

# Combined, Functional Genomic-Biochemical Approach to Intermediary Metabolism: Interaction of Acivicin, a Glutamine Amidotransferase Inhibitor, with *Escherichia coli* K-12

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**Acivicin, a modified amino acid natural product, is a glutamine analog. Thus, it might interfere with metabolism by hindering glutamine transport, formation, or usage in processes such as transamidation and translation. This molecule prevented the growth of *Escherichia coli* in minimal medium unless the medium was supplemented with a purine or histidine, suggesting that the HisHF enzyme, a glutamine amidotransferase, was the target of acivicin action. This enzyme, purified from *E. coli*, was inhibited by low concentrations of acivicin. Acivicin inhibition was overcome by the presence of three distinct genetic regions when harbored on multicopy plasmids. Comprehensive transcript profiling using DNA microarrays indicated that histidine biosynthesis was the predominant process blocked by acivicin. The response to acivicin, however, was quite complex, suggesting that acivicin inhibition resonated through more than a single cellular process.**

Interconnections among biochemical pathways remain an understudied question in modern biology. Currently, this problem is being addressed in several different bacterial systems. For example, Frodyma et al. (28) and Tsang et al. (77) investigated the metabolic integration (23) of vitamin synthetic pathways which are thought to have a rather low flux since cofactors are used in catalytic rather than structural quantities. We have chosen to explore metabolic integration by focusing on the action of an inhibitor, acivicin (Fig. 1), since it is believed to interact with glutamine amidotransferases (61, 62, 74, 84) and its antibacterial activity toward *Bacillus subtilis* is antagonized by histidine and purine nucleosides (35). These enzymes extract ammonia from glutamine prior to attaching it to an organic backbone.

In *Escherichia coli* there are at least 12 distinct glutamine amidotransferases (58, 83) involved in biosynthesis, underscoring the importance of ammonia assimilation by processes in addition to transamination. Five are involved in amino acid biosynthesis: anthranilate synthase (EC 4.1.3.27, TrpE), asparagine synthase (EC 6.3.5.4, AsnB), carbamoyl phosphate synthetase (EC 6.3.5.5, CarAB [used in arginine as well as pyrimidine nucleotide production]), glutamate synthase (EC 1.4.1.13, GltBD), and imidazole glycerol phosphate (IGP) synthase (HisHF). Along with CarAB, another four (EC 6.3.4.2, PyrG, CTP synthetase; EC 2.4.2.14, PurF, glutamine 5-phospho- $\alpha$ -1-pyrophosphate [PRPP] amidotransferase; EC 6.3.5.3, PurL, 5'-phosphoribosyl-*N*-formyl glycinamide [FGAM] synthetase; and EC 6.3.4.1, GuaA, GMP synthetase) are enzymes

of nucleotide biosynthesis. Two, PabAB (4-amino-4-deoxychorismate synthase, a component of the folate pathway) and NadE (EC 6.3.5.1, NAD synthetase), function in cofactor synthesis, while one, GlmS (EC 2.6.1.13), is involved in production of the cell wall precursor *N*-acetylglucosamine phosphate. Thus, antagonism of this enzyme family might be most revealing.

One glutamine amidotransferase, IGP synthase, is encoded by *hisH* and *hisF*; these two genes are components of the histidine operon, *hisGDCBHAFI* (Fig. 2A) (88). This amidotransferase occupies a central position in the eight-enzyme pathway from PRPP and ATP to histidine (Fig. 3). If this reaction or the immediately preceding HisA (pro-phosphoribosyl formimino-5-aminoimidazole-4-carboxamide ribonucleotide [PROFAR] isomerase)-catalyzed reaction is blocked, ATP is still condensed with PRPP and undergoes subsequent modification, including opening of its six-membered ring. Such blockages drain the purine nucleotide pools, effectively causing the metabolic economy to grind to a halt due to a lack of "currency," presumably in the form of adenylates. Normally the amidotransferase reaction of the histidine biosynthetic pathway liberates 5-aminoimidazole-4-carboxamido-1- $\beta$ -D-ribofuranosyl 5'-monophosphate (AICAR) as a by-product. The latter molecule, a purine biosynthetic intermediate, is salvaged in a process that leads to the resynthesis of ATP. This combined histidine-purine cycle is hence critical for cellular function, as demonstrated by the studies of Hartman et al. (36), Shedlovsky and Magasanik (70, 71), Johnston and Roth (44), and Taylor et al. (29, 42, 72, 73). Moreover, overproduction of HisHF has other deleterious consequences for cell division (3, 27, 57) independent of the above-mentioned adenylate drain. Thus, the HisHF enzyme is an attractive site for the study of metabolic integration.

Due to the arrangement of the *his* genes within an operon (Fig. 2A) (88), it is difficult to eliminate function of an individual gene due to the polar nature of many *his* mutations. Furthermore, draining of adenylates by such mutants might

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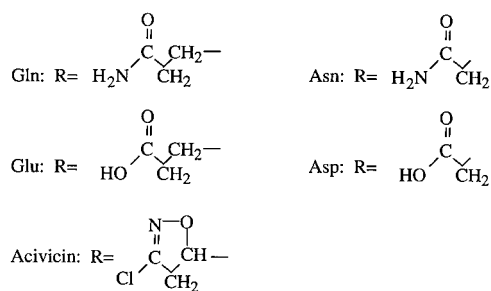


FIG. 1. Structures of some amino acids. Only R groups are depicted.

provide a strong selective pressure for true reversion or pseudo-reversion. Hence, the ability to transiently compromise HisHF or HisA activity by the addition of a specific inhibitor is desirable. We demonstrate that acivicin has such HisHF-directed antagonism. The nutrients that prevent its inhibitory action, its specificity, and the consequences of its administration are investigated by the genetic, biochemical, and enzymological analyses of *E. coli* reported here.

#### MATERIALS AND METHODS

**Abbreviations and nomenclature.** Standard bacterial nomenclature (8) is used. Biosynthetic intermediates are abbreviated as follows: PRFAR,  $N^1$ -(5'-phosphoribulose) formimino]-5-aminoimidazole-4-carboxamide ribonucleotide; IAP, imidazole acetol phosphate; HOL-P, L-histidinol phosphate; HOL, L-histidinol; and 2-KG, 2-ketoglutarate. Polypeptide nomenclature includes HisG (ATP phosphoribosyl transferase), the HisI (the bifunctional phosphoribosyl-ATP pyrophosphorohydrolase/phosphoribosyl-AMP cyclohydrolase), the HisH (glutamine amidotransferase) domain, the HisF (cyclase) domain, HisB (the bifunctional IGP dehydratase/HOL-P phosphatase), HisC (HOL-P aminotransferase), HisD (histidinol dehydrogenase), and YIP (yeast inorganic pyrophosphatase).

**Chemicals and biochemical reagents.** Acivicin, glutamine, PRPP, and yeast inorganic pyrophosphatase were purchased from Sigma (St. Louis, Mo.). Purified *E. coli* HisHF enzyme (0.4 mg/ml, 7 U/mg) was a gift from V. J. Davisson, Purdue University.

**Strains and plasmids.** Plasmids are described in Table 1. *E. coli* strains FB1 ( $\Delta$ hisGDCBHAFI750) (12) and FB1/*phisAGIE*-tac were obtained from V. J. Davisson. The set of *his* operon point mutants was obtained from P. E. Hartman and has been described previously (30, 31). *Salmonella enterica* serovar Typhimurium Tn10 mutations were backcrossed into the wild type, selecting for tetracycline resistance as described elsewhere (20).

**Inhibition assays.** Disk diffusion was performed as described for sulfometuron methyl (47, 50), a modification of a previously described scheme (75). An alternative bioluminescent technique was also used (26). Briefly, an insertion of a *recA* promoter-*Photobacterium luminescens luxCDABE* fusion within *lacZ* was crossed into strain DPD1692, selecting for kanamycin resistance. This strain, DPD1718, produces a high, baseline bioluminescence that is induced by DNA-damaging agents (82) and dampened by a wide range of metabolic inhibitors (11). Details of the construction have been described elsewhere (25). Both techniques are amenable to auxanography, a means to determine the pathway blocked by either mutation (20) or inhibitor action (47) through the supplementation with pools of nutrients. This method was used to determine those nutrients that allow metabolic function, be it growth or bioluminescence, in the presence of the inhibitor.

