0.17
yadN	b0141	0.23	*fba*	b2925	0.17
ytfA	b4205	0.23	*hdeA*	b3510	0.17
gadB	b1493	0.24	*leuB*	b0073	0.18
MUP			PRM		
yhjG	b3524	0.03	*ais*	b2252	0.02
aroF	b2601	0.11	*b1973*	b1973	0.02
trpB	b1261	0.13	*atoC*	b2220	0.03
trpE	b1264	0.16	*livJ*	b3460	0.03
gadB	b1493	0.17	*nikE*	b3480	0.03
gadA	b3517	0.18	*nuoL*	b2278	0.03
rplC	b3320	0.21	*ycdY*	b1035	0.03
trpD	b1263	0.21	*gadA*	b3517	0.05
tyrA	b2600	0.21	*gadB*	b1493	0.05
trpA	b1260	0.22	*b1284*	b1284	0.06
b1973	b1973	0.23	*gapA*	b1779	0.06
trpC	b1262	0.23	*hmpA*	b2552	0.06
fusA	b3340	0.24	*yjfQ*	b4191	0.06
gapA	b1779	0.25	*apbA*	b0425	0.07
rpsJ	b3321	0.25	*b1604*	b1604	0.07
rplE	b3308	0.26	*hdeB*	b3509	0.07
rplR	b3304	0.26	*ibpB*	b3686	0.07
tdh	b3616	0.27	*livK*	b3458	0.07
tufA	b3339	0.27	*uhpB*	b3668	0.07
cirA	b2155	0.29	*yhaB*	b3120	0.07
metE	b3829	0.29	*yiaA*	b3562	0.07
rplB	b3317	0.29	*b1045*	b1045	0.08
rpsD	b3296	0.29	*serC*	b0907	0.08
serA	b2913	0.29	*yeaG*	b1783	0.08
ybeD	b0631	0.29	*ynfM*	b1596	0.08
ptsI	b2416	0.30	*b3254*	b3254	0.09
rplF	b3305	0.3	*mraY*	b0087	0.09
rpsE	b3303	0.3	*sucA*	b0726	0.09
csgC	b1043	0.31	*udp*	b3831	0.09
hdeB	b3509	0.31	*b1057*	b1057	0.10

[a] Additional data can be found at http://trna.chem.yale.edu/supdata/sup001.

material [Table 2D; http://trna.chem.yale.edu/supdata/sup001/]). As with KAS treatment, exposure to PRM decreased expression of a subset of the *rpoS* regulon (14) and stationary-phase stimulon (55) genes such as *gadAB*, *hdeABD*, *osmEY*, *otsAB*, *treAR*, *wrbA*, and *xasA* as well as genes involved in a much broader array of energy metabolism functions, ranging from glycolysis and associated functions (*dld*, *eno*, *gapA*, *gapC*, *pgi*, *pgk*, and *ptsHI*), the TCA cycle (*gltA*, *icdA*, *sdhBC*, *sucACD*, and *aceA*), and the pentose shunt (*talA* and *tktB*) to respiration (*cydA*, *cyoD*, *fdhE*, *fdnI*, *hyfGHI*, *narVW*, *nikBDE*, *nrdEFHI*, and *pflB*). Normally highly expressed genes (55), including *aroF*, *folE*, *ilvC*, *metE*, and *livJK*, were subjected to an apparent down-regulation. Transcripts of genes of biosynthetic pathways (*aroAG*, *asd*, *glnBEGL*, *gltD*, *guaC*, *hisBDFIL*, *leuB*, *lysC*, *serAC*, *thrAC*, *trpABC*, and *tyrB*), catabolism of alternative carbon sources (*ebgAC*, *gatYZ*, *tauABCD*, *malGKM*, and *manXYZ*), and iron metabolism (*cirA*, *entABC*, *fecBR*, and *fhuBF*) also accumulated to a substantially lower level.

Differential responses of a *relA* mutant to the four inhibitors. The absence of an intact RelA protein in the cell interfered with its ability to mount an effective response to a limited supply of amino acid. Differences in the transcriptional patterns in the presence and absence of the *relA* gene product, possibly revealing response elements under stringent control, are highlighted below.

AZL. Transcription of about 9% of *E. coli* genes (381 genes) increased >2.5-fold after AZL treatment of the *relA* mutant (Table 4 and supplementary material [Table 3A; http://trna.chem.yale.edu/supdata/sup001/]); for 41 of the genes, transcription was induced >8-fold. Of this subset of 41 genes, 22 encoded products of unknown function, 3 specified amino acid biosynthetic genes (*argA*, *argG*, and *metA*), and nine (*clpB*, *dnaK*, *grpE*, *htpG*, *htpX*, *htrA*, *ibpA*, *ibpB*, and *mopA*) were involved in the heat shock response to unfolded cytoplasmic proteins (58). Moreover, this group included 12 ribosomal protein genes (16).

However, levels of transcripts of 61 ORFs decreased after AZL treatment (Table 5 and supplementary material [Table 4A; http://trna.chem.yale.edu/supdata/sup001/]). Among those were many biosynthetic genes, including several (*aroF*, *folE*, *ilvC*, and *metE*) that are known to be quite highly expressed during normal exponential growth in minimal medium. Levels of transcripts of genes known to accumulate during acidification (1) and entry into stationary phase (55) (*dps*, *gadA*, *gadB*, *hdeA*, *hdeB*, *osmC*, *osmY*, *wrbA*, *xasA*, and *yeaG*) were also found to be lowered.

