THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 12, pp. 7960–7969, March 20, 2009

A Regulatory Role of the Bateman Domain of IMP Dehydrogenase in Adenylate Nucleotide Biosynthesis*

Received for publication, November 10, 2008, and in revised form, January 16, 2009 Published, JBC Papers in Press, January 18, 2009, DOI 10.1074/jbc.M808541200

Maxim Pimkin[1], **Julia Pimkina, and George D. Markham**[2]

From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The Bateman domain (CBS subdomain) of IMP dehydrogenase (IMPDH), a rate-limiting enzyme of the *de novo* GMP biosynthesis, is evolutionarily conserved but has no established function. Deletion of the Bateman domain has no effect on the *in vitro* IMPDH activity. We report that *in vivo* deletion of the Bateman domain of IMPDH in *Escherichia coli* (*guaB*$^{\Delta CBS}$) sensitizes the bacterium to growth arrest by adenosine and inosine. These nucleosides exert their growth inhibitory effect via a dramatic increase in the intracellular adenylate nucleotide pool, which results in the enhanced allosteric inhibition of PRPP synthetase and consequently a PRPP deficit. The ensuing starvation for pyrimidine nucleotides culminates in growth arrest. Thus, deletion of the Bateman domain of IMPDH derepresses the synthesis of AMP from IMP. The growth inhibitory effect of inosine can be rescued by second-site suppressor mutations in the genes responsible for the conversion of inosine to AMP (*gsk*, *purA*, and *purB*) as well as by the *prsA1* allele, which encodes a PRPP synthetase that is insensitive to allosteric inhibition by adenylate nucleotides. Importantly, the *guaB*$^{\Delta CBS}$ phenotype can be complemented *in* trans by a mutant *guaB* allele, which encodes a catalytically disabled IMPDH^{C305A} protein containing an intact Bateman domain. We conclude that the Bateman domain of IMPDH is a negative trans-regulator of adenylate nucleotide synthesis, and that this role is independent of the catalytic function of IMPDH in the *de novo* GMP biosynthesis.

Inosine 5′-monophosphate dehydrogenase (IMPDH)[3] catalyzes the first committed reaction in the *de novo* synthetic pathway of GMP, the NAD-dependent oxidation of IMP to XMP (1). IMP is the last common precursor of both guanylate and adenylate nucleotides and also serves as a substrate for adenylosuccinate (AMPs) synthetase, which commits IMP to adenylate nucleotide biosynthesis (Fig. 1). Sources of IMP include *de novo* synthesis starting from 5-phosphoribosyl 1-pyrophosphate (PRPP), regeneration from AMP and GMP, as well as salvage of hypoxanthine and inosine by phosphoribosylation and phosphorylation, respectively (1). Inhibition of IMPDH depletes the cellular guanylate pool and is antiproliferative, which has led to the establishment of IMPDH as a target for anti-tumor, immunosuppressive, and antimicrobial therapies (2, 3).

A multitude of crystal structures of IMPDH from various sources have been reported, and these have collectively created a detailed picture of the catalytic domain structure-function relationships (for reviews see Refs. 4 and 5). IMPDH is a tetramer with each subunit consisting of two structurally discrete domains (6). The larger catalytic domain is an α–β barrel of about 400 amino acids. The approximately 120-residue subdomain is inserted within the center of the dehydrogenase sequence and is composed of two tandem repeats of an amino acid sequence motif with homology to the enzyme cystathionine β-synthase (CBS). Pairs of CBS sequences are common in many proteins of unrelated functions and are also known as Bateman domains (7, 8). Despite the nearly absolute conservation of a Bateman domain in the several hundred known IMPDH sequences, the physiological function of this structure remains a mystery. Amino acid substitutions in the Bateman domain of human IMPDH type 1 are associated with the RP10 form of autosomal dominant retinitis pigmentosa, a hereditary degenerative disease of the retina (9–11). However, extensive evidence indicates that amino acid substitutions in the Bateman domain of IMPDH, as well as a complete deletion of the structure, do not impair the *in vitro* catalytic activity of the enzyme (12–15). The subdomain therefore appears irrelevant to the catalytic function of the core domain and has been speculated to have an as yet to be discovered moonlighting role. This is in sharp contrast to several other enzymes, such as AMP-dependent protein kinase and cystathionine β-synthase, which are allosterically regulated by binding of adenosine-containing compounds to their Bateman domains (16, 17).

In our previous study we created a bacterial model that allowed us to gain initial insights into the possible *in vivo* functions of the Bateman domain of IMPDH (18). A guanine prototrophic *Escherichia coli* strain (MP101, *guaB*$^{\Delta CBS}$) was constructed in which the Bateman domain coding sequence was deleted from the chromosomal *guaB* gene for IMPDH while preserving the catalytic function of the core enzyme. The metabolic effects of this mutation allowed us to conclude that the Bateman domain of IMPDH plays an important role in main-

* This work was supported, in whole or in part, by National Institutes of Health Grants GM072425 and CA06927. This work was also supported by an appropriation from the Commonwealth of Pennsylvania. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute, or any other sponsoring organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Recipient of the Fox Chase Cancer Center Plain and Fancy Postdoctoral Fellowship.

[2] To whom correspondence should be addressed: 333 Cottman Ave., Philadelphia, PA 19111. Tel.: 215-728-2439; Fax: 215-728-3574; E-mail: gd_markham@fccc.edu.

[3] The abbreviations used are: IMPDH, inosine 5′-monophosphate dehydrogenase; AMPs, adenylosuccinate; XMP, xanthosine 5′-monophosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; ppGpp, guanosine 3′-diphosphate 5′-diphosphate; CBS, cystathionine β-synthase; MOPS, 4-morpholinepropanesulfonic acid.

taining the physiological adenylate nucleotide pool and in the regulation of the purine nucleotide turnover.

In the present study we employed metabolic phenotype screening to identify additional phenotypic manifestations of the *guaB*$^{\Delta CBS}$ mutation. We found that growth of the *guaB*$^{\Delta CBS}$ mutant on minimal media is arrested by adenosine or inosine, and that these exert their growth inhibitory effect via a dramatic increase in the adenylate nucleotide pool size. The increase in the intracellular concentrations of the adenylate nucleotides leads to enhanced allosteric inhibition of PRPP synthetase and, as a result, starvation for PRPP. An insufficient PRPP supply causes depletion of the pyrimidine nucleotide pool and culminates in growth arrest. We provide evidence that the Bateman domain of IMPDH negatively regulates the biosynthesis of AMP, possibly via inhibition of AMPs synthetase, and that this regulation is independent of the IMPDH catalytic function.

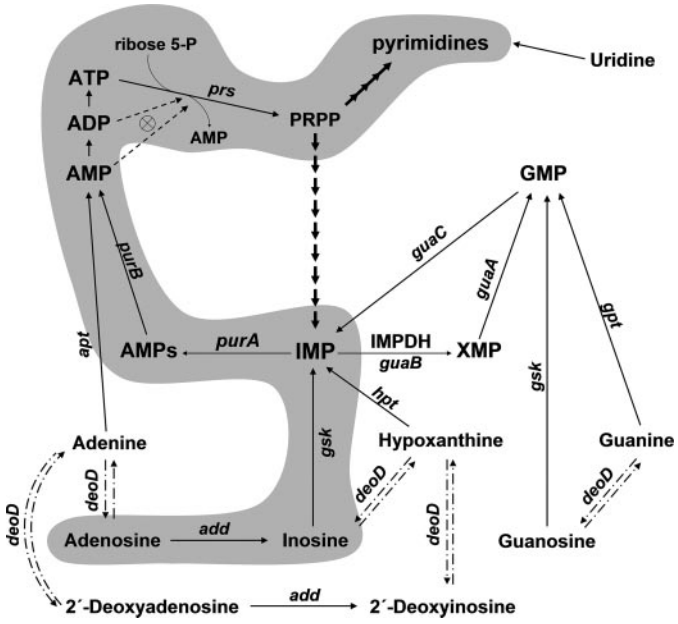

FIGURE 1. **The *de novo* and salvage pathways for purine nucleotide biosynthesis.** The pathway of adenosine/inosine toxicity is highlighted. *Dash-dot lines* indicate the reversible reactions catalyzed by purine nucleoside phosphorylase (*deoD*); most strains employed in this study are null for the enzyme. *Dashed lines* indicate feedback regulation of PRPP synthetase by ADP and AMP. The gene designations are: *purA*, adenylosuccinate synthetase; *purB*, adenylosuccinate lyase; *guaB*, IMP dehydrogenase; *guaA*, GMP synthetase; *deoD*, purine nucleoside phosphorylase; *hpt*, guanine-hypoxanthine phosphoribosyltransferase; *gpt*, guanine phosphoribosyltransferase; *gsk*, guanosine-inosine kinase; *apt*, adenine phosphoribosyltransferase; *add*, adenosine deaminase; *guaC*, GMP reductase; *prs*, PRPP synthetase.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The antibiotic concentrations used throughout this study were as follows: tetracycline, 20 μg/ml; kanamycin, 35 μg/ml; chloramphenicol, 12.5 μg/ml. All cell growth procedures were carried out at 37 °C. MOPS medium (19) supplemented with 0.4% glucose as carbon source was used as the standard minimal salts growth medium. Some growth phenotypes were also verified in M9 minimal salts medium with 0.2% glucose (data not shown). MOPS medium containing 0.0125% glucose was used for glucose-limited growth. All minimal media were routinely supplemented with 1 μg/ml thiamine.