The ability of plasmids to alter the response to acivicin was also assayed using a bioluminescence-based protocol. Transformants (59) of strain DPD1718 harboring either pUC18 or pDEW327 were obtained by selecting for resistance to ampicillin (100  $\mu\text{g}/\text{ml}$ ) on Luria-Bertani plates (20). Single-colony isolates were inoculated into minimal E medium supplemented with thiamine, 0.4% glucose, and 100  $\mu\text{g}$  of ampicillin per ml and incubated overnight at 37°C. Cultures were diluted into a modification of this medium that contained 50 instead of 100  $\mu\text{g}$  of ampicillin per ml and shaken until they reached the exponential phase of growth. They were then exposed to acivicin in microtiter plates, and the response was monitored as a function of time using a standard method as published (79) except that the microtiter plates were incubated in a luminometer chamber

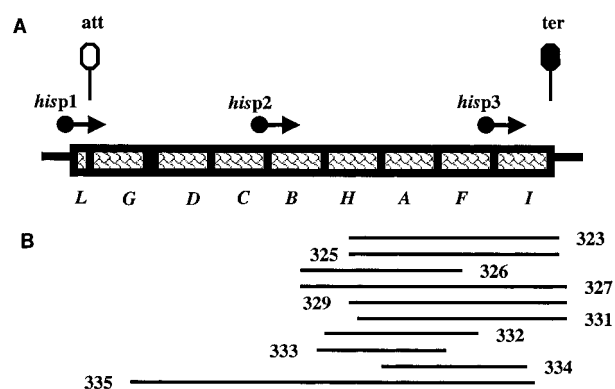


FIG. 2. (A) The histidine operon. *his* genes are indicated by boxes. Promoters are indicated by filled dots with arrows denoting direction of transcriptions. Sites of transcriptional termination are denoted by lollipops. (B) Plasmids that complement *his* point mutants, denoted by lines.

maintained at 37°C. The resultant kinetic curves (data not shown) indicated that acivicin blocked luminescence shortly after its administration. This blockage was transient if the medium was supplemented appropriately or if the genetic constitution titrated out inhibitory effects. For simplicity, an endpoint, a modified response ratio (7, 79) obtained at 400 min, was calculated from the difference in luminescence obtained after treatment with a given concentration of acivicin for this time period divided by the difference in luminescence obtained with an untreated sample.

**Genetic titration and complementation.** The method used has been described in general for *E. coli* (15). The specific plasmid libraries containing random segments of the *E. coli* W3110 genome have been described (26) and used (87; Z. Xue, D. R. Smulski, D. Delduco, S.-Y. Soon-Yong Choi, M. H. Jia, and R. A. LaRossa, unpublished data) elsewhere. The MIC of acivicin for strain DPD1675 was 1  $\mu\text{g}/\text{ml}$  on E (20) minimal agar medium supplemented with 0.2% glucose, thiamine, and proline. Selection was for those few transformants (59) of pBR322- and pUC18-based genomic libraries of *E. coli* that would support growth on the above-described medium supplemented with ampicillin (100  $\mu\text{g}/\text{ml}$ ) and acivicin (3  $\mu\text{g}/\text{ml}$ ). Plasmid DNA was isolated from resistant clones, backcrossed by transformation (59) into strain DPD1675 to ascertain that resistance was a plasmid-specified phenotype, and sequenced as described previously (26, 87; Xue et al., unpublished).

Similarly, *hisA* and *hisH* mutants were transformed with the same libraries with selection for ampicillin-resistant prototrophs. In a like manner, the *his*-complementing plasmids were backcrossed as well as being transformed into a broad set

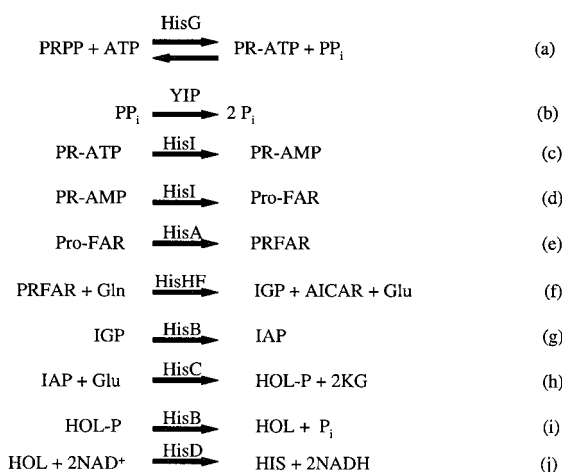


FIG. 3. Histidine biosynthesis. Also shown is the reaction (b) catalyzed by yeast inorganic pyrophosphatase that drives reaction a to the right in a coupled in vitro system.

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source (reference)
<i>E. coli</i>		
CS1562	F <sup>-</sup> λ <sup>-</sup> <i>tolC::mini-Tn10 supE42 rph-1</i>	C. Schnaitman (5)
DPD1675	<i>ilvB2101 ara thi Δ(proAB-lac) tolC::mini-Tn 10</i>	Lab strain (80)
DPD1692	<i>lac Kan<sup>r</sup></i>	Lab strain (17)
DPD1718	<i>lac Kan<sup>r</sup> lacZ::recAϕluxCDABE</i>	Lab strain (17)
FB1	<i>ΔhisGDCBHAFI750</i>	V. J. Davisson (12)
<i>hisG2243</i>	<i>hisG2243</i>	PEH, JHU <sup>a</sup> (30)
<i>hisG3857</i>	<i>hisG3857</i>	PEH, JHU (30)
<i>hisD921</i>	<i>hisD921</i>	PEH, JHU (30)
<i>hisC901</i>	<i>hisC901</i>	PEH, JHU (30)
<i>hisC904</i>	<i>hisC904</i>	PEH, JHU (30)
<i>hisB463</i>	<i>hisB463</i>	PEH, JHU (30)
<i>hisH4744</i>	F <sup>-</sup> <i>hisH4744 nadB29 thi mtl xyl ara lac</i>	PEH, JHU (31)
<i>hisA323</i>	<i>hisA323</i>	PEH, JHU (30)
<i>hisF860</i>	<i>hisF860</i>	PEH, JHU (30)
<i>hisF891</i>	<i>hisF891</i>	PEH, JHU (30)
<i>hisI903</i>	<i>hisI903</i>	PEH, JHU (30)
MG1655	F <sup>-</sup> λ <sup>-</sup> <i>rph-1</i>	D. Berg, Washington University (6)
RFM443	<i>rpsL galK2 lacΔ74</i>	Rolf Menzel (56)
<i>S. enterica</i> serovar Typhimurium		
LT2	<i>S. enterica</i> serovar Typhimurium +	K. Rudd, Miami
TT7542	<i>S. enterica</i> serovar Typhimurium <i>relA21::Tn10</i>	K. Rudd
TV101	<i>S. enterica</i> serovar Typhimurium <i>rfa</i>	Lab strain (81)
LT2 <i>relA</i>	<i>S. enterica</i> serovar Typhimurium <i>relA21::Tn10</i>	This study, P22 (TT7542) × LT2 → Tet <sup>r</sup>
Plasmids		
pBR322	Cloning vector	68
pDEW326	pUC18 + <i>his' BHAF'</i>	This work
pDEW327	pUC18 + <i>his' BHAFI</i>	This work
pDEW335	pUC18 + <i>his' GDCBHAFI'</i>	This work
<i>phisAGIE-tac</i>		V. J. Davisson (21)
pUC18	Cloning vector	68
PVV101	pBR322 + <i>trpEDC'</i>	C. Yanofsky, Stanford University

<sup>a</sup> PEH, JHU, Philip E. Hartman, Johns Hopkins University.

of *his* point mutants. The extent of chromosomal DNA carried on each plasmid was determined by complementation of auxotrophs and sequencing the vector-chromosome junctions.

**Substrate preparation.** PRFAR was synthesized using *E. coli* strain FB1/*phisAGIE-tac* based on published protocols of Davisson et al. (21) and Deras (22), with some modifications. The reaction scheme is shown in Fig. 3. PRPP (270 μmol) was reacted with excess ATP (400 μmol) in the presence of 72 U of inorganic pyrophosphatase and 9.6 mg of FB1/*phisAGIE-tac* extract in 86 mM potassium phosphate (pH 7.5)–28 mM MgCl<sub>2</sub>–3.5 mM EDTA. After the reaction flask was shaken at 30°C for 1 h, an additional 4.8 mg of FB1/*phisAGIE-tac* extract was added, and the reaction mixture was incubated for another 2 h. PRFAR was purified by applying the reaction mixture to a Q-Sepharose column (2.5 by 14 cm; Pharmacia) equilibrated with 60 mM NH<sub>4</sub>HCO<sub>3</sub>, and eluting with an NH<sub>4</sub>HCO<sub>3</sub> gradient (60 to 300 mM over 300 ml). The fractionated eluant was analyzed by UV-visible light spectroscopy, and fractions in which A<sub>290</sub>/A<sub>260</sub> was >1.0 were pooled and dried by lyophilization. The lyophilized product was redissolved in 0.1 M tetraethylammonium acetate (pH 7.0) and further purified by reverse-phase high-pressure liquid chromatography on a C<sub>18</sub> column (Supelco LC18 column; preparative scale; 2.5 by 25 cm). PRFAR was eluted isocratically by 0.1 M tetraethylammonium acetate, monitored by optical density at 300 nm. Peak fractions containing PRFAR were pooled, lyophilized, and stored at –80°C. Purified PRFAR was analyzed by UV-visible light spectroscopy and gave characteristic absorbance peaks at A<sub>220</sub> and A<sub>300</sub>.

**HisHF enzyme assay.** The standard assay for *E. coli* HisHF (55) was performed as described elsewhere (45). The assay basis is the initial rate of substrate PRFAR disappearance as monitored by A<sub>300</sub>. Briefly 100 μM PRFAR is mixed with 5 mM glutamine in 50 mM Tris-HCl (pH 8.0), and the reaction is initiated by addition of purified HisHF at 0.02 U/ml (62 nM). The mixture was incubated at 25°C for 2 to 5 min. One unit of activity is defined as formation of 1 μmol of product per minute under the specified reaction conditions.

