Specific features of L-histidine production by *Escherichia coli* concerned with feedback control of AICAR formation and inorganic phosphate/metal transport

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**Abstract**

**Background:** In the L-histidine (His) biosynthetic pathway of *Escherichia coli*, the first key enzyme, ATP-phosphoribosyltransferase (ATP-PRT, HisG), is subject to different types of inhibition. Eliminating the feedback inhibition of HisG by the His end product is an important step that enables the oversynthesis of His in breeding strains. However, the previously reported feedback inhibition-resistant mutant enzyme from *E. coli*, HisG<sup>E271K</sup>, is inhibited by purine nucleotides, particularly ADP and AMP, via competitive inhibition with its ATP substrate. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), which is formed not only during His biosynthesis but also during de novo purine biosynthesis, acts as a natural analog of AMP and substitutes for it in some enzymatic reactions. We hypothesized that AICAR could control its own formation, particularly through the His biosynthetic pathway, by negatively influencing HisG enzymatic activity, which would make preventing ATP-PRT transferase inhibition by AICAR crucial for His overproduction.

**Results:** For the first time, both the native *E. coli* HisG and the previously described feedback-resistant mutant HisG<sup>E271K</sup> enzymes were shown to be sensitive to inhibition by AICAR, a structural analog of AMP. To circumvent the negative effect that AICAR has on His synthesis, we constructed the new His-producing strain EA83 and demonstrated its improved histidine production. This increased production was particularly associated with the improved conversion of AICAR to ATP due to purH and purA gene overexpression; additionally, the PitA-dependent phosphate/metal (Me<sup>2+</sup>-Pi) transport system was modified by a pitA gene deletion. This His-producing strain unexpectedly exhibited decreased alkaline phosphatase activity at low Pi concentrations. AICAR was consequently hypothesized inhibit the two-component PhoBR system, which controls Pho regulon gene expression.

**Conclusions:** Inhibition of a key enzyme in the His biosynthetic pathway, HisG, by AICAR, which is formed in this pathway, generates a serious bottleneck during His production. The constructed His-producing strain demonstrated the enhanced expression of genes that encode enzymes involved in the metabolism of AICAR to ATP, which is a substrate of HisG, and thus led to improved His accumulation.

**Keywords:** L-Histidine, Inorganic phosphate/metal transport, ATP-phosphoribosyltransferase, AICAR (ZMP), PitA, Pho regulon, *Escherichia coli*, L-Histidine production

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Background

L-Histidine (His) is an essential amino acid that is used in the pharmaceutical industry as a component of nutritious mixes for infant and adult humans [1]. In biochemical studies, His is a growth factor involved with many primary metabolites [2, 3]. Industrial His-producing strains of Corynebacterium glutamicum, Brevis bacterium flavum, Serratia marcescens and Escherichia coli have been described [3].

The biosynthesis of His was initially well characterized in Salmonella typhimurium and E. coli as imperative model systems to study the fundamental processes connected with this function, including transcriptional attenuation, gene expression, and enzymatic feedback regulation [4–6].

In bacterial cells, the His biosynthesis pathway is associated with ten biochemical reactions and nine enzymes that contribute to the conversion of two biosynthetic precursors, phosphoribosyl pyrophosphate (PRPP) and adenosine triphosphate (ATP), into this amino acid (Fig. 1) [6].