For the strain sources and cloning strategies please refer to Table 1. For oligonucleotide sequences see Table 2. The P1*vir* transductions and molecular cloning procedures were carried out as described elsewhere (20, 21). The genetic identities of the recombinant DNA constructs and strains were verified by PCR and sequencing.

The *E. coli* strains MP255 and MP350 were constructed from BW25113 and MP101, respectively, by replacing the *deoD* gene for purine nucleoside phosphorylase with a *tet* cassette that confers resistance to tetracycline. The sequences of the DM465 and DM466 oligonucleotides used for *tet* cassette amplification from the TT25401 genomic DNA are presented in Table 2. Similarly, the DM402 strain was created by replacing the *purB* gene of BW25113 with a kanamycin resistance cassette. The DM475 and DM476 PCR primers, carrying *purB*-complementary 5′ adaptors, were used to amplify the *kan* cassette from the

TABLE 1
Strains and plasmids used in this study

Strain/plasmid	Parent/genotype	Ref./source/construction
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	22
MP101	[BW25113] *guaB*$^{\Delta CBS}$	18
JW1615	[BW25113] *add*::*kan*	Keio collection (44)
JW5401	[BW25113] *guaB*::*kan*	Keio collection (44)
JW0466	[BW25113] *gsk*::*kan*	Keio collection (44)
JW4135	[BW25113] *purA*::*kan*	Keio collection (44)
JW0101	[BW25113] *guaC*::*kan*	Keio collection (44)
TT25401	*tet*$^{+}$	Tet^{R}; J. Roth laboratory[a]
MP310	[BW25113] *guaB*$^{\Delta CBS}$ *add*::*kan*	MP101 × P1(JW1615), Kan^{r}
MP330	[BW25113] *guaB*$^{\Delta CBS}$ *gsk*::*kan*	MP101 × P1(JW0466), Kan^{r}
MP255	[BW25113] *deoD*::*tet*	Recombineering, BW25113 *deoD* → *tet,* Tet^{r}
MP350	[BW25113] *guaB*$^{\Delta CBS}$ *deoD*::*tet*	Recombineering, MP101 *deoD* → *tet,* Tet^{r}
MP2501	[BW25113] *deoD*::*tet purA*::*kan*	MP255 × P1(JW4135), Kan^{r} Tet^{r}
MP3501	[BW25113] *guaB*$^{\Delta CBS}$ *deoD*::*tet purA*::*kan*	MP350 × P1(JW4135), Kan^{r} Tet^{r}
MP402	[BW25113] *purB*::*kan*	Recombineering, BW25113 *purB* → *kan,* Kan^{r}
MP4021	[BW25113] *deoD*::*tet purB*::*kan*	MP255 × P1(MP402), Kan^{r} Tet^{r}
MP4022	[BW25113] *guaB*$^{\Delta CBS}$ *deoD*::*tet purB*::*kan*	MP350 × P1(MP402), Kan^{r} Tet^{r}
pGUAB	pCC1	*guaB*^{wt} gene for IMPDH, native promoter (18)
pGUA6	pGUAB	*guaB*$^{TGT(914–916)→GCG}$ gene for IMPDH^{C305A}, native promoter
pGSK	pCC1	*gsk* gene for guanosine-inosine kinase under control of constitutive P*bla*$_{TEM}$ promoter
pPRS	pCC1	Wild-type *prs* allele for PRPP synthetase, native promoter
pPRS-A1	pPRS	*prsA1* allele for ADP/AMP-insensitive PRPP synthetase (*prs*^{A386C}), native promoter (23)

[a] J. Roth, unpublished data.

TABLE 2
Sequences of the oligonucleotides used in this study

Primer	Sequence
DM481	5′-GCCAAAAAGGTCAATAGAAACGTTG
DM482	5′-GCATAGGTCATACAAATGGATATTACAGAC
DM483[a]	5′-CGGTGTTGACCGTGTGCTGACAGTGGCTCTGCACGCTGAACAGATTCAGGGTTTC
DM484	5′-GAAACCCTGAATCTGTTCAGCGTGCAGAGCCACTGTCAGCACACGGTCAACACCG
DM479[b]	5′-AAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGTTTCAATAATTTCACACAGGAAACAGCTATGAAATTTCCCGGTAAACGTA
DM480	5′-TTAACGATCCCAGTAAGACTCTTCC
DM429[a]	5′-CGGCATTGGCCCTGGCTCTATCGCGACAACTCGTATCGTGACTGGCG
DM430	5′-CGCCAGTCACGATACGAGTTGTCGCGATAGAGCCAGGGCCAATGCCG
DM465[c]	5′-GAGCGTTGACTCCGCCTTTGTTATGTCACAAAAAGGATAAAACACACCAAACACCCCCCAAAACC
DM466[c]	5′-CTCCCGCTCCGGCTTCACAAGGCAATCGCCTTGCAGCGAAACACAACACACAACCACACCACACCAC
DM475[c]	5′-CTCAGGCAAAACAAATTCTTGCTCATTTAACCCCGGAGTTGTGATCTGGAGCTGCTTCGAAGTTCC
DM476[c]	5′-CTTAATAAGCAGGCCGGACAGCATCGCCATCCGGCACTGATACGAGGTATGAATATCCTCCTTAGTTCC

[a] The mutagenic sequence is underlined.
[b] The 5′ extension containing the P*bla*$_{TEM}$ promoter sequence is underlined.
[c] The 5′ extension for recombination with the target gene is underlined.

pKD4 DNA. A standard recombineering protocol (22) was employed for the *deoD*::*tet* and *purB*::*kan* gene replacements.

The pGSK single-copy plasmid was created using the Copy Control PCR Cloning kit (Epicenter). The oligonucleotides DM479 and DM480 were used to amplify the *gsk* gene for guanosine-inosine kinase and place it under control of the P*bla*$_{TEM}$ promoter. Purified *E. coli* BW25113 genomic DNA was used as the template. The resulting PCR product was cloned in the pCC1 single-copy number vector and sequenced to verify the construct.

The single-copy pGUA6 plasmid, which encodes a C305A active site mutant of IMPDH, was constructed using a standard QuikChange site-directed mutagenesis procedure (Stratagene) with the DM429/DM430 oligonucleotide pair. Our previously constructed pGUAB plasmid, a single-copy pCC1 derivative containing the *guaB*^{wt} gene preceded by the P*guaBA* native promoter, was employed as a template (18).

The wild-type *prs* gene for PRPP synthetase was amplified from the *E. coli* BW25113 genomic DNA using the DM481/DM482 primer pair and cloned in pCC1, yielding the pPRS plasmid. A standard QuikChange site-directed mutagenesis procedure (Stratagene) was subsequently employed to create the pPRS-A1 plasmid harboring the previously described *prsA1* allele encoding a A386C single amino acid substitution variant of PRPP synthetase (23). The DM483 and DM484 oligonucleotides were employed for the mutagenesis.