**K<sub>i</sub> determination.** To determine the inhibition constant (K<sub>i</sub>) for acivicin, the HisHF assay was performed in the presence of 400 or 625 nM acivicin at

glutamine concentrations of 0.125, 0.25, 0.5, 1, and 2 mM. The K<sub>i</sub> for acivicin was determined from the reciprocal plot of 1/V versus 1/[glutamine].

**HisHF inactivation by acivicin.** HisHF (1.0 μM) was preincubated at 25°C in 100 μl of 50 mM Tris-HCl (pH 8.0) with (i) 10 μM acivicin, (ii) 100 μM PRFAR, (iii) 10 μM acivicin and 100 μM PRFAR, and (iv) buffer only. An aliquot (12 μl) was removed at 0, 10, 20, 40, and 60 min and diluted into a reaction mixture (288 μl) containing 100 μM PRFAR, 5 mM glutamine, and 50 mM Tris-HCl (pH 8.0), and the initial rate of the reaction was measured as described above.

**Gene expression profiling.** The basic dual-label, fluorescence-based method has been described in detail elsewhere (86). These experiments differed from those described previously in that the genes were spotted at a higher density, i.e., 9,000 spots per slide, using a generation III DNA spotter (Molecular Dynamics, Sunnyvale, Calif.) such that an entire genome was spotted in duplicate on a single slide, negating the need for slide-to-slide correction. Genes were categorized into the functional groups of Riley and Labedan (67) as has been used in other transcript profiling exercises (85, 86).

## RESULTS AND DISCUSSION

**Nutritional supplementation.** Acivicin inhibited the growth of many *E. coli* K-12 and *S. enterica* serovar Typhimurium strains when grown on solidified minimal, but not rich, media. The presence of histidine or purines (guanine plus adenine), but not tryptophan, glutamate, or glucosamine-6-phosphate, significantly lessened the inhibition as monitored by disk diffusion assays (data not shown) with *E. coli* strain CS1562 (69) and *S. enterica* serovar Typhimurium strain TV101. Similar results were obtained when nutrient pools were used in auxanography (20) with a bioluminescent tester strain, DPD1718

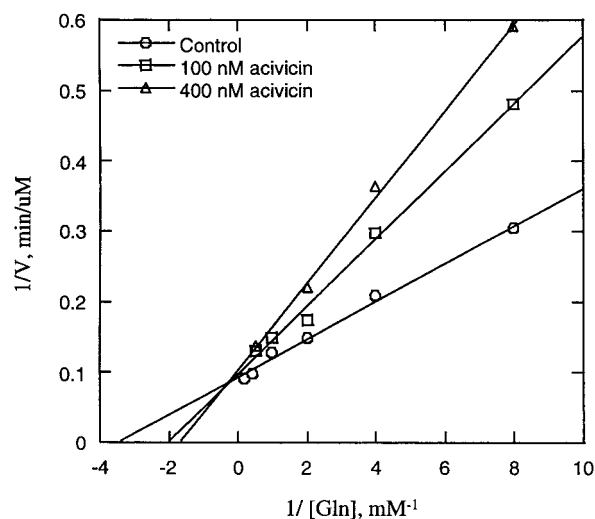


FIG. 4. Inhibition of *E. coli* IGP synthase (HisHF) by acivicin.

(data not shown). The mixture of adenosine, histidine, phenylalanine, glutamine, and thymine was completely effective at preventing inhibition by acivicin, while supplementation with histidine, lysine, and the three branched chain amino acids was almost as effective an antidote. Other pools were incapable of preventing the inhibitory action. Histidine alone was not quite as effective an antidote as either mixture. This, together with the structural similarity between acivicin and glutamine, suggested that the target of acivicin in *E. coli* might be the HisHF enzyme.

These results were somewhat surprising since acivicin has been suggested to target other enzymes, notably glutamine amidotransferases, including GMP synthetase (41, 54), CTP synthetase (54),  $\gamma$ -glutamyl transpeptidase (38), formylglycineamide ribonucleotide synthetase (24), and carbamoyl phosphate synthase (4), in mammalian and protozoan systems.  $K_i$ s as low as 2 (4) to 5 (24)  $\mu$ M and as high as 420  $\mu$ M (38) have been reported for these interactions.

**Acivicin is a competitive inhibitor of glutamine for HisHF.** HisHF catalyzes the reaction of PRFAR and glutamine to form of IGP, AICAR, and glutamate (88). The  $k_{cat}$  of *E. coli* HisHF is  $8.5 \text{ s}^{-1}$ , the  $K_m$  for glutamine is 240  $\mu$ M, and the  $K_m$  for PRFAR is 1.5  $\mu$ M (45). We assayed the activity of HisHF in the presence of 100 or 400 nM acivicin, with glutamine concentrations ranging from 0.125 to 2 mM, while keeping PRFAR at 100  $\mu$ M, a vast excess. A reciprocal plot of  $1/V$  versus  $1/[\text{glutamine}]$  was generated (Fig. 4), resulting in an estimate of 290  $\mu$ M for the glutamine  $K_m$ . This value was consistent with another determination noted above. Moreover, the  $K_i$  of acivicin was determined to be 140 nM. This indicates that acivicin is at least an order of magnitude more inhibitory in vitro toward HisHF than those enzymes that have been tested by others. Thus, acivicin was a potent inhibitor of HisHF in vitro.

In a second experiment, HisHF activity was measured with excess (5 mM) glutamine and various concentrations of PRFAR (5, 12.5, 25, and 50  $\mu$ M) in the presence or absence of 400 nM acivicin. No difference in the initial HisHF reaction rate was observed in the presence of the inhibitor (data not shown).

This indicated that acivicin was a competitive inhibitor of glutamine, but not PRFAR, binding to HisHF.

**The inactivation of HisHF by acivicin is accelerated by PRFAR.** A possible mechanism of acivicin action is that it binds competitively to the glutamine binding site on HisH and inactivates the enzyme by covalent modification of an active site cysteine residue essential for glutamine amidotransferase activity. HisH and HisF of *E. coli* are isolated as a single heterodimer (45). It thus is possible that both the glutamine amidotransferase activity of HisH and the cyclase activity of HisF are carried out at one active site shared between the two polypeptides and that the binding of glutamine and PRFAR is cooperative. A second possibility is that separate substrate binding sites exist on the HisH and HisF polypeptides. After the glutamine amidotransferase reaction is carried out on the HisH domain,  $\text{NH}_3$  might be transferred to the PRFAR binding site on the HisF domain. To test this hypothesis, acivicin was used to probe the active site of HisHF together with PRFAR. HisHF was preincubated with 10  $\mu$ M acivicin alone (1:10 ratio of enzyme to inhibitor), 100  $\mu$ M PRFAR alone (1:100 ratio of enzyme to substrate), or both 10  $\mu$ M acivicin and 100  $\mu$ M PRFAR. The preincubation time varied from 0 to 60 min. At each time point, an aliquot of the mixture was removed and assayed for remaining activity. The fraction of residual activity was plotted versus preincubation time (Fig. 5). In the presence of either acivicin or PRFAR alone, there was a time-dependent loss of HisHF activity. This indicates that both acivicin and PRFAR were irreversible inhibitors of the reaction when incubated with HisHF alone. It is rather interesting that PRFAR inhibited the reaction irreversibly when it was added to the enzyme before the addition of glutamine. Even more interesting, when both acivicin and PRFAR were preincubated with HisHF, the enzyme activity at the first time point (10 min) decreased dramatically to almost the background level. We have not yet investigated if inactivation is more than additive. In addition, binding of PRFAR to the active site may promote covalent

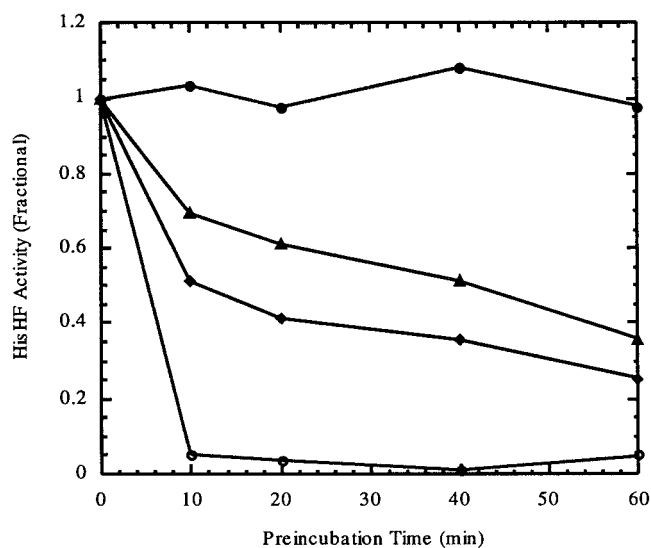


FIG. 5. Inactivation of *E. coli* IGP synthase (HisHF) by acivicin (triangles), PRFAR (diamonds), and acivicin plus PRFAR (open circles). The untreated enzyme is indicated by closed circles.

bond formation between glutamine and the catalytic cysteinyl residue of the HisH domain. These results were consistent with the proposed catalytic mechanism (66) and our hypothesis that the HisH domain and HisF domain share one active site that carries out both the glutamine amidotransferase and cyclase steps of the reaction. Moreover, they raise the possibility that the dominant factor contributing to the growth inhibition of a variety of microbial and eukaryotic cells could be the inactivation rates of various glutamine amidotransferases by acivicin as well as each enzyme's  $K_i$  for the small molecule.