The first key step in the His biosynthetic pathway, the condensation of PRPP and ATP to form phosphoribosyl-ATP (PR-ATP), is catalyzed by the ATP-PR transferase (HisG, EC 2.4.2.17), which is exposed to a variety of types of inhibition: allosteric feedback inhibition by His, competitive inhibition by adenosine mono- and diphosphates (AMP and ADP, respectively) [7], and competitive product inhibition by PR-ATP. Eliminating the His-mediated feedback inhibition of HisG plays a critical role in the regulation of His biosynthesis [6, 8]. Feedback-resistant HisG-mutant enzymes from S. typhimurium [9] and HisG-mutant enzymes from Salmonella typhimurium [10] have been previously characterized. The HisGE271K Fbr-mutant from E. coli was obtained by traditional selection methods several decades ago [12], and its properties were later investigated [13]. Despite having complete resistance to feedback inhibition by His, this mutant enzyme is still susceptible to competitive inhibition by purine nucleotides, particularly ADP and AMP [12]. This finding suggests that HisGE271K, similar to the native HisG, could be the target of inhibition by the AMP structural analog, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5’-monophosphate (AICAR). Figure 1 indicates that AICAR is formed via the His biosynthetic pathway as well as through the de novo purine biosynthetic pathway [6, 14]. In the present study, we confirmed that both the native (HisGWT) and feedback-resistant (HisGE271K) enzymes were sensitive to inhibition by AICAR. The formation of AICAR through the His biosynthetic pathway in E. coli is thus negatively controlled by end-product (AICAR) inhibition of the first key enzyme in the pathway, HisG. Considering that His

![Fig. 1](image-url)
biosynthesis is accompanied by equimolar AICAR generation, the effective metabolism of AICAR to ATP, which in turn functions as the HisG substrate, appears crucial for the production of this amino acid.

The reactions that convert AICAR into ATP include the use of bi-functional PurH (EC 2.1.2.3 + EC 3.5.4.10) [15], PurA (EC 6.3.4.4) [16], PurB (EC 4.3.2.2), Adk (EC 2.7.4.3) and H\(^+\)-ATP synthase (F\(_{i}\)F\(_{o}\)-ATP synthase) [17] (Fig. 1). Since the final step of ATP synthesis from AICAR includes ADP phosphorylation by inorganic phosphate (P\(_i\)) that is catalyzed by the proton force-dependent ATP synthase, the maintenance of intracellular P\(_i\) at the appropriate level is a significant requirement for the efficiency of the whole pathway.

The two major P\(_i\) transport systems were initially described in *E. coli* cells: Pst (PstSCAB, phosphate-specific transport) and Pit (PitA, phosphate inorganic transport) [18–21]. PstSCAB, a member of the ABC superfamily of ATP-dependent transporters, is the major high-affinity, low-velocity phosphate uptake system. Under conditions of P\(_i\) limitation, the Pst system is activated more than 100-fold, and P\(_i\) is primarily taken up by the Pst transporter. In addition, at basal expression levels, the Pst system and not PitA (see below) primarily contributes to P\(_i\) uptake when excess P\(_i\) is present [22]. *E. coli* also possesses a high-velocity, low-affinity P\(_i\) transporter, PitA, which does not belong to the phosphate (Pho) regulon; PitA is dependent on the proton motive force for energization, and P\(_i\) uptake via PitA is defined by the presence of divalent cations such as Zn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\), which form soluble metal phosphate (MeHPO\(_4\)) complexes [23]. PitA gene expression was reported to be modulated in a chemically defined medium by the availability of P\(_i\) and Zn\(^{2+}\) ions [24], and PitA functions primarily as a transporter of divalent metal cations (Zn\(^{2+}\)) complexed with P\(_i\) [22, 25]. A third P\(_i\) transport gene, *pitB*, encodes a functional P\(_i\) transporter that is a homolog of PitA, sharing 81% identity (http://blast.ncbi.nlm.nih.gov/, BLAST\(^\text{®}\) data); *pitB* is repressed at low P\(_i\) levels by the Pho regulon [26] and likely does not play a role in P\(_i\) uptake in normal cells because it is not expressed under normal growth conditions [22]. Two additional transporters, encoded by *gltT* and *uphT*, transport P\(_i\) with low affinity [27–29]; however, in the absence of Pst and Pit, these systems cannot support growth when phosphate is provided as P\(_i\) [24, 30]. Maintaining a sufficient P\(_i\) pool is extremely important for many energy-consuming cellular processes [18], particularly for His synthesis.