Measurements of Nucleotide Pools—The intracellular nucleotide pools were measured by $^{33}P_i$ labeling of exponentially growing cells as described (24). Briefly, about 10^4 freshly grown cells were inoculated into 1 ml of glucose-limited MOPS minimal medium, containing 0.0125% glucose as carbon source, up to 10 μCi/ml $H_3{}^{33}PO_4$ (40–158 Ci/mg), and the appropriate antibiotics. Purine auxotrophic strains were supplemented with 0.1 mM guanine or adenine, as appropriate. After overnight growth at 37 °C the culture typically attained a terminal A_{600} of about 0.2. To allow the cells to resume growth, 20% glucose was added to a final concentration of 0.4%. Other additives, when indicated, were also added at this point. Cell growth was monitored by measurements of A_{600} on a calibrated spectrophotometer. After incubation at 37 °C with shaking for an additional 2 h the cultures typically had an A_{600} of 0.2–0.6 depending on the strain background and additives used. For nucleotide extraction, a 150-μl portion of the culture was mixed with ice-cold 11 N formic acid to a final concentration of 0.5 N. The inorganic phosphate was precipitated along with any acid-insoluble cell debris using a precipitation reagent that was prepared on the day of use by mixing 400 mM sodium tungstate, 500 mM tetraethylamine·Cl, and 500 mM procaine in the ratio 5:4:1 (24). Of this mixture, 14.3 μl was added to each 157-μl formic acid extract. The sample was vortexed and centrifuged at >10,000 × *g* at 4 °C for 10 min. The supernatant was chromatographed two-dimensionally on polyethyleneimine-cellulose plates as described (24). The radiolabeled nucleotide spots were visualized by phosphorimaging followed by quantification using the MultiGauge software (Fujifilm). The ATP concentration in the BW25113 strain was previously estimated to be 3.5 mM (18). Concentrations of the rest of the labeled nucleotides were calculated by comparison of their intensities to the intensity of the ATP spot in BW25113. All reported nucleotide concentrations are the average of at least three independent measurements.

Assays of Enzyme Activities in Cell Extracts—The cells were grown overnight in 20-ml batches in glucose-limited MOPS medium. The terminal A_{600} of the overnight cultures was typically 0.2. Cell growth was restored by re-addition of glucose to the medium to a final concentration of 0.4%. Where indicated, 4 mM inosine was added simultaneously with glucose. The cultures were incubated in a shaking water bath at 37 °C for 2 h. The A_{600} was measured and the cells were harvested at 4 °C by centrifugation at 5,000 × *g* for 20 min. The cells were washed by resuspending in 600 μl of cold 100 mM Tris·HCl, pH 7.46, 2 mM EDTA, 0.1 mM dithiothreitol followed by spinning at 10,000 × *g*. The BugBuster Master Mix reagent (Novagen), containing 30 μg/ml phenylmethanesulfonyl fluoride was used for protein extraction. One ml of BugBuster reagent was added per 100 A_{600} of bacteria. The extracts were spun at 15,000 × *g* at 4 °C for 10 min and the supernatant was diluted 20-fold with cold 100 mM Tris·HCl, pH 7.46, 2 mM EDTA, 0.1 mM dithiothreitol. The protein concentration in the extracts was measured using the Bio-Rad Bradford protein assay. Enzyme activities were normalized to the total protein concentrations in the 20-fold diluted extracts.

IMPDH activity was assayed essentially as described (18), with minor modifications. The 20-μl reaction mixture contained 0.2 mM [8-^{14}C]IMP, 0.2 mM NAD, 50 mM Tris·HCl, pH 7.6, 150 mM KCl, 0.1 mM dithiothreitol, and 90–150 ng/μl of

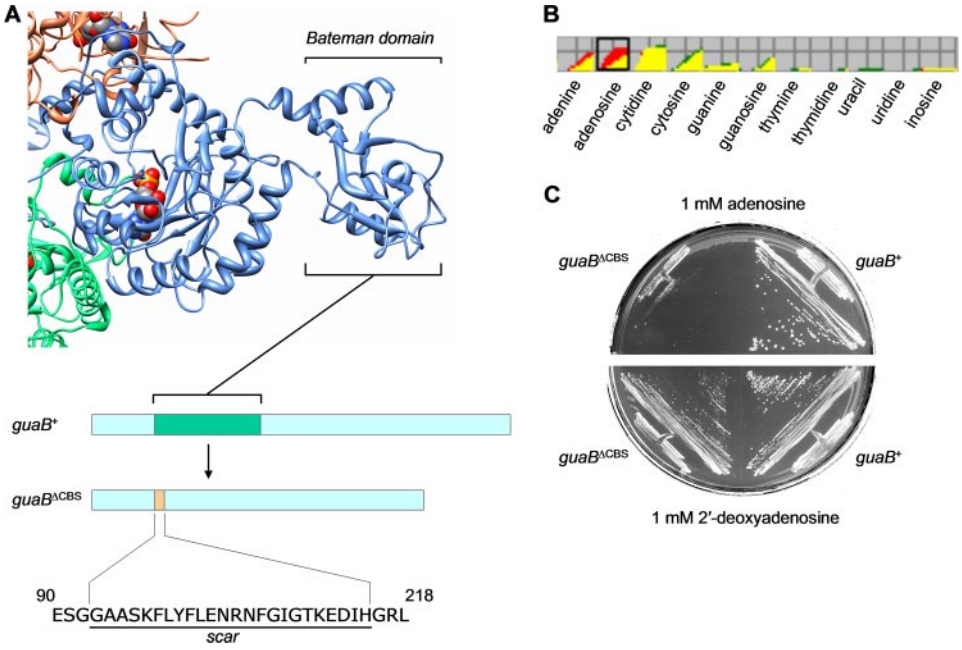

FIGURE 2. **The *guaB*$^{\Delta CBS}$ mutation sensitizes *E. coli* to growth inhibition by adenosine.** *A*, the structure of the homologous *Streptococcus pyogenes* IMPDH tetramer (Protein Data Bank code 1ZFJ). A single subunit of the tetramer is shown along with the contacting parts of the two adjacent subunits. The individual subunits are shown in *blue*, *orange*, and *green*. The substrate (IMP) is shown in a *sphere* representation. The positioning of the Bateman domain inside the dehydrogenase sequence and its replacement with a scar sequence are illustrated in a schematic bar representation. UCSF Chimera was used for structure visualization (43). *B*, results of the Biolog phenotype microarray screening: an overlay diagram of the respiration rates of the *guaB*$^{+}$ wild-type strain (BW25113, *red*) and *guaB*$^{\Delta CBS}$ mutant (MP101, *green*) using nucleosides and bases as nitrogen source. *C*, growth of the *guaB*$^{+}$ wild-type strain and *guaB*$^{\Delta CBS}$ mutant on MOPS minimal media supplemented with 0.4% glucose as carbon source and 1 mM adenosine or 2′-deoxyadenosine.

protein. The reaction was incubated at 37 °C for 30 min and stopped by addition of 5.5 N formic acid to a final concentration of 0.6 N. The production of [8-^{14}C]XMP was monitored by TLC as previously described (24).

Measurements of the AMPs synthetase activity were carried out using a modification of a previously described procedure (25). The reaction was started by combining 2.5 μl of cell extract (1–1.75 μg of protein) with 20.5 μl of assay mixture containing 0.4 mM [8-^{14}C]IMP, 2 mM GTP, 8 mM $Mg(CH_3COO)_2$, 50 mM HEPES, pH 7.0, and 8 mM aspartic acid. After incubation at 37 °C for 30 min, the reaction was quenched by addition of 2.5 μl of cold 5.5 N formic acid. The production of [8-^{14}C]AMPs was monitored by thin-layer chromatography. Two microliters of the reaction mixture were spotted on a polyethyleneimine-cellulose plate and air-dried. The plate was then immersed in methanol for 10 min, dried, and developed in the Ta buffer (24).

The activity assays for guanosine-inosine kinase were performed essentially as described (26). Ten μl of cell extract (4–7 μg of protein) was mixed with 10 μl of assay mixture, giving final concentrations in the assay of 80 mM Tris·HCl, pH 7.6, 35 mM $MgCl_2$, 35 mM KCl, 2 mM ATP, and 0.25 mM [8-^{14}C]guanosine. The reaction mixture was incubated at 37 °C for 30 min and quenched by addition of 2.5 μl of 5.5 N formic acid. Two μl of the reaction was spotted on a polyethyleneimine-cellulose thin layer plate that was developed in methanol up to the application line and then further developed in deionized water.

Anti-IMPDH Antibody and Western Blot Analysis—Polyclonal anti-IMPDH antibodies to the *E. coli* enzyme were custom-made by 21st Century Biochemicals (USA) using rabbit immunizations with the following two synthetic peptides: CLPNTADLSTQLTKTIRL and CVHDVTITKESPNYRLGS. These amino acid sequences are not part of the Bateman domain sequence. The antiserum was affinity purified and used for a standard immunoblot analysis of the crude BugBuster *E. coli* extracts. The protein load in each lane was standardized using the Bradford Bio-Rad protein assay.