MUP. The *relA* mutant's response to the MUP inhibitor was quite different from that observed for the parental strain. Transcripts of 105 genes attained moderately (between 2.5- and 10-fold) elevated levels (Table 4 and supplementary material [Table 3B; http://trna.chem.yale.edu/supdata/sup001/]). One-third of the elevated transcripts emanated from ribosomal protein operons (16). Thus, the *relA* mutation was responsible for an apparent inversion in the expression of ribosomal protein gene transcripts; these mRNAs accumulated after MUP treatment of the *relA* strain, while they did not do so after inhibition of the parental strain.

Transcripts of 94 ORFs were down-regulated >2.5-fold by MUP treatment of the *relA* strain, while the expression levels of 43 were decreased >4-fold (Table 5 and supplementary material [Table 4B; http://trna.chem.yale.edu/supdata/sup001/]). Included in this more stringent subset were nine genes (*aceA*, *aceB*, *aceK*, *eno*, *gapA*, *gltA*, *icdA*, *pflB*, and *ptsI*) of central carbon metabolism and two genes (*cycA* and *cyoB*) of respiration as well as many amino acid biosynthetic genes. Four genes (*aroF*, *cysK*, *ilvC*, and *metE*) classified as amino acid biosynthetic genes have been previously noted to be highly expressed (55), while eight others (*aroF*, *pheA*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, and *tyrA*) belong to the family of genes specifying synthesis of the aromatic amino acids. Thus, the patterns of reduced transcript accumulation were similar for the *relA* and *relA*$^+$ pair challenged with MUP; the striking though expected difference (9) was that expression of the genes specifying the translational machinery did not cease in the *relA* mutant.

KAS. Challenge with the KAS inhibitor resulted in at least a 2.5-fold-increased abundance of transcripts corresponding to 339 ORFs in the *relA* strain (Table 4 and supplementary material [Table 3C; http://trna.chem.yale.edu/supdata/sup001/]), echoing the broad impact of this inhibitor upon the *relA*$^+$ strain reported above. Of these genes, 32 specify ribosomal proteins (16). Further inspection revealed that 10 of the 63 ORFs (overexpressed >7-fold) encoded ribosomal proteins. This challenge also reduced transcript levels for 120 ORFs (Table 5 and supplementary material [Table 4C; http://trna.chem.yale.edu/supdata/sup001/]), including genes involved in stress responses (*mopA*, *mopB*, *hdeB*, *gadA*, *gadB*, and *otsB*) (43), biosynthetic genes (both those previously noted to be highly expressed [*aroF*, *cysK*, *folE*, *glnA*, and *ilvC*, and *metE*] [55] and those that do not appear to be extraordinary [*aroB*, *aroG*, *cysA*, *cysN*, *ilvE*, *ilvG*, *ilvI*, *lysC*, *serA*, *thrB*, *thrC*, *trpA*, *trpB*, *trpC*, *trpD*, and *trpE*]), and genes of central carbon metabolism (*aceA*, *fba*, *gapA*, *gltA*, *icdA*, *pgk*, *ppc*, *ptsI*, *sucA*, and *tpiA*).

PRM. Transcripts of 259 ORFs accumulated in the *relA* strain after PRM administration (Table 4 and supplementary material [Table 3D; http://trna.chem.yale.edu/supdata/sup001/]). The pattern seen was reminiscent of that observed with the parental strain. The accumulated transcripts were produced from 45 genes specifying ribosomal proteins and 17 genes (*cmk*, *dnaA*, *dnaG*, *dnaN*, *himD*, *infB*, *insA_1*, *insB_1*, *mviM*, *nusA*, *priB*, *prlA*, *rnpA*, *rpoA*, *rpoD*, *trmD*, and *tsf*) that were adjacent to ribosomal protein-specifying genes (16). Down-regulated transcripts fell into the same general classes seen with the parental strain; transcripts from genes involved in energy metabolism (*aceA*, *aceK*, *adhP*, *atpA*, *atpD*, *atpG*, *eno*, *fba*, *gapA*, *pflB*, *pgk*, *ppc*, *ptsI*, *sdhB*, *sucA*, *sucB*, and *sucC*), iron metabolism (*entB, entE* and *fur*), stress responses (*cadA*, *gadA*, *gadB*, and *hdeA*) (43), and biosynthesis (*aroF*, *aroG*, *ilvC*, *leuA*, *leuB*, *lysC*, *metE*, *serA*, *thrB*, *thrC*, *trpA*, and *trpB*) and highly expressed genes (*aroF*, *ilvC*, and *metE*) (55) were less abundant in the mutant after PRM incubation (Table 5 and supplementary material [Table 4D; http://trna.chem.yale.edu/supdata/sup001/]).

Independent measurements of inhibitor effects. Microarray data can reveal an organism's transcriptional response to stimuli on a genomic scale. However, to reinforce this system-wide profile, two alternative measures of monitoring a gene's transcription were used: real-time PCR and assays of an *E. coli* strain harboring the *lux* operon fused to the *grpE* promoter.