Oversynthesis of any cell metabolite, e.g., His, alters the carbon fluxes and pools of intermediate compounds, possibly resulting in consequences that are generally unpredictable. In the present study, some effects were revealed to be related to His overproduction by *E. coli*, and the elucidation of these effects has begun. AICAR negatively influenced His biosynthesis in *E. coli*, and a strategy for the efficient conversion of this intermediate to ATP by enhancing the de novo purine biosynthetic pathway genes, *purH* and *purA*, was developed that improved His production. The influence of AICAR on the activity of HisG was studied and revealed that AICAR is a structural analog of AMP; AICAR was even a stronger inhibitor of HisG ($K_i = 0.65$ mM) than AMP ($K_i = 2.15$ mM). His oversynthesis was accompanied with a change in the functioning of the Pho regulon that was explained by changes in the AICAR pool. Furthermore, *pitA* gene deletion positively influenced His overproduction, and although the precise mechanism underlying this effect remains unclear, several explanations based on biological role of PitA as a Me\(^{2+}\)-P\(_i\) importer were supposed.

**Methods**

**Strains, plasmids and media**

All bacterial strains and plasmids used in this study are listed in Table 1.

The following media were used to culture bacteria: Luria–Bertani (LB), M9, SOB, SOC [31], and MOPS, and the MOPS medium was supplemented with 0.25 mM KH\(_2\)PO\(_4\). Glucose (0.4%) was added to minimal media as a carbon source. The antibiotics ampicillin (Ap, 100 mg/L), chloramphenicol (Cm, 20 mg/L), tetracycline (Tc, 20 mg/L), and kanamycin (Km, 50 mg/L) were used when necessary.

**Test tube cultivation conditions**

For His accumulation, the strains were grown in LB medium at 30 °C overnight; 0.1 mL of each culture was then inoculated into 2 mL of fermentation medium in a test tube, which was then cultivated for 72 h at 30 °C with shaking on a rotary shaker (250 rpm), until all of the glucose was consumed. The composition of the fermentation medium (g/L) was as follows: soybean meal hydrolysate, 0.1 g; l-aspartate, 0.5 g; (NH\(_4\))\(_2\)SO\(_4\), 9 g; KCl, 0.5 g; KH\(_2\)PO\(_4\), 0.25 g; MgSO\(_4\)·7H\(_2\)O, 0.2 g; FeSO\(_4\)·7H\(_2\)O, 0.01 g; MnSO\(_4\)·5H\(_2\)O, 0.01 g; ZnSO\(_4\)·7H\(_2\)O, 0.01 g; adenosine, 0.1 g; vitamin B1, 0.0005 g; betaine, 1 g; CaCO\(_3\), 30 g; and glucose, 25 g, as the carbon source; the pH was adjusted to 6.0.

**DNA manipulation**

Genetic manipulation of *E. coli* and techniques for the isolation and manipulation of nucleic acids were performed according to standard protocols [31]. Restriction enzymes, T4 DNA ligase, High Fidelity PCR Enzyme Mix, Taq polymerase, and 1-kb DNA Ladder were purchased from Thermo Scientific Inc. (USA). Plasmids
Table 1  *E. coli* strains and plasmids used in this study