**Reconstruction experiments.** Several salient features of *his* operon expression must be noted to aid in the interpretation of the following experiments. Three promoters (12) (Fig. 2A) are present in the operon although the primary promoter (*his*p1) is more than 10-fold stronger than *his*p2, which in turn is far stronger than *his*p3. Furthermore, transcription from *his*p1 occludes usage of *his*p2 (88). Initiation at *his*p1 is stimulated by the product of RelA, ppGpp (89), produced in response to starvation for any amino acid (13). Transcriptional read-through past *att* (43), the attenuator site, occurs when the *in vivo* level of histidyl-tRNA<sup>his</sup> is low (88). Thus, the operon is subject to both global and specific regulatory circuitry.

Plasmids capable of complementing *hisA* and *hisH* mutants were found by selecting for prototrophic recombinants after transformation with genomic libraries. The vector junctions with the chromosomal inserts were determined by sequencing. These results are summarized in Fig. 2B.

The following experiments were performed with the sequenced plasmids (Fig. 2B) to corroborate the presumption that HisHF was the primary target of acivicin within *E. coli*. Two micrograms of acivicin created a zone of inhibition with a diameter of  $38 \pm 3$  mm ( $n = 2$ ) on the control strain. RFM443/pBR322. Strain RFM443/pUC18 yielded a zone of  $34 \pm 0$  mm ( $n = 2$ ). Strain RFM443/pDEW335 (*his'*GDCBHAFI') had a greater tolerance; its zone of inhibition was  $19 \pm 1$  mm. RFM443/pDEW327 (*his'*BHAFI') was somewhat tolerant, having a zone of  $27 \pm 0$  mm ( $n = 2$ ) while RFM443/pDEW326 (*his'*BHAF') was sensitive, displaying a diameter of  $42 \pm 2$  mm ( $n = 2$ ). pDEW335 (*his'*GDCBHAFI') contains the nonoccluded *his*p2 promoter, while the other two *his* plasmids lack this internal promoter. Further, pDEW326 (*his'*BHAF') cannot specify an increased content of IGP synthase since only HisH and HisA polypeptides can be elevated. Thus, elevated expression of HisH, HisA, and HisF was sufficient for development of an acivicin-tolerant phenotype. Amplification of the *trp* control region, *trpE* and *trpD* expressing the two subunits of another glutamine amidotransferase, anthranilate synthase, did not result in tolerance; strain RFM443/pVV101 (*trpEDC'*) displayed a diameter of  $39 \pm 4$  mm ( $n = 2$ ). This result suggests that a glutamine amidotransferase cannot protect cells by simply acting as a macromolecular sponge (49) absorbing acivicin. Thus, amplification of the genes specifying the histidine biosynthetic glutamine amidotransferase, HisHF, conferred tolerance to the inhibitory agent.

This result was further confirmed with a bioluminescence experiment (Fig. 6). A plasmid expressing only HisH, HisA, and HisF (pDEW327) and a control vector (pUC18) were placed in a bioluminescent *E. coli* strain, DPD1718. The bioluminescence of each recombinant was titrated with acivicin. The response ratio obtained 400 min after the administration of

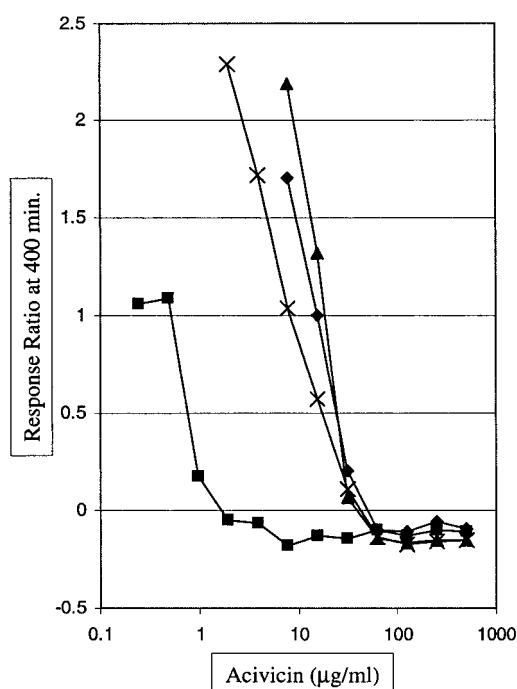


FIG. 6. Titration of a bioluminescent response by acivicin. The bioluminescent host strain DPD1718 was transformed with either pUC18 or pDEW327. Squares, the strain contained pUC18 and the medium was not supplemented with histidine; diamonds, the strain contained pUC18 and the medium was supplemented with histidine; crosses, the strain contained pDEW327 (*his'*BHAFI') and the medium was not supplemented with histidine; triangles, the strain contained pDEW327 (*his'*BHAFI') and the medium was supplemented with histidine.

acivicin was plotted as a function of inhibitor concentration. As can be seen from Fig. 6, much more acivicin was needed to inhibit bioluminescence from the strain in which *hisHAF* was amplified, again suggesting that HisHF was the primary target of acivicin. The protection afforded by *hisHAF* amplification was similarly supplied by supplementation with L-histidine (Fig. 6; compare the curve of the control strain treated with L-histidine to that of the strain carrying the *hisHAF* amplification in the absence of L-histidine). Evidence for L-histidine supplementation enhancing the protective effect of *hisHAF* amplification was not obtained (Fig. 6); such effects strongly indicate that HisHF or HisA was the *in vivo* target of acivicin.

**Genetic titration.** Libraries of random fragments of the *E. coli* genome in either pUC18 or pBR322, harbored in strain DPD1675, served as a source of genetic variation. Clones resistant to acivicin were selected, plasmids conferring the resistance phenotype were sequenced, and the precise locations of the resistance elements were thus determined. These resistance elements mapped to two regions distinct from *his*.

Nine plasmids mapped to one of these regions (9). Although each encompassed several genes in this region (Fig. 7), only a single gene, *yedA*, was present in each plasmid. The function of this gene has not yet been described, although homology to the PecM protein of *Erwinia chrysanthemi* ( $E$  value of  $7e-19$ ) has been noted by computational searches (1, 2). *yedA* is predicted to encode an integral membrane protein with nine regions spanning the lipid bilayer. It is tempting to speculate that YedA is a component of an acivicin export system.

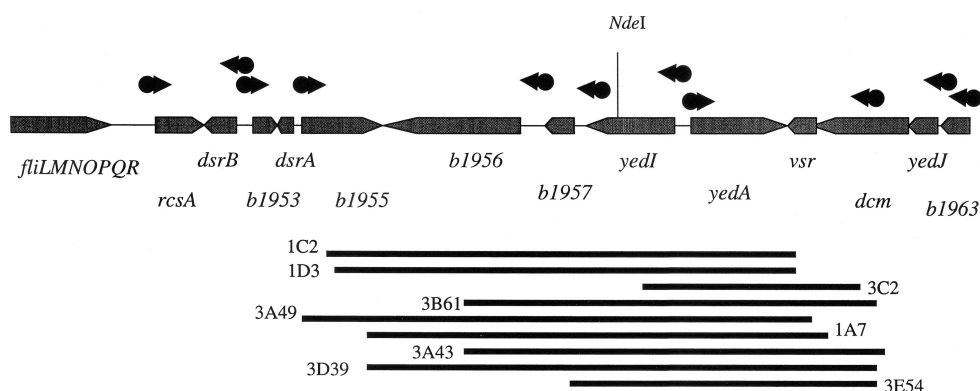


FIG. 7. *yedA* region of the *E. coli* chromosome. Lines below the map show the extents of various plasmids that confer acivicin resistance. Directions of transcription are indicated by arrowheads on symbols denoting genes; dots indicate promoters.

One other plasmid, mapping to a different region and containing intact sequences of *iciA* and *yqfE*, a divergently transcribed pair of genes, conferred resistance to acivicin (Fig. 8). Neither *iciA* nor *yqfE* alone resulted in an acivicin-resistant phenotype (data not shown). The function of *yqfE* is unknown, while those of IciA have been defined by *in vivo* and *in vitro* studies. IciA is a DNA binding protein that is a member of the LysR family of transcriptional regulators (76). It binds to a set of three double-stranded 13-mers within *oriC*, the origin of chromosomal replication. Such binding apparently antagonizes the creation of a bubble in the double helix; that bubble, stabilized by the specific binding to the same 13-mers in one single strand, is a prerequisite for the initiation of DNA synthesis (40). IciA is a pleiotropic regulator stimulating expression of *nrpD* (33), *dnaA* (53), and perhaps other genes. Moreover, IciA in the presence of arginine inhibits its own synthesis (14). *iciA* transcription is also positively regulated by PhoB when phosphate is depleted (34). Further, IciA is a substrate for proteolytic degradation by the *htrA*-encoded protease Do (91), providing a mechanism in addition to its synthesis early in the cell cycle (93) for its periodic pattern of accumulation. Hence, amplification of a complex sensing system may confer resistance to acivicin. The relationship between *yedA* and the *iciA* region has not yet been addressed. Studies combining chromosomal mutations in one region and amplification of the second region on a multicopy plasmid may be informative. Thus, amplification of three distinct genetic regions (two, *yedA* and *iciA-yqfE*, defined by genetic titration and one, *hisHAF*, proven by reconstruction) gave rise to acivicin resistance.