TABLE 4. Transcripts accumulated after inhibitor challenge of a *relA* strain[a]

Inhibitor and gene	b no.	Expression ratio
AZL		
ibpB	b3686	210
ibpA	b3687	160
yfiE	b2577	35
metA	b4013	33
ybfB	b0702	30
clpB	b2592	25
mgtA	b4242	22
cpsB	b2049	22
b2451	b2451	20
b1776	b1776	17
b2656	b2656	17
b2681	b2681	16
yhcN	b3238	15
yshA	b3875	14
yfiA	b2597	14
ygfS	b2886	14
b2680	b2680	13
ybfH	b0691	13
cyoD	b0429	13
ybcU	b0557	13
argA	b2818	12
b1627	b1627	12
sdaA	b1814	12
htpG	b0473	12
yiaB	b3563	11
htpX	b1829	11
htrA	b0161	11
dnaK	b0014	10
yfiD	b2579	10
ylcB	b0572	9.9
MUP		
sdaB	b2797	9.7
sdaC	b2796	8.3
mhpC	b0349	6.9
ybfA	b0699	6.3
b1759	b1759	5.8
rplK	b3983	5.7
yafU	b0218	5.6
rplA	b3984	5.4
yhbE	b3184	5.4
ylcD	b0574	5.2
b1445	b1445	5.1
ycdY	b1035	5.1
yjeR	b4162	5.1
rplL	b3986	4.8
rpsS	b3316	4.8
rplJ	b3985	4.7
rplP	b3313	4.4
rpsC	b3314	4.4
ybgD	b0719	4.4
rplB	b3317	4.3
rplV	b3315	4.3
rplW	b3318	4.3
pin	b1158	4.2
rplD	b3319	4.1
b2667	b2667	3.9
rplC	b3320	3.9
rplY	b2185	3.9
rpmC	b3312	3.9
rpsJ	b3321	3.9
trmD	b2607	3.9
KAS		
yaiL	b0354	46
mobA	b3857	29
wbbI	b2034	27
yeeT	b2003	27
b1983	b1983	26
yjfM	b4185	23
rpsR	b4202	23
fadA	b3845	20
b2447	b2447	19
rpsM	b3298	18
b1601	b1601	18
yfbM	b2272	17
rpsF	b4200	17
rplP	b3313	17
bacA	b3057	16
ybdJ	b0580	16
rpmC	b3312	15
b0833	b0833	15
glvC	b3683	15
yjeP	b4159	14
priB	b4201	14
uhpC	b3667	14
b2451	b2451	12
rpsU	b3065	11
rpmJ	b3299	11
wbbJ	b2033	11
rplO	b3301	10
b1593	b1593	10
gapC_2	b1416	9.9
dadA	b1189	9.5
PRM		
yehQ	b2122	85
b0011	b0011	16
ylcB	b0572	16
dksA	b0145	14
wecD	b3790	13
yfhB	b2560	13
ygfR	b2885	12
yfiD	b2579	12
purL	b2557	12
ygaA	b2709	11
yfiK	b2578	11
metA	b4013	11
seqA	b0687	11
sgaB	b4194	11
ybcU	b0557	11
ycjF	b1322	10
yjfR	b4192	10
phoQ	b1129	10
malG	b4032	9.6
b1628	b1628	8.8
yciR	b1285	8.3
b1445	b1445	8.2
b1045	b1045	8.1
priB	b4201	8.1
ybdM	b0601	7.9
rplO	b3301	7.7
rpsR	b4202	7.6
trmD	b2607	7.5
glnB	b2553	7.4
insA_1	b0022	7.4

[a] Additional data can be found at http://trna.chem.yale.edu/supdata/sup001.

TABLE 5. Transcripts diminished after inhibitor challenge of a *relA* strain[a]