RESULTS

The guaB$^{\Delta CBS}$ *Allele Sensitizes a Wild-type E. coli Strain to Adenosine and Inosine*—In our previous study, we created and characterized an *E. coli* strain carrying a chromosomal mutation in the *guaB* gene for IMPDH (18). Specifically, the nucleotide sequence encoding the Bateman domain of the dehydrogenase was excised and replaced with an in-frame 24-amino acid "scar" sequence, as illustrated in Fig. 2*A*. The resulting strain, MP101 (*guaB*$^{\Delta CBS}$), was prototrophic for guanine and grew with a wild-type rate on minimal salts media, indicating that the IMPDH enzymatic function is sustained. However, the mutation resulted in a 1.7-fold increase in the intracellular ATP pool as well as alteration of the purine nucleotide turnover rates of the mutant, indicating for the first time that the Bateman domain of IMPDH may play a regulatory role in the purine nucleotide homeostasis (18).

In search for unexpected manifestations of the *guaB*$^{\Delta CBS}$ mutation we employed Biolog phenotype microarray technology, which couples cell respiration on various nitrogen and carbon sources to reduction of a tetrazolium dye and production of purple color (27). Microarrays PM1–PM8 were used, which allowed testing of 768 growth conditions in a single experiment. Compared with the *guaB*$^{+}$ isogenic strain, the *guaB*$^{\Delta CBS}$ mutant respired significantly more slowly with adenosine as the nitrogen source (Fig. 2*B*). To confirm the results obtained on Biolog plates, we grew the wild-type (BW25113) and *guaB*$^{\Delta CBS}$ strain (MP101) on a minimal salts medium supplemented with 0.4% glucose as carbon source and 1 mM adenosine as nitrogen source (data not shown) as well as on standard MOPS minimal medium (which contained ammonia as nitrogen source and 0.4% glucose as carbon source) supplemented with 1 mM adenosine. The *guaB*$^{\Delta CBS}$ mutant was unable to grow on minimal media in the presence of adenosine, irrespective of whether ammonia was present as a source of nitrogen, suggesting that the lack of growth results from adenosine toxicity rather than a

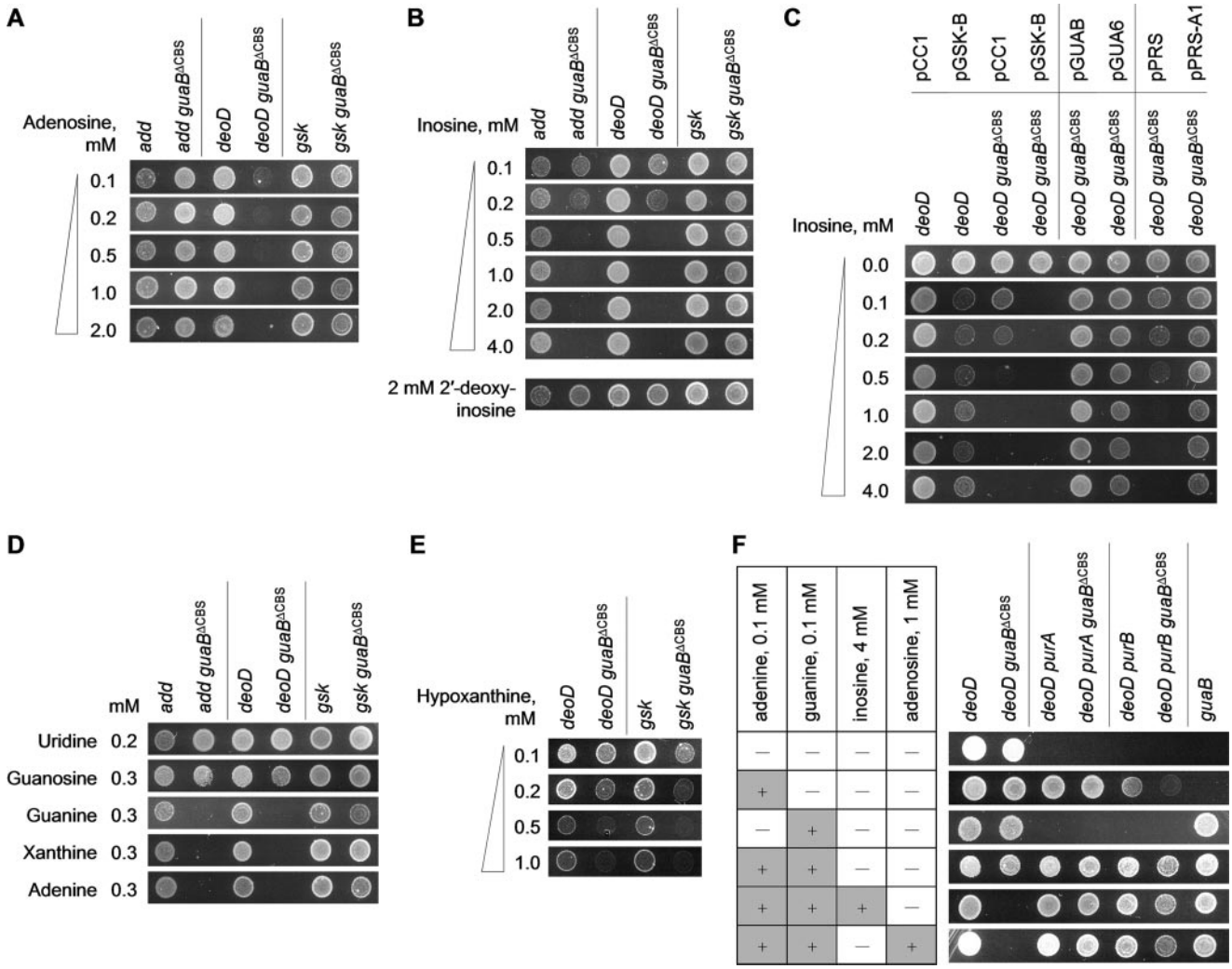

FIGURE 3. **Agar dilution spot assay on MOPS glucose minimal media.** Appropriate antibiotics were used as described under "Experimental Procedures." Freshly grown cells were spotted on the agar surface (10^4 colony forming units per spot) and the plates were incubated for 36–48 h at 37 °C. Most phenotypes were verified in MOPS liquid batch cultures with similar results. *A*, sensitivity testing of various concentrations of adenosine and second-site suppressors of adenosine toxicity. *B*, sensitivity testing of various concentrations of inosine. *C*, effects of *gsk* overexpression and the *prsA1* and *guaB*^{C305A} alleles on inosine toxicity. The strain genotypes are shown in combination with the plasmids used for gene expression and complementation. *D*, the effect of nucleosides and bases on growth in the presence of 4 mM inosine. *E*, growth on minimal media supplemented with hypoxanthine. *F*, growth phenotypes of purine-auxotrophic derivatives of the *deoD guaB*$^{+}$ and *deoD guaB*$^{\Delta CBS}$ strains. The presence of purine additives in each lane are indicated by *plus signs*.

defect in utilization of adenosine as the sole nitrogen source. Surprisingly, the *guaB*$^{\Delta CBS}$ strain was not affected by the presence of equivalent concentrations of 2′-deoxyadenosine (Fig. 2*C*). Similar results were obtained with liquid MOPS cultures (data not shown).

In *E. coli*, adenosine and 2′-deoxyadenosine are metabolized in the same way and can be either deaminated to produce ammonia and either inosine or 2′-deoxyinosine, respectively, or reversibly phosphorylyzed into adenine and the respective sugar phosphate (1). The selective toxicity of adenosine but not 2′-deoxyadenosine indicates that adenosine does not cause growth inhibition via adenine. In support of such an interpretation, a *guaB*$^{\Delta CBS}$ *deoD* strain, which lacks purine nucleoside phosphorylase and is therefore unable to cleave adenosine to form adenine, retained susceptibility to adenosine (Fig. 3*A*). In contrast, an adenosine deaminase knock-out mutation rendered the subdomain deletion mutant (*guaB*$^{\Delta CBS}$ *add*) resistant to adenosine (Fig. 3*A*), which indicated that adenosine has to be converted to inosine to cause growth arrest. In agreement with this result, the adenosine-resistant *guaB*$^{\Delta CBS}$ *add* strain was susceptible to growth arrest by 0.5 mM inosine, but not 2 mM 2′-deoxyinosine (Fig. 3*B*).