**Gene expression profiling.** The growth of *E. coli* strain MG1655 in minimal medium with glucose as a carbon source was inhibited about 80% by 0.5  $\mu\text{g}$  of acivicin per ml; such inhibition was completely prevented by coexposure to histidine. Histidine also reversed a greater acivicin challenge (2  $\mu\text{g}/\text{ml}$ , 90% inhibition); the culture exposed to both the antagonist and the antidote grew at about 90% of the uninhibited rate (data not shown). Results from a representative DNA microarray experiment, in which *E. coli* MG1655 was challenged with acivicin at 2  $\mu\text{g}/\text{ml}$  in minimal medium for 60 min, are presented in Tables 2 and 3. This treatment lowered the growth rate by about 85%. The structural similarity among acivicin, glutamine, and asparagine is illustrated in Fig. 1. This

similarity was reflected in the gene expression profile described below.

**Indications that acivicin serves as an imposter of certain natural amino acids.** Transcription of *glnA*, encoding glutamine synthetase, was lowered more than sixfold. Expression of *asnS*, coding for asparaginyl-tRNA synthetase, was decreased more than fivefold; unfortunately, the spotted *glnS* PCR product was of poor quality (Y. Wei and R. LaRossa, unpublished data), precluding insight into its transcriptional response to acivicin.

**Evidence that HisHF is the major target *in vivo*.** If the HisHF enzyme is inhibited by acivicin *in vivo*, then transcription should initiate frequently at the *his* promoter (75, 89), pass through the leader/attenuator (43), and traverse the structural genes. This expectation was indeed realized, as the eight structural genes, *hisGDCBHAFI*, and the leader *hisL* were up-regulated 6- to 16-fold by administration of 2  $\mu\text{g}$  of acivicin per ml. Ranking of open reading frames (ORFs) by fold induction with acivicin placed *his* operon genes in the 4th (*hisC*), 5th (*hisL*), 6th (*hisB*), 7th (*hisI*), 8th (*hisG*), 9th (*hisD*), 11th (*hisH*), 12th (*hisF*), and 28th (*hisA*) positions.

**Metabolic mayhem.** The term “metabolic mayhem” has been applied to the action of sulfonylurea herbicides in *S. enterica* serovar Typhimurium, which causes the cell to (i) signal methionine sufficiency (10) in the face of methionine limitation (51) and (ii) skew the ratios of 2-ketoacids (52) and acyl coenzyme A's (78), two classes of central precursor metabolites. Together, about 60% of the organic content of *E. coli* is derived from these two sets of central building blocks (48). A similar case may be evident when HisHF activity was limiting. The ATP pool was compromised (29, 42). The cell, however,

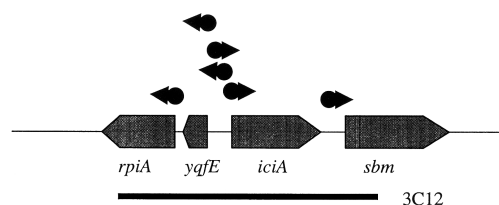


FIG. 8. *iciA* region of the *E. coli* chromosome, denoted as described for Fig. 7.