Inhibitor and gene	b no.	Expression ratio	Inhibitor and gene	b no.	Expression ratio
AZL			KAS		
uhpB	b3668	0.06	*ybeK*	b0651	0.01
aroF	b2601	0.07	*ynfM*	b1596	0.02
trpE	b1264	0.07	*b0263*	b0263	0.02
gadB	b1493	0.08	*ycjW*	b1320	0.04
gadA	b3517	0.10	*b1644*	b1644	0.06
ilvC	b3774	0.11	*gapA*	b1779	0.07
ycjD	b1289	0.13	*ilvC*	b3774	0.09
glnB	b2553	0.15	*b1565*	b1565	0.09
kdpA	b0698	0.15	*lysC*	b4024	0.11
wrbA	b1004	0.15	*b2653*	b2653	0.11
lpxD	b0179	0.16	*b1513*	b1513	0.15
tyrA	b2600	0.16	*yagY*	b0292	0.15
trpA	b1260	0.17	*yagU*	b0287	0.15
hdeB	b3509	0.18	*ymfM*	b1148	0.15
trpB	b1261	0.18	*b0165*	b0165	0.15
b2342	b2342	0.20	*b1153*	b1153	0.16
fucA	b2800	0.20	*ycgX*	b1161	0.16
livJ	b3460	0.22	*aceA*	b4015	0.18
livK	b3458	0.22	*gloB*	b0212	0.19
trpD	b1263	0.23	*b1462*	b1462	0.19
yeaG	b1783	0.23	*glnA*	b3870	0.19
serC	b0907	0.24	*yrfD*	b3395	0.19
ybaJ	b0461	0.24	*serA*	b2913	0.19
talA	b2464	0.25	*gltA*	b0720	0.20
dps	b0812	0.26	*malS*	b3571	0.21
pflB	b0903	0.26	*trpB*	b1261	0.21
trpC	b1262	0.26	*gadA*	b3517	0.21
yadF	b0126	0.26	*aroF*	b2601	0.22
aceA	b4015	0.27	*smpA*	b2617	0.22
nrdI	b2674	0.28	*trpD*	b1263	0.22
MUP			PRM		
aroF	b2601	0.04	*yhcO*	b3239	0.01
aceA	b4015	0.07	*yi81_1*	b0016	0.02
b1973	b1973	0.08	*b1547*	b1547	0.03
gapA	b1779	0.08	*yfaE*	b2236	0.03
ilvC	b3774	0.08	*b2386*	b2386	0.03
trpE	b1264	0.09	*ykgG*	b0308	0.04
aceB	b4014	0.11	*eutB*	b2441	0.04
eno	b2779	0.11	*glpR*	b3423	0.04
trpB	b1261	0.11	*b1172*	b1172	0.05
b2350	b2350	0.14	*cadA*	b4131	0.05
gadB	b1493	0.14	*ybhI*	b0770	0.06
livJ	b3460	0.14	*flhB*	b1880	0.06
hisD	b2020	0.15	*rfe*	b3784	0.06
lysC	b4024	0.15	*aceA*	b4015	0.06
gadA	b3517	0.16	*yhcK*	b3226	0.08
hdeB	b3509	0.16	*b1759*	b1759	0.09
hisG	b2019	0.16	*aceK*	b4016	0.09
mobB	b3856	0.16	*ybcK*	b0558	0.10
trpA	b1260	0.16	*b1503*	b1503	0.11
leuA	b0074	0.17	*celF*	b1734	0.11
metE	b3829	0.17	*cirA*	b2155	0.11
pheA	b2599	0.17	*b2361*	b2361	0.11
ompC	b2215	0.18	*b2650*	b2650	0.11
yrbG	b3196	0.18	*ybbT*	b0505	0.12
cycA	b4208	0.19	*ybeJ*	b0655	0.13
cyoB	b0431	0.19	*sucA*	b0726	0.13
trpD	b1263	0.19	*eno*	b2779	0.13
aceK	b4016	0.20	*atpD*	b3732	0.14
gltA	b0720	0.20	*sucB*	b0727	0.16
livK	b3458	0.20	*modA*	b0763	0.16

[a] Additional data can be found at http://trna.chem.yale.edu/supdata/sup001.

TABLE 6. Transcript changes measured by RT-PCR and microarrays

Gene	b no.	Expression ratio for indicated *E. coli* K-12 strain and treatment															
		WT + AZL		relA + AZL		WT + MUP		relA + MUP		WT + KAS		relA + KAS		WT + PRM		relA + PRM	
		Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR
argA	b2818	38	64	12	16	1.4	1.0	0.68	1.5	0.95	1.6	1.9	0.21	1.2	1.5	0.72	0.28
argB	b3959	9.2	17	3.5	7.2	0.87	0.90	0.83	1.0	0.99	4.6	0.60	0.54	1.1	3.4	0.82	0.15
aroF	b2601	0.38	0.063	0.065	0.015	0.11	0.59	0.041	0.75	0.093	0.016	0.21	0.0041	0.21	0.029	0.26	0.0063
dnaK	b0014	13	12	10	7.1	1.2	0.95	0.30	1.8	0.30	0.15	0.43	0.26	0.57	0.98	1.1	2.2
ilvC	b3774	0.48	0.013	0.11	0.0042	0.40	0.86	0.078	0.72	0.11	0.0035	0.088	0.0062	0.21	0.012	0.16	0.068
lacZ	b0344	1.3	0.046	0.75	0.59	1.0	0.56	1.2	0.84	1.2	0.22	0.36	0.70	1.3	0.83	0.85	1.2
leuS	b0642	1.0	1.0	1.0	1.6	0.85	0.52	0.98	0.83	0.94	0.85	0.94	0.58	0.64	0.86	0.97	0.70
livJ	b3460	0.4	0.20	0.22	0.19	0.37	1.6	0.14	0.97	0.062	0.017	0.34	0.029	0.035	0.022	0.26	0.016
mopA	b4143	7.9	25	8.7	14	0.45	0.93	0.31	1.1	0.24	0.10	0.35	0.25	0.37	0.36	0.54	0.64
rplV	b3315	3.0	4.6	4.0	2.3	0.37	0.70	4.3	0.93	3.0	5.1	7.5	2.1	6.1	7.3	4.1	4.2
rpmD	b3302	2.0	2.4	2.0	5.4	0.59	0.32	2.9	1.1	3.1	1.7	4.1	2.0	7.9	2.5	3.9	3.2
rpsL	b3342	0.80	2.5	0.62	2.5	0.88	0.62	0.82	1.4	0.64	1.6	1.1	1.7	0.79	1.8	0.88	1.6
sfsA	b0146	1.5	0.36	1.2	0.064	0.83	0.47	0.21	0.65	0.49	0.16	7.7	0.79	0.13	0.16	4.2	0.79
soxS	b4062	4.0	45	6.4	15	1.1	0.94	1.3	1.3	1.6	1.7	1.8	1.2	3.3	42	1.5	13
trmD	b2607	1.2	0.95	1.3	0.94	0.35	0.24	3.9	0.60	2.4	0.97	1.1	1.0	4.7	4.4	7.5	3.1
trpC	b1262	0.39	0.056	0.26	0.023	0.23	0.86	0.20	0.51	0.37	0.040	0.34	0.025	0.40	0.039	0.50	0.013
wrbA	b1004	0.22	0.61	0.15	2.1	0.85	1.1	0.42	1.0	0.16	0.032	0.28	0.12	0.31	0.065	0.81	0.14