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| MG1655            | *Escherichia coli* K-12 wild-type | VKPM\* B-6195 |
| BL21(DE3)         | *E. coli B* F–ompT gal dcm lon hisD5 (r3m7m9) λDE3 [lacI P_T7gene1 and 1 am7 min3] [molB\*1, recA\*3] | [32] |
| KF37              | MG1655+ [ΔpurR:Cm\*] | [13] |
| MG1655 [ΔpurR:Cm\*] | MG1655 wild-type *E. coli* K-12, but ΔpurR::λattR-cat-λattl | Laboratory collection |
| BW25113 [ΔpurH:Km\*] | lac\* mB\_{14} ΔlacZ\_{1916} hisR514 ΔaraBAD\_{1533} ΔrhaBAD\_{1578} ΔpurH::FRT-kan-FRT | [33] |
| MG1655 [Δ(p80-attB)] | MG1655 with deleted native (p80-attB) site | [34] |
| MG1655 [Δ(p80-attB) IS 5.11::p80-attB] | MG1655 with deleted native p80 attB site and reconstruction of attB site in IS 5.11 locus | [34] |
| MG1655 [Δ(p80-attB) IS 5.11::p80-attB] | MG1655 [Δ(p80-attB) IS 5.11::p80-attB], but IS 5.11::(λ-attB) P\_{φ80} purA | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔpurH::λattR-cat-λattl and ΔpurH::FRT-kan-FRT | This study |
| KF37 [Δ(p80-attB)] | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| KF37 [Δ(p80-attB)] | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| EA79              | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| EA83              | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| CC118 λpir+      | Host strain for maintenance of pir-dependent recombinant plasmids | [35] |
| MG1655 [Δ(p80-attB) ΔyibH::p80-attB] | MG1655 with deleted native (p80-attB) site and reconstruction of attB site instead of yibH locus | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| MG1655 [Δ(p80-attB)] | MG1655, but ΔpurH::λattR-cat-λattl and ΔpurH::FRT-kan-FRT | This study |
| KF37 [Δ(p80-attB)] | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| KF37 [Δ(p80-attB)] | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| KF37 [Δ(p80-attB)] | KF37, but ΔlacI-cat-λattl-P\_{φ80} purA | This study |
| Plasmids          |              |        |
| pKD46             | oriR101, repA101ts, oarC, P\_{φ80} purA, β, exo of phage λI, Ap\^\^, used as a donor of λRed-genes to provide λRed-dependent recombination | [36] |
| pMWts-lnt/Xis     | oriR101, repA101ts, λcIts857, X\_φ80→λxis-int, Ap\^\^, used as a helper plasmid for thermodurable expression of the λxis-int genes | [34] |
| pAH123            | oriR101, repA101ts, λcIts857, X\_φ80→λxis-int, Ap\^\^, used as a helper plasmid for thermodurable expression of the φ80-int gene | [37], GenBank accession number AJ048726 |
| pET15b            | Ap\^\^, pBR322 origin, P\_{φ80}-lacI, and T7 promoter/O\_{φ80}, T7 transcription start, His6-Tag coding sequence, T7 terminator | Novagen |
| pMW188-Km\*      | oriR101, repA, MCS, Ap\^\^, λattIR-kan-λattl—donor of λXis/int-excisable Km\* marker | [38] |
| pET15b-hisG       | Ap\^\^, pET15b containing hisG gene | This study |
| pET15b-hisG\^{279K} | Ap\^\^, pET15b containing the mutant hisG\^{279K} gene | This study |
| pAH162-2Ter       | attP φ80, pAH162, λattIR-κattR | [34], GenBank accession number AJ048738 |
| pMW119-P\_φ80-lacI | Ap\^\^, low-copy-number vector pMW119, containing P\_φ80-lacI | Laboratory collection |
| pMW119-P\_φ80-lacI-purA | Ap\^\^, low-copy-number vector pMW119, containing P\_φ80-lacI-purA | This study |
| pAH162-2Ter-purA  | oriR101, φ80-attR, λattIR-κattR, purA | This study |
and genomic DNA were isolated using QIAGEN Plasmid Mini Kits (QIAGEN GmbH, Germany) and Bacterial Genomic DNA Kits (Sigma), respectively. QIAquick Gel Extraction kits (QIAGEN GmbH, Germany) were used to isolate DNA from agarose gels. Oligonucleotides were purchased from Evrogen (Russia). The sequences of the oligonucleotide primers are presented in Additional file 1: Table S1.

Quantitative determination of the L-histidine concentration
The amount of His that accumulated in the medium was determined by thin-layer chromatography (TLC) using plates coated with silica gel (Merck, Germany). Samples were applied to the TLC plates using Linomat 5 (Camag, Switzerland). The plates were developed with a mobile phase consisting of propan-2-ol:acetone:25% aqueous ammonia:water = 12.5:12.5:3:2 (v/v). A solution of ninhydrin (1%) in acetone was used as the visualizing reagent; the plates were dried and then scanned at 520 nm using a Linomat 5 scanner (Camag, Switzerland).

pET15b-hisG plasmid construction
The native hisG gene was cloned into a pET15b plasmid vector after PCR amplification (see Additional file 1: Table S1 for details, primers 4, 5). The obtained PCR fragments were treated with BamHI and NdeI endonucleases and ligated into a pET15b vector that had been treated with the same enzymes. The obtained pET15b-hisG plasmid carries the gene encoding HisG with an N-terminal cleavable His6-Tag (HT-HisG) for affinity purification. The obtained plasmid was introduced into the strain E. coli BL21(DE3) for HT-HisG induced biosynthesis followed by tagged-protein purification. Plasmid pET15b-hisG(E271K), which harbors the mutant feedback-resistant HisG (HT-HisG(E271K)) gene, was obtained in a similar manner.