TABLE 2. Transcripts induced by acivicin treatment

Gene	Location	Fold induction	Gene	Location	Fold induction	Gene	Location	Fold induction	Gene	Location	Fold induction
<i>ackA</i>	b2296	2.0	<i>metB</i>	b3939	2.1	<i>b0305</i>	b0305	2.2	<i>b2300</i>	b2300	3.2
<i>acrA</i>	b0463	2.4	<i>metJ</i>	b3938	4.7	<i>b0358</i>	b0358	2.2	<i>b2302</i>	b2302	3.6
<i>acrR</i>	b0464	2.6	<i>metK</i>	b2942	4.3	<i>b0458</i>	b0458	3.5	<i>b2303</i>	b2303	2.8
<i>ahpC</i>	b0605	3.4	<i>metR</i>	b3828	2.8	<i>b0485</i>	b0485	2.6	<i>b2442</i>	b2442	2.2
<i>aldH</i>	b1300	4.4	<i>mobB</i>	b3856	2.2	<i>b0572</i>	b0572	3.5	<i>b2529</i>	b2529	2.1
<i>amyA</i>	b1927	2.3	<i>mrda</i>	b0635	2.3	<i>b0573</i>	b0573	2.6	<i>b2530</i>	b2530	2.7
<i>appA</i>	b0980	4.3	<i>mvp</i>	b2113	3.4	<i>b0581</i>	b0581	2.7	<i>b2597</i>	b2597	23.6
<i>araA</i>	b0062	5.5	<i>mscL</i>	b3291	3.8	<i>b0600</i>	b0600	2.1	<i>b2664</i>	b2664	3.2
<i>argA</i>	b2818	3.6	<i>msrA</i>	b4219	2.7	<i>b0607</i>	b0607	6.8	<i>b2665</i>	b2665	4.7
<i>argB</i>	b3959	5.7	<i>mtr</i>	b3161	20.6	<i>b0643</i>	b0643	5.3	<i>b2856</i>	b2856	2.6
<i>argC</i>	b3958	8.5	<i>murE</i>	b0085	2.4	<i>b0662</i>	b0662	2.2	<i>b2886</i>	b2886	2.1
<i>argD</i>	b3359	2.8	<i>nadC</i>	b0109	4.2	<i>b0707</i>	b0707	3.7	<i>b2889</i>	b2889	2.1
<i>argE</i>	b3957	6.4	<i>nagB</i>	b0678	2.6	<i>b0753</i>	b0753	2.5	<i>b2900</i>	b2900	2.0
<i>argG</i>	b3172	7.7	<i>narK</i>	b1223	7.5	<i>b0786</i>	b0786	2.4	<i>b2922</i>	b2922	2.3
<i>argH</i>	b3960	2.2	<i>nfo</i>	b2159	3.4	<i>b0789</i>	b0789	2.5	<i>b2941</i>	b2941	5.0
<i>argR</i>	b3237	4.0	<i>nfrA</i>	b0568	2.2	<i>b0790</i>	b0790	2.7	<i>b2959</i>	b2959	3.5
<i>aroG</i>	b0754	2.2	<i>nrdD</i>	b4238	7.8	<i>b0800</i>	b0800	2.7	<i>b2960</i>	b2960	2.6
<i>aspC</i>	b0928	7.5	<i>nrdG</i>	b4237	5.6	<i>b0806</i>	b0806	2.2	<i>b3010</i>	b3010	2.1
<i>betA</i>	b0311	2.4	<i>nrdH</i>	b2673	3.0	<i>b0836</i>	b0836	2.9	<i>b3011</i>	b3011	2.1
<i>betB</i>	b0312	2.4	<i>nrdI</i>	b2674	2.2	<i>b0865</i>	b0865	7.0	<i>b3021</i>	b3021	2.2
<i>betI</i>	b0313	4.3	<i>osmE</i>	b1739	2.6	<i>b0897</i>	b0897	3.1	<i>b3022</i>	b3022	3.1
<i>betT</i>	b0314	8.0	<i>osmY</i>	b4376	3.8	<i>b0964</i>	b0964	2.1	<i>b3024</i>	b3024	3.1
<i>bfr</i>	b3336	6.4	<i>otsA</i>	b1896	4.4	<i>b0966</i>	b0966	3.8	<i>b3029</i>	b3029	2.1
<i>blc</i>	b4149	2.3	<i>otsB</i>	b1897	3.4	<i>b1003</i>	b1003	2.5	<i>b3068</i>	b3068	4.5
<i>cdsA</i>	b0175	2.2	<i>panB</i>	b0134	4.4	<i>b1045</i>	b1045	2.6	<i>b3097</i>	b3097	2.6
<i>chaA</i>	b1216	2.4	<i>pckA</i>	b3403	2.5	<i>b1050</i>	b1050	4.7	<i>b3098</i>	b3098	2.9
<i>clpB</i>	b2592	8.6	<i>phnB</i>	b4107	7.8	<i>b1060</i>	b1060	2.5	<i>b3099</i>	b3099	2.2
<i>cls</i>	b1249	3.5	<i>phnG</i>	b4101	2.2	<i>b1103</i>	b1103	3.2	<i>b3160</i>	b3160	2.5
<i>csgA</i>	b1042	3.2	<i>proB</i>	b0242	2.7	<i>b1104</i>	b1104	3.1	<i>b3190</i>	b3190	2.8
<i>cspE</i>	b0623	2.8	<i>pta</i>	b2297	2.3	<i>b1105</i>	b1105	3.0	<i>b3203</i>	b3203	2.2
<i>cydA</i>	b0733	2.8	<i>ptsG</i>	b1101	2.3	<i>b1107</i>	b1107	2.5	<i>b3263</i>	b3263	2.9
<i>cynX</i>	b0341	4.2	<i>purA</i>	b4177	7.7	<i>b1108</i>	b1108	6.1	<i>b3292</i>	b3292	2.3
<i>dapB</i>	b0031	5.2	<i>qor</i>	b4051	2.4	<i>b1111</i>	b1111	4.2	<i>b3293</i>	b3293	2.5
<i>djf</i>	b3639	2.0	<i>recN</i>	b2616	3.2	<i>b1112</i>	b1112	3.2	<i>b3399</i>	b3399	2.5
<i>eaeH</i>	b0297	2.7	<i>ribE</i>	b1662	2.1	<i>b1128</i>	b1128	2.2	<i>b3400</i>	b3400	3.4
<i>entE</i>	b0594	2.7	<i>rna</i>	b0611	4.8	<i>b1145</i>	b1145	2.0	<i>b3401</i>	b3401	2.0
<i>fabB</i>	b2323	2.9	<i>rob</i>	b4396	2.8	<i>b1168</i>	b1168	2.1	<i>b3446</i>	b3446	2.7
<i>flgI</i>	b1080	2.2	<i>rpoH</i>	b3461	3.2	<i>b1178</i>	b1178	2.0	<i>b3448</i>	b3448	4.3
<i>frdB</i>	b4153	2.7	<i>sdaA</i>	b1814	3.0	<i>b1195</i>	b1195	2.2	<i>b3472</i>	b3472	2.5
<i>frdC</i>	b4152	2.0	<i>slp</i>	b3506	3.0	<i>b1205</i>	b1205	5.3	<i>b3494</i>	b3494	3.7
<i>frr</i>	b0172	3.4	<i>slyA</i>	b1642	2.6	<i>b1256</i>	b1256	3.5	<i>b3515</i>	b3515	2.7
<i>ftn</i>	b1905	10.6	<i>slyD</i>	b3349	2.1	<i>b1257</i>	b1257	3.8	<i>b3516</i>	b3516	2.0
<i>fucU</i>	b2804	4.5	<i>sohA</i>	b3129	2.4	<i>b1273</i>	b1273	2.0	<i>b3522</i>	b3522	3.5
<i>glgS</i>	b3049	3.3	<i>soxS</i>	b4062	2.8	<i>b1285</i>	b1285	3.1	<i>b3548</i>	b3548	3.8
<i>glpD</i>	b3426	2.2	<i>sseA</i>	b2521	2.1	<i>b1321</i>	b1321	2.7	<i>b3555</i>	b3555	2.3
<i>grpE</i>	b2614	2.5	<i>sugE</i>	b4148	2.5	<i>b1333</i>	b1333	3.0	<i>b3574</i>	b3574	2.2
<i>hemN</i>	b3867	2.1	<i>tdh</i>	b3616	3.7	<i>b1376</i>	b1376	7.0	<i>b3581</i>	b3581	2.8
<i>hepA</i>	b0059	4.8	<i>thdF</i>	b3706	2.3	<i>b1378</i>	b1378	2.0	<i>b3596</i>	b3596	4.0
<i>hisA</i>	b2024	6.2	<i>thrC</i>	b0004	2.6	<i>b1414</i>	b1414	2.2	<i>b3655</i>	b3655	2.6
<i>hisB</i>	b2022	14.3	<i>thrL</i>	b0001	6.6	<i>b1446</i>	b1446	3.1	<i>b3698</i>	b3698	2.6
<i>hisC</i>	b2021	16.4	<i>tolR</i>	b0738	6.6	<i>b1454</i>	b1454	2.3	<i>b3818</i>	b3818	3.1
<i>hisD</i>	b2020	11.8	<i>torD</i>	b0998	2.7	<i>b1586</i>	b1586	6.6	<i>b3827</i>	b3827	2.0
<i>hisF</i>	b2025	9.6	<i>treF</i>	b3519	2.4	<i>b1598</i>	b1598	2.1	<i>b3861</i>	b3861	2.1
<i>hisG</i>	b2019	12.1	<i>trg</i>	b1421	8.2	<i>b1667</i>	b1667	3.7	<i>b3875</i>	b3875	2.4
<i>hisH</i>	b2023	10.4	<i>tus</i>	b1610	2.3	<i>b1678</i>	b1678	2.0	<i>b3923</i>	b3923	3.7
<i>hisI</i>	b2026	13.6	<i>ugpB</i>	b3453	2.0	<i>b1725</i>	b1725	2.1	<i>b3928</i>	b3928	2.2
<i>hisJ</i>	b2309	2.8	<i>umuD</i>	b1183	2.2	<i>b1778</i>	b1778	2.5	<i>b3937</i>	b3937	2.7
<i>hisL</i>	b2018	15.7	<i>uspA</i>	b3495	4.2	<i>b1783</i>	b1783	2.7	<i>b3995</i>	b3995	2.0
<i>hslS</i>	b3686	34.4	<i>uxaB</i>	b1521	7.7	<i>b1816</i>	b1816	2.1	<i>b4030</i>	b4030	6.7
<i>hslT</i>	b3687	22.3	<i>xseB</i>	b0422	3.2	<i>b1869</i>	b1869	2.0	<i>b4126</i>	b4126	2.7
<i>htpX</i>	b1829	2.6	<i>xylE</i>	b4031	2.4	<i>b1870</i>	b1870	2.2	<i>b4127</i>	b4127	2.3
<i>hyaF</i>	b0977	2.0	<i>yfiB</i>	b2605	2.3	<i>b1871</i>	b1871	2.3	<i>b4135</i>	b4135	3.4
<i>ilvI</i>	b0077	2.4	<i>ygiG</i>	b3073	2.1	<i>b1953</i>	b1953	4.7	<i>b4178</i>	b4178	3.8
<i>insB_I</i>	b0264	2.1	<i>yhaH</i>	b3103	2.5	<i>b1955</i>	b1955	3.7	<i>b4189</i>	b4189	2.6
<i>intA</i>	b2622	2.5	<i>yihP</i>	b3877	2.2	<i>b2007</i>	b2007	2.3	<i>b4199</i>	b4199	2.8
<i>kbl</i>	b3617	2.1	<i>b0058</i>	b0058	4.1	<i>b2080</i>	b2080	7.2	<i>b4206</i>	b4206	2.6
<i>lacI</i>	b0345	2.2	<i>b0105</i>	b0105	2.8	<i>b2098</i>	b2098	2.4	<i>b4234</i>	b4234	2.0
<i>lpdA</i>	b0116	2.2	<i>b0119</i>	b0119	2.6	<i>b2112</i>	b2112	2.5	<i>b4255</i>	b4255	2.5
<i>melA</i>	b4119	2.1	<i>b0163</i>	b0163	2.1	<i>b2122</i>	b2122	2.5	<i>b4311</i>	b4311	2.0
<i>melR</i>	b4118	2.0	<i>b0233</i>	b0233	2.2	<i>b2127</i>	b2127	4.9	<i>b4325</i>	b4325	2.6
<i>menC</i>	b2261	2.1	<i>b0286</i>	b0286	2.2	<i>b2135</i>	b2135	2.1	<i>b4326</i>	b4326	9.4
<i>metA</i>	b4013	5.6	<i>b0288</i>	b0288	2.0	<i>b2299</i>	b2299	3.2			