As shown in Fig. 1, metabolism of inosine can proceed in two directions: phosphorylation to IMP by inosine-guanosine kinase (*gsk*) or phosphorolysis to hypoxanthine and ribose 1-phosphate by the action of purine nucleoside phosphorylase (*deoD*), the same enzyme that metabolizes adenosine and guanosine. As shown in Fig. 3*B*, the toxic effect of inosine was preserved in the *guaB*$^{\Delta CBS}$ *deoD* strain. In the absence of *deoD*, the only route of inosine metabolism is phosphorylation to IMP by guanosine-inosine kinase (Fig. 1). Indeed, as demonstrated in Fig. 3, *A* and *B*, transduction of a *gsk* mutation into the *guaB*$^{\Delta CBS}$ mutant rendered the resulting strain (*guaB*$^{\Delta CBS}$ *gsk*) completely adenosine- and inosine-insensitive. Collectively, these results indicate that to impose growth arrest adenosine must be converted to

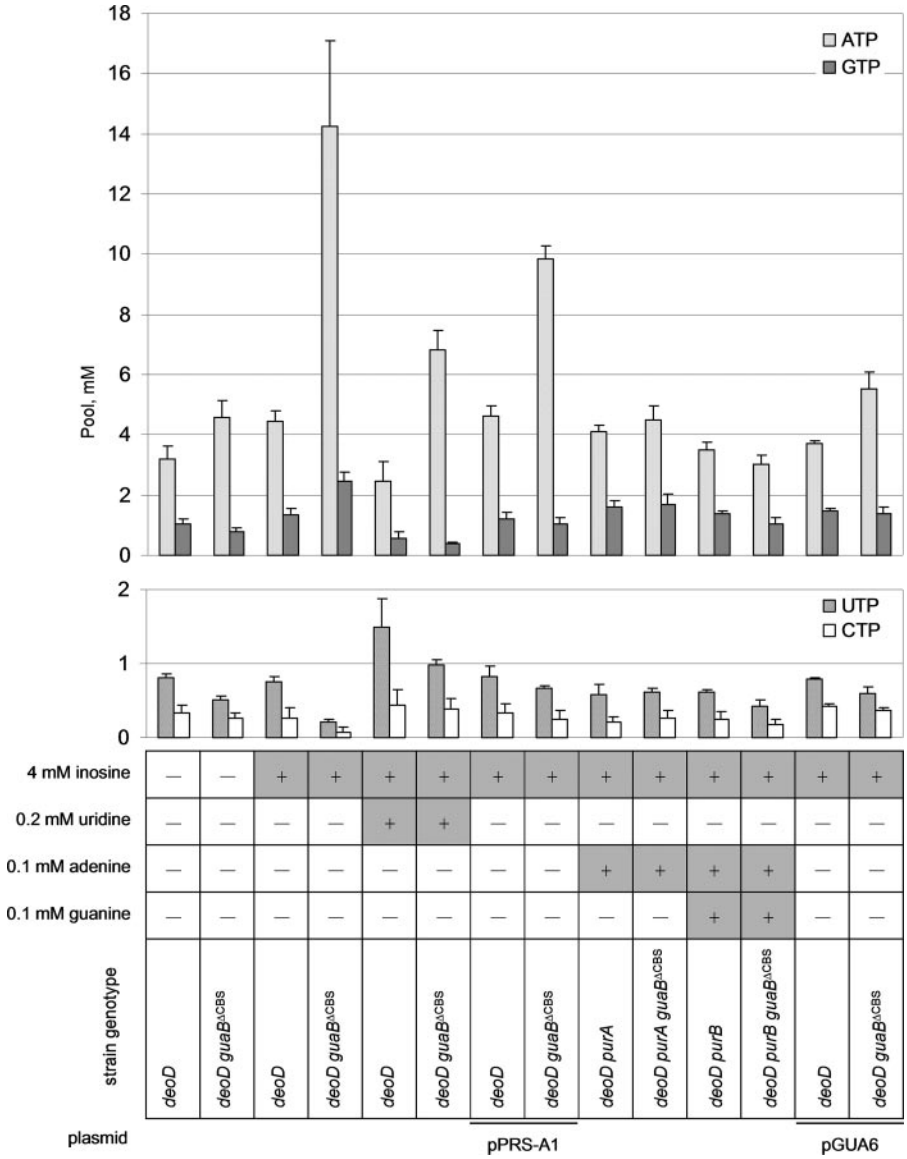

FIGURE 4. **Nucleotide pools in strains grown on minimal media supplemented with the compounds indicated.** The average of 3 independent measurements is given. *Error bars* are standard deviations.

IMP via a consecutive action of adenosine deaminase and guanosine-inosine kinase (Fig. 1).

Notably, the *guaB*$^{\Delta CBS}$ mutant could also be rescued from the toxic effects of inosine by addition of guanosine to the growth media, but not guanine (Fig. 3*D*). The *gsk* gene product is a bifunctional guanosine-inosine kinase with guanosine being a much better substrate than inosine (28), and guanosine appears to prevent the conversion of inosine to IMP by substrate competition. The lack of growth inhibition by 2′-deoxyinosine is probably due to a poor, if any, utilization of this substrate by guanosine-inosine kinase (29, 30).

The guaB$^{\Delta CBS}$ *Allele Relaxes Control over Adenylate Nucleotide Synthesis*—Metabolic effects of a nucleoside are most readily studied in a system where it cannot be interconverted with the nucleobase. Consequently, all further experiments were carried out using derivatives of the wild-type and *guaB*$^{\Delta CBS}$ strains carrying a deletion of the *deoD* gene for purine nucleoside phosphorylase, termed MP255 (*deoD*) and MP350 (*deoD guaB*$^{\Delta CBS}$), respectively (Table 1).

To trace the mechanism of growth inhibition by the IMP precursors, we measured how the nucleotide pools of the wild-type and subdomain deletion strains responded to inosine addition. As shown in Fig. 4, the "wild-type" *guaB*$^{+}$ *deoD* stain responded to added inosine by only a slight increase in the ATP and GTP levels. In contrast, in the *deoD guaB*$^{\Delta CBS}$ mutant the ATP pool increased up to 4-fold and the GTP pool increased about 3-fold after a 2-h incubation with 4 mM inosine, compared with a culture where no inosine was used. A modest accumulation of IMP over the wild-type levels was detected in inosine-treated *deoD guaB*$^{\Delta CBS}$ cells ($[IMP]_{MP255} = 0.13 \pm 0.06$ mM; $[IMP]_{MP350} = 0.24 \pm 0.05$ mM); the statistical significance of this observation was marginal ($p = 0.08$). In contrast, the pyrimidine nucleotide pools of the inosine-challenged *deoD guaB*$^{\Delta CBS}$ mutant were almost undetectable (at least 3.5-fold lower than in the *guaB*$^{+}$ strain), suggesting that pyrimidine starvation may be the underlying cause of the growth arrest. Indeed, addition of 0.2 mM uridine rescued the inosine-mediated growth inhibition of the *deoD guaB*$^{\Delta CBS}$ strain (Fig. 3*D*) and reversed the pyrimidine nucleotide pool change (Fig. 4). Uridine also lessened the inosine-induced swelling of the ATP pool although it was unable to bring it back to the wild-type value (Fig. 4). This suggests that pyrimidine starvation is secondary to the adenylate nucleotide pool increase in the mechanism of inosine toxicity.

It has been reported that an increase in the adenylate nucleotide pool leads to an enhanced allosteric inhibition of PRPP synthetase (*prs*) by ADP and AMP, which results in a decreased availability of PRPP for pyrimidine nucleotide biosynthesis (26). If such an interpretation were correct, the *guaB*$^{\Delta CBS}$ mutant would be insensitive to inosine if it carried the previously described *prsA1* allele encoding a PRPP synthetase mutant that is insensitive to allosteric inhibition by adenylates (23). As expected, introduction of a single-copy pPRS-A1 plasmid containing the *prsA1* allele rendered the recipient *deoD guaB*$^{\Delta CBS}$ mutant inosine-resistant (Fig. 3*C*), although it did not prevent the inosine-induced swelling of the ATP pool (Fig. 4). In contrast, pPRS, the parent plasmid harboring the wild-

type allele for PRPP synthetase, did not confer inosine resistance upon the subdomain deletion mutant (Fig. 3*C*). Although we did not conduct direct measurements of the PRPP pools due to a low stability of PRPP in acid extracts, suppression of inosine toxicity by *prsA1* strongly suggests that the PRPP pools are depleted in the *guaB*$^{\Delta CBS}$ mutant following inosine addition. Collectively, these results indicate that the *guaB*$^{\Delta CBS}$ mutation relaxes control over the adenylate nucleotide biosynthesis in the presence of IMP precursors (adenosine and inosine), which leads to increased intracellular concentrations of adenylate nucleotides, PRPP deficit, and thus starvation for pyrimidine nucleotides.