Real-time PCR. Verification of the major trends suggested by the microarray data was sought, using real-time PCR to measure relative changes in the abundance of selected transcripts (Table 6) between different RNA preparations. The direction of change, or lack thereof, observed with this technique generally confirmed results obtained by global profiling with microarrays, although variations in magnitude between the two technologies were observed (see results for *aroF* in Table 6). Overall, there was more than 80% concordance of the data generated by the microarray with that generated by the real-time PCR procedure with regard to the direction of the expression level change. Transcripts of genes involved in amino acid biosynthesis and uptake, a major functional group involved in the cell's responses to all inhibitors tested, showed consistent agreement between the trends suggested by both techniques (see results for *argA*, *argB*, *aroF*, *ilvC*, *livJ*, and *trpC* in Fig. 2). Also, the data from genes tested, which are involved in the cell's response to stresses and entry into stationary phase, exhibited firm agreement (see results for *dnaK*, *mopA*, *soxS*, and *wrbA* in Fig. 2). Disagreement was apparent between the microarray and real-time reverse transcriptase PCR (RT-PCR) data for two genes involved in sugar metabolism (*lacZ* and *sfsA*) and the gene coding for ribosomal protein S12 (*rpsL*). While the reasons for the discrepancies between the two data sets for these three genes are not clear, the overall trends suggested by the real-time PCR data for the set of genes shown in Table 6 were in good agreement with expression changes inferred from the genome-wide data set (see above).

Bioluminescence of the *grpE*::*lux* operon fusion strain. The heat shock response (58) is thought to be triggered by the presence of unfolded proteins in the cytoplasm (23). To determine whether such a response can be triggered by the translation inhibitors studied here, we used *E. coli* strain DPD3084, harboring the *rpoH*-controlled *grpE* promoter fused to the *lux* operon. As seen in Fig. 3, the expected result (that exposure to high concentrations of ethanol elevated expression from this fusion) was revealed by bioluminescent monitoring. At moderate concentrations (16 to 250 μg/ml), AZL treatment elevated *grpE*::*luxCDABE* expression. At higher concentrations (data not shown), light emission was lowered by this treatment, presumably because protein synthesis needed to make Lux polypeptides was blocked. Concentrations lower than 16 μg/ml had no effect on light output. MUP concentrations of more than 0.4 μg/ml compromised light emission, while the strain was unresponsive to lower concentrations. Treatment with KAS lowered bioluminescence at concentrations higher than 63 μg/ml. A robust bioluminescent response indicative of heat shock was observed at 2 to 31 μg/ml of the antibiotic, amounts well below that needed for growth inhibition, while lower doses were ineffectual. The response to PRM was more complicated. Reduced light emission was seen over a wide range of concentrations (from 8 to 250 μg/ml); at lower concentrations, DPD3084 appeared indifferent to the challenge. During the time course studied, increased heat shock expression was seen in a narrow window of PRM concentrations of 63 to 125 μg/ml (consistent with a previously reported modest response to a related *grpE*::*lux* fusion at 200 μg/ml [52]).

Characterization of abundant biosynthetic transcripts often lost after inhibitor challenge. Several such transcripts were lost after inhibitor challenges (Table 3 and Table 5) (39). To examine this phenomenon more thoroughly, *cysK*, *glnA*, *ilvC*, and *metE* transcripts were studied.

RT-PCR was used to measure the chemical half-lives of several transcripts in an early-exponential-phase culture (A_{600} = 0.4) of *E. coli* strain MG1655 subjected to shaking at 37°C in Luria-Bertani medium after blocking of transcription initiation with rifampin (0.15 mg/ml) (Fig. 4). As controls, the long-lived *ompA* transcript and the labile *trxA* mRNA (17) were also examined. The half-life of the *trxA* mRNA was found to be 1.9 min, while that of the *ompA* transcript was 8.8 min. Those for *cysK*, *glnA*, *ilvC*, and *metE* were determined to be 1.8, 0.5, 1.4, and 2.3 min, respectively. Thus, the chemical half-lives of these transcripts were not remarkable.