HisG expression and purification
Escherichia coli BL21(DE3)/pET15-hisG was grown in 50 mL of LB medium until the OD_{560} was 0.8. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, which was followed by incubation for 4 h. The cells were centrifuged, washed twice with 50 mL of 100 mM NaCl solution, centrifuged again, resuspended in 50 mL of buffer I (300 mM Tris–HCl, 300 mM KCl, and 1 mM PMSE, pH 8.1) and disrupted by two passages through a cold French press cell at 2000 psi. Unbroken cells and cell debris were removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant was applied to a 1-mL HiTrap® column (Pharmacia, Sweden); this column was then washed with 10 ml of buffer I, and bound protein was eluted with a linear gradient of buffer I and buffer II (20 mM Tris–HCl and 400 mM imidazole, pH 8). The resulting HT-HisG preparation was further purified by gel filtration using a 10-mL BioGel P10 column (Pharmacia, Sweden) equilibrated with buffer III (20 mM potassium phosphate buffer, pH 7, 1 mM DTT, 10 μM PLP, and 10% (w/v) glycerol). This method was used to purify two ATP-phosphoribosyltransferases (ATP-PRTs): native HT-HisG and mutant HT-HisG(E271K).

ATP-phosphoribosyltransferase assay
The HisG enzymatic activity and initial velocity of the forward phosphoribosyltransferase reaction were measured by monitoring the formation of PR-ATP (ε_{290} = 3600/M/cm [40]) in the presence of E. coli inorganic pyrophosphatase (PPase, Sigma-Aldrich, USA). Reactions were performed in UV-star 96-well microplates (Greiner Bio-One, Germany). The reaction mixture consisted of 100 mM Tris–HCl (pH 8.1), 100 mM KCl, 10 mM MgCl₂, 5 mM ATP, 1 mM PRPP, 10 μU of pyrophosphatase and 500 nM E. coli HisG [41]. The reaction was initiated by the addition of ATP. The absorbance at 290 nm was monitored for 30 min in 2-min intervals (Tecan, Switzerland). A reaction mixture containing water instead of ATP substrate was used as a blank control. To test the inhibition of HisG by AMP and AICAR, these compounds were added to the initial reaction mix. Notably, the molar extinction coefficients of AICAR and AMP are ε_{290, AICAR} = 2700/M/cm and ε_{290, AMP} = 240/M/cm. AICAR concentrations higher than 1 mM were therefore undesirable for the measurements, but AMP concentrations as high as 5 mM could be used without significant interference. To obtain the values of K_{I, AMP}
and $K_{\text{ICAR}}$, AMP or AICAR was added to the reaction mix to a final concentration of 20 and 1 mM, respectively. To calculate the inhibition constants, the following formula was used [42]: $K_i = \frac{[I]}{(K_M/[M] - 1)}$, where $K_i$ is the inhibition constant, $[I]$ is the concentration of the inhibitor, $K_M$ is the Michaelis constant, and $K_M$ is the $K_M$ value in the presence of inhibitor.