TABLE 3. Transcripts repressed by acivicin treatment

Gene	Location	Fold induction	Gene	Location	Fold induction	Gene	Location	Fold induction	Gene	Location	Fold induction
<i>accC</i>	b3256	0.45	<i>fmu</i>	b3289	0.37	<i>minC</i>	b1176	0.44	<i>rplW</i>	b3318	0.11
<i>aceA</i>	b4015	0.37	<i>gadA</i>	b3517	0.31	<i>moaA</i>	b0781	0.26	<i>rplX</i>	b3309	0.29
<i>aceK</i>	b4016	0.48	<i>gadB</i>	b1493	0.41	<i>msbA</i>	b0914	0.07	<i>rplY</i>	b2185	0.14
<i>add</i>	b1623	0.21	<i>gatA</i>	b2094	0.13	<i>mukE</i>	b0923	0.16	<i>rpmA</i>	b3185	0.43
<i>ampE</i>	b0111	0.45	<i>gatB</i>	b2093	0.47	<i>mutT</i>	b0099	0.48	<i>rpmB</i>	b3637	0.22
<i>apaG</i>	b0050	0.39	<i>gatC</i>	b2092	0.27	<i>nadE</i>	b1740	0.49	<i>rpmD</i>	b3302	0.46
<i>appY</i>	b0564	0.35	<i>gatD</i>	b2091	0.49	<i>narG</i>	b1224	0.37	<i>rpmG</i>	b3636	0.25
<i>apt</i>	b0469	0.31	<i>gatY</i>	b2096	0.36	<i>narL</i>	b1221	0.49	<i>rpmH</i>	b3703	0.38
<i>aroA</i>	b0908	0.20	<i>gatZ</i>	b2095	0.10	<i>nhaB</i>	b1186	0.31	<i>rpmJ</i>	b3299	0.46
<i>aroF</i>	b2601	0.46	<i>gdhA</i>	b1761	0.27	<i>nohB</i>	b0560	0.47	<i>rpoA</i>	b3295	0.41
<i>aroH</i>	b1704	0.45	<i>gidB</i>	b3740	0.42	<i>nusA</i>	b3169	0.28	<i>rpsC</i>	b3314	0.13
<i>asd</i>	b3433	0.41	<i>glhA</i>	b3870	0.15	<i>ompA</i>	b0957	0.11	<i>rpsD</i>	b3296	0.41
<i>asnS</i>	b0930	0.19	<i>glhS</i>	b0680	0.00	<i>pdxH</i>	b1638	0.27	<i>rpsE</i>	b3303	0.47
<i>asr</i>	b1597	0.50	<i>glpE</i>	b3425	0.49	<i>pheA</i>	b2599	0.25	<i>rpsF</i>	b4200	0.18
<i>atpA</i>	b3734	0.44	<i>gltD</i>	b3213	0.22	<i>phoP</i>	b1130	0.20	<i>rpsG</i>	b3341	0.44
<i>atpC</i>	b3731	0.50	<i>gltF</i>	b3214	0.44	<i>pncB</i>	b0931	0.45	<i>rpsH</i>	b3306	0.27
<i>atpD</i>	b3732	0.45	<i>gltJ</i>	b0654	0.42	<i>ppn</i>	b3164	0.34	<i>rpsI</i>	b3230	0.13
<i>atpF</i>	b3736	0.47	<i>gpt</i>	b0238	0.47	<i>ppa</i>	b4226	0.36	<i>rpsJ</i>	b3321	0.10
<i>avtA</i>	b3572	0.48	<i>gst</i>	b1635	0.07	<i>pqiA</i>	b0950	0.19	<i>rpsK</i>	b3297	0.39
<i>bioD</i>	b0778	0.44	<i>guaA</i>	b2507	0.38	<i>prc</i>	b1830	0.33	<i>rpsM</i>	b3298	0.39
<i>btuR</i>	b1270	0.28	<i>guaB</i>	b2508	0.42	<i>priB</i>	b4201	0.18	<i>rpsN</i>	b3307	0.26
<i>cfa</i>	b1661	0.22	<i>gusC</i>	b1615	0.13	<i>priC</i>	b0467	0.46	<i>rpsP</i>	b2609	0.17
<i>cirA</i>	b2155	0.03	<i>gyrA</i>	b2231	0.47	<i>prtA</i>	b3300	0.29	<i>rpsQ</i>	b3311	0.24
<i>cspA</i>	b3556	0.12	<i>hdhA</i>	b1619	0.41	<i>proX</i>	b2679	0.32	<i>rpsR</i>	b4202	0.15
<i>cspC</i>	b1823	0.14	<i>hsdS</i>	b4348	0.41	<i>pyrB</i>	b4245	0.05	<i>rpsS</i>	b3316	0.15
<i>cybB</i>	b1418	0.47	<i>iclR</i>	b4018	0.41	<i>pyrI</i>	b4244	0.20	<i>rpsT</i>	b0023	0.40
<i>cyoC</i>	b0430	0.42	<i>ilvB</i>	b3671	0.46	<i>rbsA</i>	b3749	0.35	<i>rpsU</i>	b3065	0.18
<i>cysA</i>	b2422	0.27	<i>ilvC</i>	b3774	0.04	<i>rhsD</i>	b0497	0.44	<i>secG</i>	b3175	0.36
<i>cysC</i>	b2750	0.43	<i>ilvD</i>	b3771	0.35	<i>ribB</i>	b3041	0.43	<i>sfsA</i>	b0146	0.50
<i>cysD</i>	b2752	0.48	<i>ilvE</i>	b3770	0.35	<i>rimL</i>	b1427	0.23	<i>sodA</i>	b3908	0.45
<i>cysK</i>	b2414	0.35	<i>ilvG</i>	b3767	0.09	<i>rmpA</i>	b3704	0.22	<i>speD</i>	b0120	0.46
<i>cysM</i>	b2421	0.29	<i>ilvM</i>	b3769	0.28	<i>rpiR</i>	b4089	0.43	<i>suhB</i>	b2533	0.25
<i>cysN</i>	b2751	0.28	<i>infB</i>	b3168	0.47	<i>rplA</i>	b3984	0.15	<i>surA</i>	b0053	0.31
<i>cysP</i>	b2425	0.42	<i>insA_3</i>	b0275	0.33	<i>rplB</i>	b3317	0.13	<i>thrA</i>	b0002	0.32
<i>dnaG</i>	b3066	0.39	<i>insB_2</i>	b0274	0.49	<i>rplC</i>	b3320	0.09	<i>trmD</i>	b2607	0.09
<i>dppA</i>	b3544	0.48	<i>ksgA</i>	b0051	0.25	<i>rplD</i>	b3319	0.11	<i>trpL</i>	b1265	0.46
<i>dsbB</i>	b1185	0.42	<i>lepB</i>	b2568	0.47	<i>rplE</i>	b3308	0.25	<i>tyrB</i>	b4054	0.45
<i>efp</i>	b4147	0.33	<i>leuC</i>	b0072	0.44	<i>rplF</i>	b3305	0.29	<i>tyrS</i>	b1637	0.17
<i>evgA</i>	b2369	0.21	<i>leuD</i>	b0071	0.24	<i>rplI</i>	b4203	0.37	<i>uidA</i>	b1617	0.13
<i>exbB</i>	b3006	0.11	<i>livG</i>	b3455	0.36	<i>rplJ</i>	b3985	0.18	<i>uidB</i>	b1616	0.05
<i>exbD</i>	b3005	0.10	<i>livJ</i>	b3460	0.13	<i>rplK</i>	b3983	0.25	<i>umuC</i>	b1184	0.50
<i>fecA</i>	b4291	0.27	<i>livK</i>	b3458	0.18	<i>rplL</i>	b3986	0.17	<i>upp</i>	b2498	0.15
<i>fecI</i>	b4293	0.11	<i>lysC</i>	b4024	0.41	<i>rplM</i>	b3231	0.16	<i>vacJ</i>	b2346	0.43
<i>fecR</i>	b4292	0.16	<i>malF</i>	b4033	0.50	<i>rplN</i>	b3310	0.30	<i>xseA</i>	b2509	0.42
<i>fepB</i>	b0592	0.47	<i>mall</i>	b1620	0.42	<i>rplO</i>	b3301	0.37	<i>ygiC</i>	b3038	0.35
<i>fhuE</i>	b1102	0.25	<i>manX</i>	b1817	0.43	<i>rplP</i>	b3313	0.15	<i>yjiR</i>	b4366	0.08
<i>fimA</i>	b4314	0.42	<i>marR</i>	b1530	0.36	<i>rplR</i>	b3304	0.41	<i>yjiS</i>	b4367	0.12
<i>fis</i>	b3261	0.13	<i>mepA</i>	b2328	0.48	<i>rplS</i>	b2606	0.15	<i>yjiT</i>	b4371	0.40
<i>fixX</i>	b0044	0.49	<i>metE</i>	b3829	0.13	<i>rplU</i>	b3186	0.48	<i>zwf</i>	b1852	0.48
<i>flgD</i>	b1075	0.32	<i>mgtA</i>	b4242	0.46	<i>rplV</i>	b3315	0.12			

did not respond by elevating the F<sub>0</sub>-F<sub>1</sub> ATP synthase-specifying transcripts; rather, the *atp* operon was mildly repressed (Table 3), indicating that the capacity to convert ADP to ATP was not enhanced. Most purine biosynthetic transcripts were not affected appreciably by the acivicin administration, suggesting that the PurR regulon (92) was indifferent to this treatment. Evidence for modulating expression of two purine-related operons, however, was found. The bicistronic *guaBA* operon was down-regulated about twofold (Table 3), while the *purA* transcript was elevated more than sevenfold (Table 2). The latter result contradicted the thought (37) that the PurR-independent regulation of *purA* is posttranscriptional. Both the *purA* and *guaBA* operons are regulated by multiple regulatory circuits (92). *guaBA* is responsive to PurR (92), cyclic AMP receptor protein (39), and DnaA (92), while *purA* is controlled

by both PurR and an adenine-dependent mechanism (92). These transcriptional data suggested that flux from IMP to AMP was being encouraged at the expense of forming GMP from the common intermediate IMP. Thus, the apparent inconsistencies in the transcriptional regulation of the purine regulon might be indicative of a baroque regulatory mechanism designed to maintain balance between the GTP and ATP pools.

**Acivicin triggers the stringent response.** The just-mentioned induction and repression of gene expression suggest that the in vivo levels of histidyl-tRNA, glutamyl-tRNA, and/or asparaginyl-tRNA may be lowered by acivicin treatment. Any such a drop would trigger the stringent response. This response has two basic elements, conservation of amino acid reserves by shutting off synthesis of ribosomes and other translational ma-



chinery (13) and a redirection of resources toward increasing amino acid biosynthesis (75, 89). Aspects of each are apparent in the gene expression profile of acivicin-treated cells. As expected for a treatment resulting in amino acid starvation, expression of the translational apparatus was decreased; 49 distinct ORFs encoding proteins involved in translation were down-regulated by a factor of 2 or more (Table 3).