The loss of *ilvC*, *glnA*, and *metE* mRNAs after different inhibitor challenges was determined by microarray analyses (see Table 7 for details). RT-PCR analysis of the same mRNA

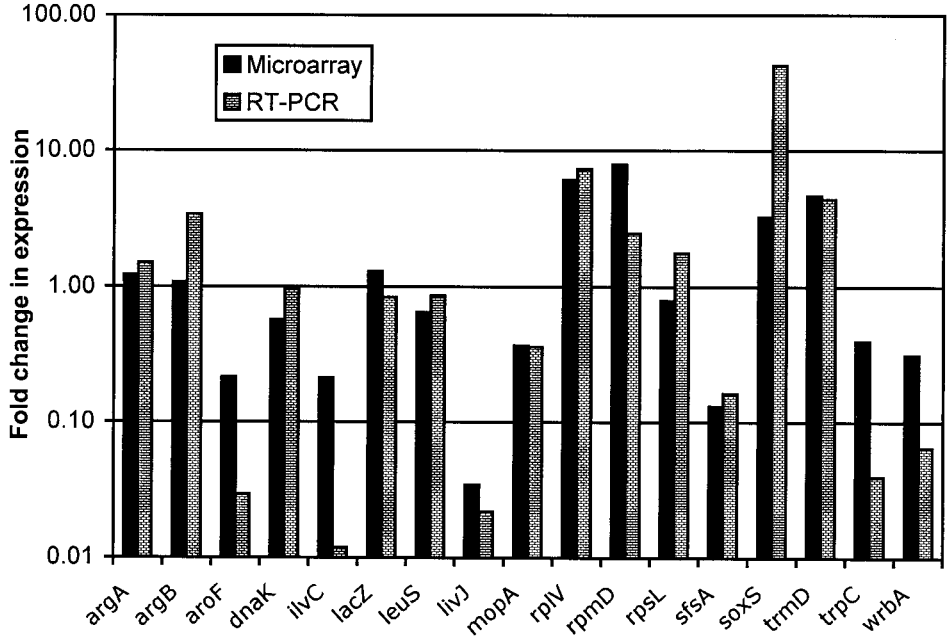

FIG. 2. Severalfold changes in levels of selected mRNAs for *relA*$^+$ strain CF1943 treated with PRM, as determined by two distinct methods (RT-PCR and hybridization to DNA microarrays). The strain was challenged, RNA was prepared from challenged and control cultures, and the RNA samples were used to determine the changes in RNA level after challenge. Distinct cultures were challenged for the RT-PCR and DNA microarray analyses.

preparations confirmed the observations. Treatment with SM, 2,4-dinitrophenol, *p*-hydroxybenzoate, and acivicin was shown by both methodologies to cause substantial loss of these mRNAs consistent with the RT-PCR studies of inhibitors arresting translation (Table 6).

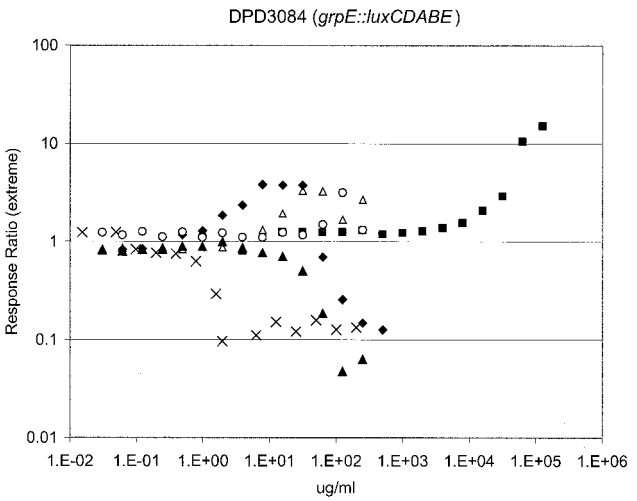

FIG. 3. Dose responses obtained with a bioluminescent sensor that detects both induction of a heat shock-regulated promoter and compromise of a "healthy" metabolism. An exponential-phase culture of *E. coli* strain DPD3084 was challenged individually with the indicated amount of ethanol (■), PRM (▲ and ○), KAS (♦), AZL (△), or MUP (×), and bioluminescence was recorded as a function of time after treatment. Maximal (■, ○, ♦, △, and ×) and minimal (▲) response ratios observed during the period following exposure to each concentration of the different chemicals are reported as extreme response ratios.

DISCUSSION

RNA polymerase activity is limiting in *E. coli*. The 4,641 predicted promoters responsible for expression of the 2,326 transcription units (37) are serviced by approximately 2,800 molecules of RNA polymerase in a cell that doubles once every hour, a rate typical of growth using glucose as a carbon source in minimal medium (the standard, uninhibited conditions used in this study). Of these enzymes, only 500 are actively engaged in transcription at any instant, with 300 producing mRNA and 200 synthesizing tRNA and rRNA (8). Even when *E. coli* is growing at its maximal rate, only about 700 RNA polymerase