Alkaline phosphatase (PhoA) assay

The enzymatic activity of *E. coli* alkaline phosphatase (the *phoA* gene protein product, E.C. 3.1.3.1) was measured according to the method of Brickman and Beckwith [43] with modifications. Cells were grown in a flask for 24 h in MOPS medium supplemented with 0.4% glucose and 0.25 mM KH$_2$PO$_4$ and were then washed with 0.9% NaCl. The cells were then lysed by sonication, and cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The protein concentration in the supernatant was determined using a standard Bradford Protein assay [44]. PhoA enzymatic activity was analyzed 2 or 24 h after $P_i$ exhaustion. Enzyme reactions were performed in 96-well UV-star microplates (Greiner Bio-One, Germany) and consisted of 500 mM Tris–HCl, pH 8.0, 1 mM MgCl$_2$, and supernatant (or supernatant diluted in 0.9% NaCl) containing from 0.02 to 0.10 mg of protein. p-Nitrophenyl-phosphate (pNPP) was used as the substrate. After pNPP addition, the reaction mixture was incubated at 37 °C for 3–4 min, by which time it had turned yellow. The reaction was stopped by the addition of 1 M KH$_2$PO$_4$, and the absorbance at 410 nm was measured in a UV-star microplate cell against a control reaction that did not contain protein.

Inorganic P$_i$ measurement

The amount of P$_i$ in culture media was determined using a common method based on phosphomolybdate reduction to molybdenum blue [45, 46]. In 96-well microplates containing 0.1 mL of each sample to be analyzed, 0.075 mL of ammonium molybdate-ferrous sulfate (a colored mixture) was added, and the resulting solution was vortexed and incubated at room temperature for 10 min. The colored mixture was prepared daily by mixing 4 vol of 2.5% ammonium molybdate solution with 1 vol of 2.5% ferrous sulfate solution. For the calibration curve, the colored mixture was added to samples containing standard solutions of KH$_2$PO$_4$. The absorbance was measured at 700 nm. A sample without added P$_i$ served as a blank.

Construction of strains and plasmids

Insertions and deletions in the chromosome of *E. coli*, usually the MG1655K-12 strain, were prepared via λ-Red modification according to the method of Datsenko and Wanner [36], exploiting the λXis/Int system for marker excision [34]. The plasmid pKD46, carrying the arabinose-inducible λ-Red genes [36] and kindly gifted by Dr. Wanner, was used. ϕ80-mediated site-specific integration was carried out according to the method of Haldimann and Wanner with the thermod inducible ϕ80-Int gene in the pAH123 plasmid [37]. Specially designed cassettes with Km$^R$, Km$^R$ and Tc$^R$ markers bracketed by hybrid λattP/λattB-sites (λattL and λattR) were transferred into *E. coli* strains by P1-mediated general transduction (P1-dution) [47]. Finally, Km$^R$, Km$^R$ and Tc$^R$ were eliminated from the *E. coli* chromosome using a λXis/Int site-specific recombination system with pMWts-λInt/Xis as a helper plasmid [34].

**Construction of *E. coli* strain MG1655 [Δ(ϕ80-attB) IS 5.11::Cm$^R$-P$_{lac21}$-purA]**

To construct *E. coli* strain MG1655 [Δ(ϕ80-attB) IS5.11::Cm$^R$-P$_{lac21}$-purA], the native purA gene was PCR amplified with primers 6–7 (see Additional file 1: Table S1 for details) using the MG1655 DNA as a template and was then cloned into a pMW119-P$_{lac}$-lacI vector that had been treated with SmaI. Plasmid pMW119-P$_{lac}$-lacI-purA was treated with BamHI and KpnI endonucleases, and the resulting purA-containing fragment was recloned into a pAH162-Tc$^R$-2Ter [34] integrative plasmid. The resulting plasmid, pAH162-Tc$^R$-2Ter-purA, was used for ϕ80-mediated integration of the promoter-less purA gene into the artificial ϕ80-attB site of the MG1655 [Δ(ϕ80-attB)] IS 5.11::ϕ80-attB] strain constructed earlier [34]. Expression of the purA gene was activated by λRed-mediated insertion of the P$_{lac21}$ promoter region with two point mutations in the -35 region (ttgaca of P$_{lac}$ was replaced with ttgaca) upstream of the gene using primers 8–9 (see Additional file 1: Table S1 for details). The obtained strain MG1655 [Δ(ϕ80-attB) IS5.11::Cm$^R$-P$_{lac21}$-purA] was used as the donor of a purA-carrier expression cassette, marked by an excisable Cm$^R$-marker, for P1-dution of the corresponding gene in the different strains of interest.