Notably, PRPP is also a precursor of the amino acids tryptophan and histidine. Depletion of PRPP has been shown to elicit starvation for these amino acids and result in stringent response with marked accumulation of ppGpp (26). However, we did not observe accumulation of ppGpp in any of our strains following addition of inosine, and supplementation of the growth media with amino acids did not rescue the inosine-induced growth arrest of the *guaB*$^{\Delta CBS}$ mutant (data not shown). We did not investigate the reason behind this observation, but other reports have suggested that amino acid synthesis may be relatively resistant to a partial depletion of the PRPP pool (31).

Mutations in the Adenylate Biosynthetic Genes Are Second-site Suppressors of Inosine Toxicity—To obtain additional evidence of the relaxed control over the ATP synthesis in the *guaB*$^{\Delta CBS}$ mutant and to provide insights into a possible mechanism of this dysregulation, we introduced into the *deoD guaB*$^{\Delta CBS}$ strain additional mutations that inactivated the genes responsible for the *de novo* synthesis of AMP from IMP. As shown in Fig. 1, in *E. coli*, as well as in all other organisms capable of *de novo* AMP synthesis, IMP is converted to adenylate nucleotides in a two-step process, catalyzed by the consecutive action of AMPs synthetase (*purA*) and AMPs lyase (*purB*). As expected, deletion of either of the two genes in the *deoD guaB*$^{\Delta CBS}$ background rendered the resulting strains (*deoD purA guaB*$^{\Delta CBS}$ and *deoD purB guaB*$^{\Delta CBS}$, respectively) auxotrophic for adenine as well as completely insensitive to growth inhibition by inosine (Fig. 3*F*). This result confirms that inosine-dependent swelling of the adenylate nucleotide pool in the *guaB*$^{\Delta CBS}$ strain proceeds through direct conversion of IMP to AMP.

The Bateman Domain of IMPDH as a Possible Trans-regulator of AMPs Synthetase—In light of the above data, a central question remains: which enzyme in the chain of reactions leading from inosine to AMP is responsible for the uncontrolled increase of the pool of adenylate nucleotides? First, we measured the activities of AMPs synthetase, guanosine-inosine kinase, and IMPDH in crude extracts of the *deoD guaB*$^{+}$ and *deoD guaB*$^{\Delta CBS}$ strains in the presence or absence of inosine (Fig. 5). The measured activity of guanosine-inosine kinase in the *deoD guaB*$^{\Delta CBS}$ strain was 1.5-fold higher than in the *deoD guaB*$^{+}$ strain grown on the minimal medium, although the two activities were virtually indistinguishable when measured during growth in the presence of inosine. In agreement with the results of our previous study (18), the activity of AMPs synthetase in the *deoD guaB*$^{\Delta CBS}$ mutant was about 40% of the *deoD*

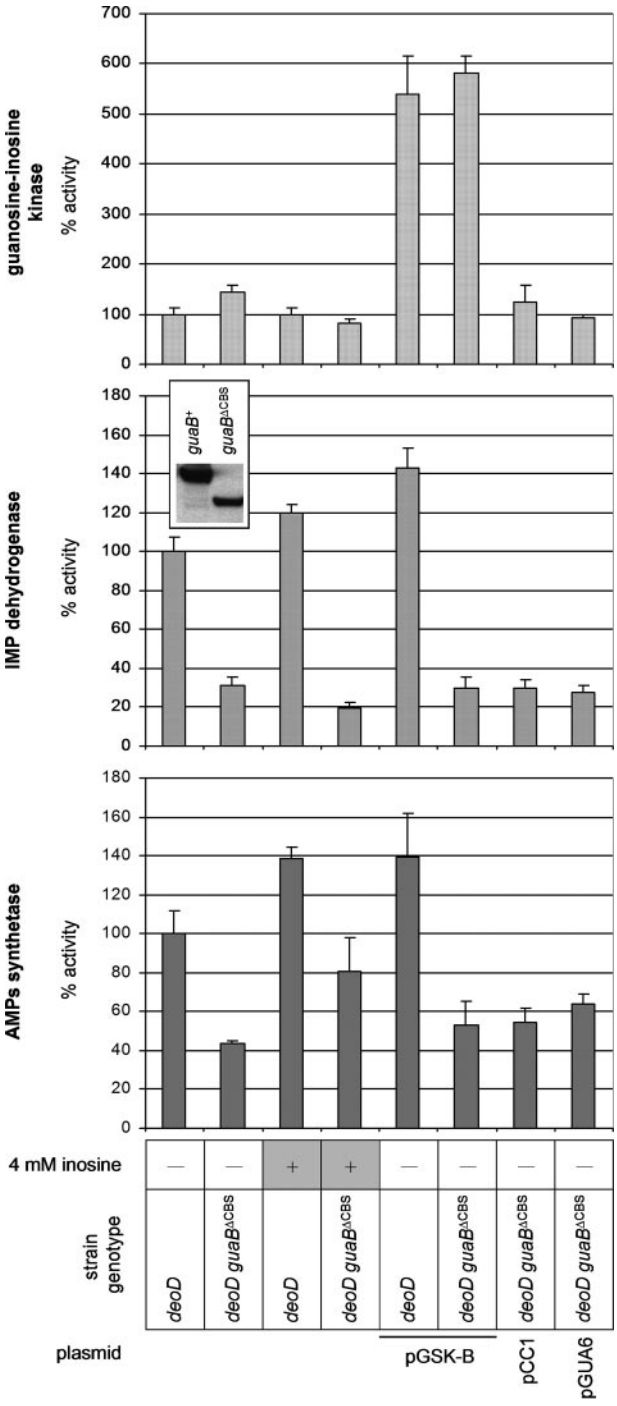

FIGURE 5. **Enzyme activities in crude cell extracts.** The measured activities of IMPDH, AMPs synthetase, and guanosine-inosine kinase were normalized with respect to the protein load in each reaction and compared with the wild-type values. The average of 4 measurements is given. *Error bars* are standard deviations. The *inset* shows an immunoblot analysis of the wild-type and *guaB*$^{\Delta CBS}$ cell extracts with a polyclonal anti-IMPDH antibody demonstrating a change in the quantity and electrophoretic mobility of IMPDH following subdomain deletion. Thirty μg of protein was loaded onto each lane. A representative of three independent experiments is shown.

guaB^{+} wild-type strain value, and we now find that it increased about 2-fold following inosine addition. Finally, the activity of IMPDH in the *deoD guaB*$^{\Delta CBS}$ strain extract was one-third of the wild-type strain value and decreased even further when inosine was added to the minimal culture medium. The 1.5-fold

increase in the guanosine-inosine kinase activity, albeit modest, raises suspicion that it may be the principal factor behind the enhanced conversion of inosine to AMP and the resulting growth inhibition. However, even a 5-fold overexpression of guanosine-inosine kinase from a constitutive P*bla*$_{TEM}$ promoter did not significantly sensitize the wild-type strain to inosine (Figs. 3*C* and 5). In addition, compared with the *deoD guaB*$^{+}$ wild-type strain, the *deoD guaB*$^{\Delta CBS}$ mutant displayed an increased sensitivity to hypoxanthine, which is converted to IMP in a *gsk*-independent fashion (although the difference in growth rates was less pronounced in this case due to a significant inhibition of the wild-type strain). This result suggests that the biosynthetic route to IMP is less important than an upstream event in the mechanism of the ATP pool expansion (Fig. 3*E*).

We previously observed a decrease in the *in vivo* activity of IMPDH following the subdomain deletion (18), and this decrease is sustained in the *deoD* background (Fig. 4). As demonstrated by an immunoblot analysis with a polyclonal anti-IMPDH antibody (Fig. 4, *inset*), the change in the *in vivo* enzyme activity is mirrored by a decreased total IMPDH concentration in the cell extract. Additionally, MP101 extracts probed with the anti-IMPDH antibody routinely demonstrated an accumulation of lower molecular weight proteins that were not observed in the wild-type *E. coli* lysates and likely represent IMPDH proteolytic fragments (data not shown). This observation supports the interpretation that the reduction of IMPDH activity accompanying our replacement of the subdomain with the "scar" sequence resulted from an enhanced *in vivo* degradation of the mutant IMPDH protein rather than a catalytic defect. In any case, a decreased IMPDH enzymatic activity *per se* does not confer inosine sensitivity, as clearly demonstrated by the fact that the JW5401 (*guaB*::*kan*) strain, a guanine auxotroph carrying a complete deletion of the *guaB* gene, is insensitive to inosine (Fig. 3*F*). Additionally, we observed that the guanylate nucleotide precursors guanine and xanthine are incapable of reversing the toxic effects of inosine (Fig. 3*D*), which further suggests that starvation for guanylate nucleotides does not play a role in the mechanism of the inosine-induced increase in the adenylate nucleotide pool.