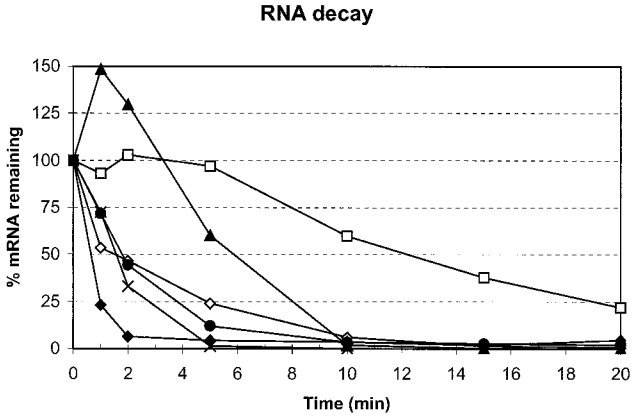

FIG. 4. mRNA decay after rifampin inhibition. The inhibitor was added to exponential-phase cultures at the initiation of the experiment. At the indicated times, RNA was prepared prior to being subjected to RT-PCR analyses to determine the amounts of *trxA* (◇), *ompA* (□), *metE* (▲), *ilvC* (×), *glnA* (♦), and *cysK* (●) mRNA that remained.

TABLE 7. Loss of abundant transcripts after distinct challenges

Transcript	Expression ratio after challenge with:							
	SM[a]		Acivicin[b]		*p*-Hydroxybenzoate[c]		2,4-Dinitrophenol[d]	
	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR
cysK	0.5	1.4	0.39	0.19	1	1	0.56	0.71
glnA	0.14	0.12	0.15	0.01	0.14	0.001	0.1	0.015
ilvC	0.21	0.03	0.04	0.001	0.06	0.003	0.05	0.014
metE	0.08	0.0031	0.13	0.033	0.3	0.067	0.13	0.13

[a] RNA isolated from strain DPD1675 challenged with inhibitor (8 μg/ml) for 45 min with shaking (250 rpm) at 37°C.
[b] RNA isolated from an exponential-phase culture of strain MG1655 treated with inhibitor (2 μg/ml) for 60 min with shaking (250 rpm) at 37°C in medium E (12) supplemented with thiamine and 0.4% glucose.
[c] RNA isolated from an exponential-phase culture of strain DE112 (49) incubated with inhibitor (3,453 μg/ml) for 60 min with shaking (250 rpm) at 37°C.
[d] RNA isolated from an exponential-phase culture of strain DE112 (49) treated with inhibitor (75 μg/ml) for 60 min with shaking (250 rpm) at 37°C in medium E (12) supplemented with thiamine and 0.4% glucose.

molecules are engaged in the production of mRNA at any one time, with 2,600 RNA polymerase molecules devoted to stable RNA synthesis.

We have previously used a parts-per-million scale (55) to estimate mRNA content in *E. coli.* Under standard conditions, at any instant >300 RNA polymerase molecules are engaged in mRNA synthesis, with a transit time of 21 s (8) for an average 951-bp gene (6). Thus, transcription across some ORF occurs about 55,000 times per generation. This is equivalent to between 10 and 15 transcription events per generation for a gene whose expression is average (223 ppm [0.0223%]). Deviations from this value are large; many ORFs may be transcribed less than once per cell division (18 ppm) while others are estimated to be transcribed about 900 times per doubling (16,200 ppm) (55). This range is consistent with other thoughts on gene expression, including that the range of protein expression is from <1 to 10^5 molecules per cell (29, 46).

These experiments provide an approximation of the distribution of RNA polymerase among those promoters that produce mRNA. It is conceivable, however, that distribution of RNA polymerase between mRNA and stable RNA synthesis can change greatly upon inhibition (3, 4); that would have caused us to underestimate induction of gene expression in response to some inhibitors.

The broad effects of each inhibitor on gene expression levels are evident when the distribution of induced and repressed genes over the spectrum of functional classifications in the cell is examined. Major trends and large effects become easily discernible using this approach. One can immediately see that the total number of gene transcripts affected varies greatly with the inhibitor used (for an example, see the results of MUP inhibition of the wild-type *relA* strain versus those of KAS inhibition of the wild-type *relA* strain [Fig. 5]). This property of the response may illustrate the inherent differential complexity of the inhibitors' targets. In the case of MUP, the primary effect is that of blocking one amino acid's incorporation into protein (i.e., MUP inhibits Ile charging onto $tRNA^{Ile}$). KAS, on the other hand, targets a process involving a higher order of complexity, interfering with the central machinery of protein synthesis itself (i.e., KAS blocks formation of the 70S ribosomal particle). The *relA*-dependent inversion of the expression of ribosomal proteins and those associated with the translation apparatus is easily recognizable for MUP treatment as discussed above (Fig. 5). Another striking feature of the various expression patterns is that a large fraction of the cell's response to each inhibitor is derived from hypothetical or unknown ORFs (Fig. 5). It is therefore clear that a large part of any inhibitor's effects on cellular physiology cannot yet be rationalized.