Evidence for elevation of amino acid biosynthetic capacity was also found (Table 2). Seven *arg* genes were induced three- to ninefold by this treatment. Also highly induced were biosynthetic genes corresponding to the aspartate-derived family of amino acids. The gene, *aspC*, specifying the major transaminase responsible for aspartate formation was elevated eightfold. The leader transcript of the *thr* operon was elevated sevenfold, while expression of five *met* ORFs was enhanced two- to sixfold. Acivicin exposure also induced the lysine synthesis-involved *dapB* mRNA by a factor of 5. This subdivision of induced transcripts by metabolic origin of amino acids is unexpected. It suggests, moreover, that the global response to amino acid starvation may be more complex than suggested by current dogma or that acivicin's targets include factors other than HisHF.

In contrast, transcription of only a single amino acid transport gene, *mtr*, was elevated (Table 2). This elevation, by a factor of 21, was dramatic; *mtr* was the third most highly induced gene. Amino acid transport had been suggested to be under stringent control (13), based primarily on studies of branched chain amino acid transport (63). That suggestion may need to be reevaluated in light of these findings. The reported pleiotropic effects of the stringent response are quite broad (13). The transcriptional responses elicited by other amino acid antagonists as well as the dependence of these responses on *relA* will further define this regulon.

#### Other stress responses triggered by the acivicin challenge.

The two most highly induced genes were *hslS* and *hslT*, heat shock loci (65), whose transcripts were elevated 20- to 30-fold (Table 2). Other stress-responsive transcripts (Table 2) were highly induced, including *clpB* (8.6-fold), *appA* (4.3-fold), *uspA* (4.2-fold), *ahpC* (3.4-fold), *rpoH* (3.2-fold), *slp* (3-fold), *rob* (2.8-fold), *cspE* (2.8-fold), *soxS* (2.8-fold), *htpX* (2.6-fold), *osmE* (2.6-fold), *sugE* (2.5-fold), *grpE* (2.5-fold), *sohA* (2.4-fold), and *slyD* (2.1-fold). Also within this group of induced mRNA species were transcripts involved in osmotolerance, including *betT* (8-fold), *otsA* (4.4-fold), *betI* (4.3-fold), *osmY* (3.8-fold), *otsB* (2.7-fold), *osmE* (2.6-fold), *treF* (2.4-fold), *betA* (2.4-fold), and *betB* (2.4-fold). Expression of some genes involved in DNA and RNA metabolism was also heightened; elevated levels of *hepA*, *ma*, *nfo*, *recN*, and *xseB* transcripts were observed.

Expression of genes involved in iron metabolism was also elevated by acivicin treatment. Induced genes included *fn* (11-fold), *bfr* (6.4-fold), and *entE* (2.7-fold). Transcripts of *cydA*, *frdB*, *frdC*, *nrdD*, *nrdG*, *nrdH*, *nrdI*, *qor*, and *torD*, involved in respiratory activity, were also elevated. Cause and effect are difficult to separate. The implied increase in iron metabolism could elevate the superoxide content, as suggested by the increased *soxS* mRNA titer. Such puzzles, as well as that involving the interplay between acivicin and respiration, await further study.

**Unanticipated repression by acivicin.** Many changes were observed that were not predictable. Strikingly, the level of several amino acid biosynthetic transcripts was not up-regulated but rather reduced by histidine starvation (Table 3), including transcripts for genes specifying components of the aromatic pathways (*aroA*, *aroF*, *aroH*, *pheA*, *trpL*, and *tyrB*), the pyruvate family (*avt*, *ilvB*, *ilvC*, *ilvD*, *ilvE*, *ilvG*, *ilvM*, *leuC*, and *leuD*), sulfur amino acids (*cysA*, *cysC*, *cysD*, *cysK*, *cysM*, *cysN*, and *metE*), and the aspartate family (*lysC*). Greater than fivefold repression was observed for *aroA*, *ilvC*, *ilvG*, and *metE*. Each of these genes is distinctive; *aroA* (16) and *ilvG* (47) specify enzymes targeted by commercially important herbicides, while *ilvC* and *metE* encode enzymes that must be highly expressed (86) due to their poor performance as catalysts (32, 60, 90). Interestingly, expression of another most highly expressed, pyrimidine biosynthetic operon (86) was down-regulated by the acivicin challenge; *pyrBI* (specifying aspartate transcarbamylase) transcripts were greatly reduced. Surprisingly, genes implicated in uptake of amino acids or their precursors were often down-regulated. Included in this category were *cysP*, *livJK*, and *proX*. Certain carbon utilization transcripts were down-regulated. Repression of six *gat* and three *uid* genes was observed.

Like the data concerning expression of amino acid permease systems in response to acivicin treatment discussed earlier, the unexpected and strong repression of many highly expressed biosynthetic genes by acivicin treatment suggests that the response to amino acid starvation is not a uniform induction of appropriate defenses. Rather, the cellular logic appears to be more selective; the cost of synthesizing a poor catalyst like IlvC or MetE may outweigh the benefits associated with their action. Thus, we are surprised that the decision to correct the perceived imbalance or to wait for the insult to pass may be made on a gene-by-gene basis.

**Unanticipated elevation of gene expression by acivicin administration.** Expression of three genes, *ack* (78), *pta* (78), and *mnp* (D. R. Smulski and R. A. LaRossa, unpublished data), whose inactivation leads to a sulfometuron methyl-sensitive phenotype in *S. enterica* serovar Typhimurium LT2 (52), was elevated after acivicin treatment. Other transcripts increased by this treatment were specified by the *tdh-kbl* operon. The corresponding gene products specified by this operon degrade threonine to pyruvate and ammonia through glycine and serine. Thus, the overall pathway involves *tdh*, *kbl*, *gcvTHP*, *lpdA*, *glyA*, and *sdaA* or *metC* (64). The *lpdA* and *sdaA* mRNA titers, as well as the *tdh-kbl* transcript levels, were elevated by acivicin treatment, suggesting increased flux through this pathway.

**Summation.** Acivicin is a natural product produced by streptomycetes. Its ecological role may be to defend the home turf of the producing species, encouraging other microbes to emigrate toward less hostile environments. Such use in antibacterial warfare may be explained by theories concerning the evolution of translation (18, 19) and biosynthetic pathways. Hence, adoption of acivicin for use in cancer therapy represents its introduction into a novel niche, one occupied by cells lacking its intended target, HisHF. Thus, studies of the action of acivicin with other glutamine amidotransferases could represent more general aspects of the binding of glutamine to the amidotransferase enzyme family. In contrast, acivicin interacted much more avidly with HisHF from *E. coli*. This specific interaction

is not limited to the *E. coli* enzyme; inhibition of the homologous eukaryotic enzyme, His7 from yeast, has been found (M. McCluskey and L. Huang, unpublished data). The relative contributions of acivicin's competition with glutamine and its inactivation of glutamine amidotransferases to the observed *in vivo* effects are worthy of further study.

That acivicin was targeted toward HisHF *in vivo* was demonstrated in several ways. Nutritional reversal of acivicin action by inclusion of histidine in the test medium was most suggestive. Amplification of the *hisHAF* portion of the histidine operon resulted in a resistance phenotype, also supporting the presumption that HisHF was the *in vivo* target. Finally, the gene expression profiling demonstrated that the cell was limited for histidine. Such starvation can both drain adenylates and limit histidyl-tRNA formation. In analogy to the detailed physiological and genetic studies of sulfometuron methyl action (48, 52), separation of these two consequences can be accomplished by determining the acivicin-induced change in the transcriptional profile of a feedback-insensitive *hisG* mutant grown in the presence of histidine. Given these results, it was somewhat surprising that a *hisHAF*-containing plasmid was not obtained from the multicopy libraries when acivicin resistance was used as a genetic selection. The large (>7-kbp) size of the operon may have contributed to this failure to obtain the expected result. Alternatively, the attenuation mechanism, unlike that of repression, may not be titrated out by the presence of the *his* operon on a multicopy plasmid.

Thus, several sorts of experiments were brought to bear on the interaction of *E. coli* and acivicin. Collectively, they supported the conclusion that acivicin inhibited HisHF while suggesting an intricate interaction between cell and inhibitor. Much of the gene expression profile could be understood by reference to the vast, preceding study of *E. coli* physiology. Without combining such knowledge with the realization that acivicin is a mimic of the natural amino acid L-glutamine, interpretation would be more difficult. Since inhibitor binding does not have to coincide with enzyme active sites, such mimicry may not always be as obvious as in the case of acivicin. Thus, we suggest that a multifaceted approach to inhibitor action remains the most likely path to definition of macromolecular targets. Especially informative may be the selection of missense mutants having a resistance phenotype; this approach has defined both inhibitor targets and the means by which these compounds are imported into the cell (46).

In addition, the gene expression profile also suggested several novel regulatory circuits. Each of these is worthy of further study. Two examples are noted. Transcripts of more than 150 genes of unknown function were elevated by exposure to acivicin; the dependence of these changes on various global regulatory mechanisms can be readily evaluated. The massive and apparently selective loss of highly expressed transcripts upon treatment is also provocative. Moreover, this global view suggested that the mRNA population is dramatically remodeled in response to acivicin. This unanticipated remodeling indicates that gene expression profiling will become a most important means for uncovering the pleiotropic responses to inhibitor action. Thus, comprehensive transcript profiling is an important tool for the biological detective; its findings, however, must be vigorously pursued by other methodologies if their authenticity is to be established.

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