As mentioned above, deletions of the *purA* gene encoding AMPs synthetase and *purB* gene for AMPs lyase rendered the *guaB*$^{\Delta CBS}$ mutant unable to convert IMP to AMP and, consequently, inosine-resistant. Indeed, as shown in Fig. 4, no significant increase in the ATP pool or decrease in the pyrimidine nucleotide pools was detected in the *deoD purA guaB*$^{\Delta CBS}$ and *deoD purB guaB*$^{\Delta CBS}$ strains growing in the presence of inosine. However, a significant difference in the AMPs accumulation patterns was observed between MP4022 (*deoD purB guaB*$^{\Delta CBS}$) and its wild-type counterpart MP4021 (*deoD purB guaB*$^{+}$). These strains are deficient in the last reaction of the *de novo* AMP synthesis, the conversion of AMPs to AMP by AMPs lyase, but retain a functional AMPs synthetase and are therefore proficient in the synthesis of AMPs from IMP. Following inosine addition, a very large, millimolar scale, accumulation of AMPs was observed in MP4022 ($[AMPs]_{MP4022} = 8.1 \pm 1.8$ mM), up to 3-fold higher compared with the MP4021 wild-type strain ($[AMPs]_{MP4021} = 2.8 \pm 1.1$ mM). In contrast, no inosine-induced accumulation of IMP was noted in the *deoD purA guaB*$^{\Delta CBS}$ strain that lacks the enzyme that is one step earlier in the AMP biosynthetic pathway (data not shown). It should be noted that measurements of IMP are complicated and generally somewhat less reliable than the rest of nucleotides. The use of a P_i precipitation reagent improves the visibility of nucleoside monophosphates by removing the phosphate front and results in a more reliable IMP quantification (24), allowing us to conclude with reasonable confidence that no significant accumulation of IMP takes place in any of our strains following addition of inosine. The much greater inosine-induced accumulation of AMPs, rather than IMP, suggests that the swelling of the adenylate nucleotide pool in the *guaB*$^{\Delta CBS}$ mutant may result from an increased *in vivo* activity of AMPs synthetase, despite the fact that the *in vitro* activity of this enzyme in crude extract is lowered by the *guaB*$^{\Delta CBS}$ mutation. The lack of correlation between *in vitro* enzyme activities and nucleotide pools has been previously reported (32) and is usually ascribed to *in vivo* regulation of enzyme activity by the components of a crowded cellular milieu.

Trans-complementation of Subdomain Deletion with a Catalytically Deficient Full-length IMPDH—We speculated that if the Bateman domain of IMPDH played a regulatory role that was not directly associated with the core domain catalysis, the phenotypic traits of the *guaB*$^{\Delta CBS}$ mutation might be rescued by a catalytically deficient IMPDH containing an intact Bateman domain and supplied in *trans*. The chemical mechanism of the IMPDH reaction proceeds through a covalent adduct between the 2-position of the IMP purine ring and the sulfur of an active site cysteine (Cys^{305} in the *E. coli* enzyme). In the subsequent steps, hydride transfer from the covalent enzyme-IMP species to NAD yields a thioimidate intermediate that is then hydrolyzed (5). A C305A mutant of IMPDH would therefore be defective in the first chemical step of the reaction but would likely keep its native structure. We cloned the wild-type *E. coli guaB* in the pCC1 single-copy vector under control of the native P*guaB* promoter and used the resulting plasmid, pGUAB, as the template for a site-directed mutagenesis procedure. The new vector carried a *guaB*$^{TGT(914-916)\rightarrow GCG}$ gene for IMPDH^{C305A} and was termed pGUA6. As expected, transformation of MP350 (*deoD guaB*$^{\Delta CBS}$) with pGUA6 failed to increase the IMPDH activity of the strain, measured in a crude extract (Fig. 5). This confirms that the C305A amino acid substitution renders IMPDH catalytically ineffective. However, in contrast to an MP350/pCC1 "empty" vector control, the MP350/pGUA6 strain was resistant to growth inhibition by inosine (Fig. 3*C*) and demonstrated only very modest changes of the purine and pyrimidine nucleotide pools following inosine addition (Fig. 4). Thus, the presence of an intact Bateman domain is both necessary and sufficient for the negative regulation of adenylate nucleotide biosynthesis, irrespective of whether it is attached to an enzymatically intact or a catalytically disabled dehydrogenase core domain.

DISCUSSION

The realization that the Bateman domain of IMP dehydrogenase is dispensable for the *in vitro* catalytic activity of the enzyme has generated the now predominant view that the

physiological importance of IMPDH may extend beyond its primary role in the *de novo* biosynthesis of GMP (4, 11). The recent discovery that point mutations in the Bateman domain of IMPDH type 1 cause human retinitis pigmentosa yet confer no detectable catalytic defect reinforced this view, renewed the interest in IMPDH biology, and suggested that further *in vivo* studies were necessary (14, 33–38). Having only one gene for IMPDH and being the most easily genetically manipulated system, *E. coli* seems to be an ideal organism for *in vivo* studies of IMPDH. We have previously demonstrated that replacement of the subdomain of *E. coli* IMPDH with a short scar sequence results in dysregulation of the purine nucleotide pool sizes and their turnover rates (18). The present study identified additional phenotypes associated with the $guaB^{\Delta CBS}$ mutation and has provided evidence that the Bateman domain of IMPDH is a negative regulator of the adenylate nucleotide synthesis.

We demonstrate that deletion of the Bateman domain of IMPDH sensitizes *E. coli* to growth arrest by adenosine and inosine via their enhanced conversion to ATP, as illustrated by the highlighted area in Fig. 1. The accumulation of adenylate nucleotides results in an increased allosteric inhibition of PRPP synthetase by AMP and ADP, which depletes cellular stores of PRPP, a common precursor of both purine and pyrimidine nucleotides. Starvation for pyrimidine nucleotides ensues which culminates in growth arrest. The toxic effect of adenosine and inosine can be rescued by uridine (which replenishes the pyrimidine nucleotide pool), guanosine (which competes with inosine for guanosine-inosine kinase, reducing the conversion of inosine to IMP), or by second-site suppressor mutations that inactivate the enzymes responsible for the conversion of inosine to AMP. Additionally, the $guaB^{\Delta CBS}$ mutation can be complemented with either the *prsA1* allele for a PRPP synthetase mutant that is insensitive to allosteric inhibition by adenylate nucleotides, or by the full-length, catalytically disabled $IMPDH^{C305A}$.

Several observations suggest that an increased utilization of IMP by AMPs synthetase, rather an enhanced IMP production by guanosine-inosine kinase, is the main driving force behind the inosine-induced swelling of the $guaB^{\Delta CBS}$ adenylate nucleotide pool. First, overexpression of guanosine-inosine kinase does not sensitize the wild-type $guaB^{+}$ strain to inosine. Second, the $guaB^{\Delta CBS}$ mutant is also sensitive to growth inhibition by hypoxanthine, which is converted to IMP by a different enzyme. Finally, only modest accumulation of IMP over the wild-type levels is detected in the *deoD* $guaB^{\Delta CBS}$ strain following inosine addition (probably attributable to the slightly higher activity of guanosine-inosine kinase in this strain), whereas high levels of AMPs accumulate in the inosine-treated *deoD purB* $guaB^{\Delta CBS}$ mutant, indicative of an inappropriately high *in vivo* activity of AMPs synthetase.

Although both ATP and GTP pools increase in the $guaB^{\Delta CBS}$ mutant following inosine treatment, only ATP plays a role in growth inhibition. This result is consistent with the reported observation that an excess of guanylate nucleotides is growth inhibitory only so long as the pathway of conversion of GMP to AMP is intact, permitting accumulation of both purines (26). It should be noted that inhibition of *de novo* AMP synthesis by *purA* and *purB* mutations in the $guaB^{\Delta CBS}$ background restricts the inosine-induced accumulation of *both* ATP and GTP. The mechanism underlying this observation is unclear, but it indicates that the increase in the GTP pool is secondary to the ATP accumulation. Moreover, it provides additional evidence that IMP accumulation is not a central factor in the inosine-induced swelling of the ATP pool. Indeed, if that were the case, a mutation in the *purA* gene would increase (not decrease) the GTP pool by further increasing IMP availability. In contrast, the IMP pool was barely detectable in the *purA* $guaB^{\Delta CBS}$ mutant following inosine addition, suggesting that salvage of inosine is tightly coupled with IMP utilization. This is in agreement with other reports that suggest that purine precursor assimilation is coupled to the synthesis of pathway end products, and that phosphorylated intermediates, such as IMP, do not accumulate under most circumstances (18, 39, 40). We note that the ATP concentration in the $guaB^{\Delta CBS}$ mutant has already increased during growth on minimal media with no purine supplementation (18). Apparently, supplementation with adenosine or inosine provides an additional source of IMP that is converted to adenylate nucleotides by a derepressed AMPs synthetase.