The four inhibitors elicited different patterns of elevated gene expression from the *relA*$^+$ strain. AZL caused elevated expression of many stress-induced genes (including those of the heat shock regulon) as well as increased content of amino acid, though not branched-chain amino acid, biosynthetic transcripts. The thought that AZL is not charged to tRNA and incorporated into protein within *E. coli* (40) needs to be reconsidered in light of these results and those found with *Salmonella enterica* serovar Typhimurium (42) and *E. coli* (26). Those mRNAs elevated after MUP treatment were quite different, and the increased amount of *thr*, *leu*, and *ilv* operon mRNAs detected conformed to expectations consistent with deattenuation caused by a limitation for isoleucyl-tRNA (18, 45). That the responses to these two inhibitors differed from those caused by treatment with agents acting on ribosomes was not surprising. Blocking initiation of translation with KAS and causing premature release of polypeptides with PRM resulted in elevated expression of ribosomal protein and other mRNAs specifying the translational machinery, as if the cell sensed that there was insufficient protein synthesis relative to other cellular activities. Surprisingly, PRM treatment did not cause an elevated heat shock response.

Shared patterns of reduced gene expression after inhibition of the *relA*$^+$ strain were also observed. MUP, KAS, and PRM treatment elicited reduced accumulation of transcripts previously identified as being highly expressed in exponentially growing cells or cells transitioning to the stationary phase (55). In addition, KAS and PRM, but not AZL or MUP, treatment resulted in diminished titers of transcripts specifying several enzymes involved in central carbon metabolism. MUP treatment resulted in a decrease in mRNAs specifying ribosomal proteins, as would be expected for the stringent response (9).

The *relA* mutation changed the observed pattern of gene expression in several ways. Most notably, each of the four treatments of a *relA* mutant resulted in elevated levels of ribosomal protein mRNAs; AZL and MUP did not cause elevated titers in the *relA*$^+$ strain. These observations reinforce the concept of RelA acting as a "brake" to subvert the inclination to increase the titer of the translational machinery when protein synthesis is limited. Such a hypothesis could not be reached without comparisons of the various responses of a

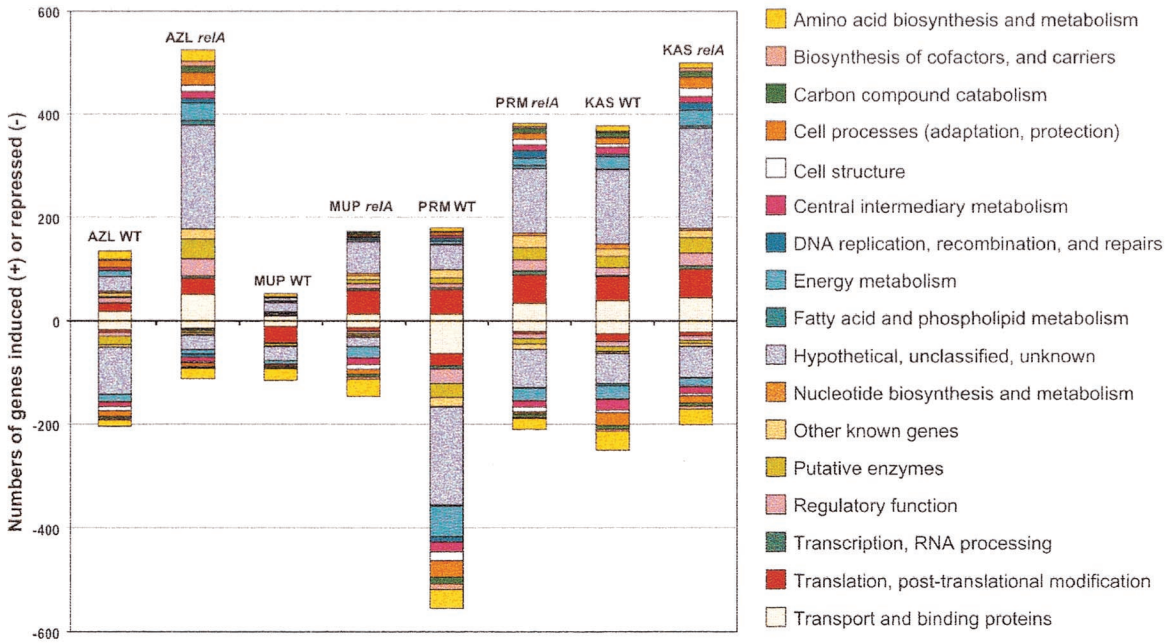

FIG. 5. A global view of changes in mRNA distribution. Each bar represents a separate inhibitor-strain combination. The number of genes whose expression was elevated or diminished is represented by the height of each bar. The contribution of each functional class of genes to the observed change is signified by the area of the colored block within each bar. WT, wild-type strain W3110; *relA*, W3110 Δ*relA251*::*kan* strain.

mutant strain challenged with a series of inhibitors blocking various points in the process of translation (Fig. 1). While a more restricted set of translational inhibitors has been used to generate transcriptional profiles with another species (31), a broader set of chemicals, a global regulatory mutant, and prior quantitative knowledge of steady-state mRNA levels (55) have contributed to the concept suggested here.

The stringent response is complicated and has been operationally linked to *relA* function. Others have presented an expanded stringent response model (10) and have linked ppGpp, *lrp*, and *rpoS* to adaptation to famine (44). Proper utilization of terminology and concepts surrounding the words stimulon, regulon, and modulon (30) may reveal considerable commonality among those results, the work reported in this article, and the results of studies presented elsewhere (39, 55). Such convergence suggests that systems biology will contribute a great deal to the understanding of microbial physiology.

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