The location of both IMPDH and AMPs synthetase at a major branch point in the purine nucleotide synthesis has generated the so far unsubstantiated speculation that the two enzymes are allosterically regulated by the intracellular levels of nucleotides or other stimuli. Several cellular metabolites have been shown to act as competitive inhibitors of AMPs synthetase, but the *in vivo* significance of these observations is typically unclear (41). Likewise, an *in vivo* importance of the classical competitive feedback inhibition of IMPDH by GMP is doubtful because of the large GMP concentrations needed for such inhibition to occur (18, 42). No allosteric regulation of either enzyme has been reported. Our observation that a catalytically disabled full-length IMPDH can complement the $guaB^{\Delta CBS}$ mutation in *trans* indicates that the catalytic function of IMPDH in *de novo* GMP biosynthesis and its regulatory role in ATP homeostasis may indeed be independent. Whether this regulation involves direct interactions of IMPDH with AMPs synthetase remains to be elucidated. The possibility of influencing growth by inhibiting the function of the Bateman domain of IMPDH suggests that it may represent a novel pharmacological target for drug development.

Acknowledgments—We thank Drs. W. Kruger, M. Andrake, and S. Karakashev for critical reading of this manuscript. We are grateful to Dr. A. T. Yeung, M. Hussein, and Dr. M. Andrake for helpful technical discussions. We thank Drs. S. Moore and R. T. Sauer for a gift of P1vir and the J. Roth laboratory for a gift of E. coli TT25401.

REFERENCES

1. Zalkin, H., and Nygaard, P. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 561–579, ASM Press, Washington, D. C.
2. Pankiewicz, K. W., and Goldstein, B. M. (2003) *Inosine Monophosphate Dehydrogenase: A Major Therapeutic Target*, Oxford University Press, Washington, D. C.
3. Pankiewicz, K. W., Patterson, S. E., Black, P. L., Jayaram, H. N., Risal, D., Goldstein, B. M., Stuyver, L. J., and Schinazi, R. F. (2004) *Curr. Med. Chem.* **11,** 887–900
4. Hedstrom, L., and Gan, L. (2006) *Curr. Opin. Chem. Biol.* **10,** 520–525

5. Pimkin, M., and Markham, G. D. (2009) *Adv. Enzymol. Relat. Areas Mol. Biol.* **76,** 1–53
6. Zhang, R., Evans, G., Rotella, F. J., Westbrook, E. M., Beno, D., Huberman, E., Joachimiak, A., and Collart, F. R. (1999) *Biochemistry* **38,** 4691–4700
7. Ignoul, S., and Eggermont, J. (2005) *Am. J. Physiol.* **289,** C1369–C1378
8. Bateman, A. (1997) *Trends Biochem. Sci.* **22,** 12–13
9. Bowne, S. J., Sullivan, L. S., Blanton, S. H., Cepko, C. L., Blackshaw, S., Birch, D. G., Hughbanks-Wheaton, D., Heckenlively, J. R., and Daiger, S. P. (2002) *Hum. Mol. Genet.* **11,** 559–568
10. Kennan, A., Aherne, A., Palfi, A., Humphries, M., McKee, A., Stitt, A., Simpson, D. A., Demtroder, K., Orntoft, T., Ayuso, C., Kenna, P. F., Farrar, G. J., and Humphries, P. (2002) *Hum. Mol. Genet.* **11,** 547–557
11. Hedstrom, L. (2008) *Nucleosides Nucleotides Nucleic Acids* **27,** 839–849
12. Nimmesgern, E., Black, J., Futer, O., Fulghum, J. R., Chambers, S. P., Brummel, C. L., Raybuck, S. A., and Sintchak, M. D. (1999) *Protein Expression Purif.* **17,** 282–289
13. Gan, L., Petsko, G. A., and Hedstrom, L. (2002) *Biochemistry* **41,** 13309–13317
14. Mortimer, S. E., and Hedstrom, L. (2005) *Biochem. J.* **390,** 41–47
15. Bowne, S. J., Sullivan, L. S., Mortimer, S. E., Hedstrom, L., Zhu, J., Spellicy, C. J., Gire, A. I., Hughbanks-Wheaton, D., Birch, D. G., Lewis, R. A., Heckenlively, J. R., and Daiger, S. P. (2006) *Investig. Ophthalmol. Vis. Sci.* **47,** 34–42
16. Townley, R., and Shapiro, L. (2007) *Science* **315,** 1726–1729
17. Kemp, B. E. (2004) *J. Clin. Investig.* **113,** 182–184
18. Pimkin, M., and Markham, G. D. (2008) *Mol. Microbiol.* **68,** 342–359
19. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) *J. Bacteriol.* **119,** 736–747
20. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Datsenko, K. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6640–6645
23. Bower, S. G., Harlow, K. W., Switzer, R. L., and Hove-Jensen, B. (1989) *J. Biol. Chem.* **264,** 10287–10291
24. Bochner, B. R., and Ames, B. N. (1982) *J. Biol. Chem.* **257,** 9759–9769
25. Juang, R. H., McCue, K. F., and Ow, D. W. (1993) *Arch. Biochem. Biophys.* **304,** 392–401
26. Petersen, C. (1999) *J. Biol. Chem.* **274,** 5348–5356
27. Bochner, B. R., Gadzinski, P., and Panomitros, E. (2001) *Genome Res.* **11,** 1246–1255
28. Mori, H., Iida, A., Teshiba, S., and Fujio, T. (1995) *J. Bacteriol.* **177,** 4921–4926
29. Kawasaki, H., Shimaoka, M., Usuda, Y., and Utagawa, T. (2000) *Biosci. Biotechnol. Biochem.* **64,** 972–979
30. Nygaard, P. (1983) in *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms* (Munch-Petersen, A., ed) pp. 27–93, Academic Press, London
31. Jochimsen, B. U., Hove-Jensen, B., Garber, B. B., and Gots, J. S. (1985) *J. Gen. Microbiol.* **131,** 245–252
32. Kelln, R. A., Kinahan, J. J., Foltermann, K. F., and O'Donovan, G. A. (1975) *J. Bacteriol.* **124,** 764–774
33. Gunter, J. H., Thomas, E. C., Lengefeld, N., Kruger, S. J., Worton, L., Gardiner, E. M., Jones, A., Barnett, N. L., and Whitehead, J. P. (2008) *Int. J. Biochem. Cell Biol.* **40,** 1716–1728
34. Kennan, A., Aherne, A., and Humphries, P. (2005) *Trends Genet.* **21,** 103–110
35. Aherne, A., Kennan, A., Kenna, P. F., McNally, N., Lloyd, D. G., Alberts, I. L., Kiang, A. S., Humphries, M. M., Ayuso, C., Engel, P. C., Gu, J. J., Mitchell, B. S., Farrar, G. J., and Humphries, P. (2004) *Hum. Mol. Genet.* **13,** 641–650
36. McLean, J. E., Hamaguchi, N., Belenky, P., Mortimer, S. E., Stanton, M., and Hedstrom, L. (2004) *Biochem. J.* **379,** 243–251
37. Xu, D., Cobb, G., Spellicy, C. J., Bowne, S. J., Daiger, S. P., and Hedstrom, L. (2008) *Arch. Biochem. Biophys.* **472,** 100–104
38. Mortimer, S. E., Xu, D., McGrew, D., Hamaguchi, N., Lim, H. C., Bowne, S. J., Daiger, S. P., and Hedstrom, L. (2008) *J. Biol. Chem.* **283,** 36354–36360
39. Yuan, J., Fowler, W. U., Kimball, E., Lu, W., and Rabinowitz, J. D. (2006) *Nat. Chem. Biol.* **2,** 529–530
40. Roy-Burman, S., and Visser, D. W. (1975) *J. Biol. Chem.* **250,** 9270–9275
41. Stayton, M. M., Rudolph, F. B., and Fromm, H. J. (1983) *Curr. Top. Cell Regul.* **22,** 103–141
42. Gilbert, H. J., Lowe, C. R., and Drabble, W. T. (1979) *Biochem. J.* **183,** 481–494
43. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) *J. Comput. Chem.* **25,** 1605–1612
44. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) *Mol. Syst. Biol.* **2,** 2006 0008