**Construction of *E. coli* strain MG1655 [Cm$^R$-P$_{purH}$]**

To construct *E. coli* strain MG1655 [Cm$^R$-P$_{purH}$], the upstream region of the purH gene, which is associated with purD in a single operon, was modified by replacement of the native regulatory region with the λP$_L$ promoter by λRed recombination as mentioned above. To construct the PCR fragment for λRed recombination with an excisable cat marker and flanking sequences homologous to the purHD regulatory region, we used primers 10 and 11 (see Additional file 1: Table S1 for details).

**Construction of strain MG1655 [ΔpitA::Km$^R$]**

To delete pitA in *E. coli* strain MG1655, the λRed integration method was used as described above. To construct
the PCR fragment for λRed recombination with an excisable kan marker and flanking sequences homologous to the pitA gene, we used primers 12 and 13 (see Additional file 1: Table S1 for details) with the pMW118-KmR plasmid as a template. The chromosomal deletion of pitA was confirmed by PCR.

Construction of E. coli strain MG1655 [CmR·Plac·pitA]
To construct E. coli strain MG1655 [CmR·Plac·pitA], the upstream region of the pitA gene, was modified by replacement of the native regulatory region with the λPlac promoter gcgggt-ttgaca-attaatagctgataggtgcatt [hybrid of λPlacl and λPtac (attatatagctgataggtgcatt)] promoters. λPtac promoter-35 sequence and λPlac promoter -35 sequence, shared by the two promoters (ttgaca). To construct the PCR fragment for λRed recombination with an excisable cat marker and flanking sequences homologous to the pitA regulatory region, we used primers 14 and 15 (see Additional file 1: Table S1 for details).

Construction of strain MG1655 [ΔpurR·CmR ΔpurH·KmR]
To design an E. coli strain of MG1655 with double deletions of the purR and purH genes, MG1655 [ΔpurR·CmR] (Table 1) was used as the recipient for P1-duction of the ΔpurH·KmR cassette from the Keio collection [33]. The presence of two chromosomal modifications, ΔpurR·CmR and ΔpurH·KmR, was confirmed by PCR with primers 16–17 and 18–19, respectively (see Additional file 1: Table S1 for details).

Construction of the KF37-based strain
with PhoBR-independent activation of Pho regulon genes
The sequence of phoBDBD, which encodes the C-terminal DNA-binding domain of PhoB [48] (specifically, (aa 1–3 of PhoB)-(Glu-Phe)-(aa 126–229 of PhoB)], was amplified by PCR using primers 20–21 (see Additional file 1: Table S1 for details) using the MG1655 chromosome as a template. Both the PCR product and integrative plasmid pAH162-TcR-2Ter (Table 1) were treated with SalI and SmaI restriction enzymes and then ligated with T4 DNA ligase. The ligation mixture was transformed into the CC118 λpir+ strain (Table 1), and the resulting pAH162-TcR-2Ter-phoBDBD plasmid, containing the promoter-less phoB gene, was inserted into the yibH locus of the MG1655 [Δ(q80-attB)]ΔyibH::q 80-attB] chromosome by q80-Int-mediated integration. The MG1655 [ΔyibH::TcR·phoBDBD] strain was cured of the TcR marker using a pMWts-Alnt/Xis helper plasmid. A PlacUV5 promoter with the KmR marker was inserted upstream of the phoBDBD gene using λRed recombination. The integrative DNA fragment was obtained by overlap extension PCR. To achieve this goal, the KmR marker was amplified from a pMW118-KmR template using primers 22–23 (see Additional file 1: Table S1 for details). Promoter PlacUV5 was amplified in parallel from a pML-PlacUV5-lacI [39] template using primers 24–25 (see Additional file 1: Table S1 for details). Extension PCR was performed by mixing the two initially amplified DNA fragments and using primers 22 and 26 (see Additional file 1: Table S1 for details). λRed recombination was applied to the MG1655 [ΔyibH::phoBDBD] strain, and the resulting MG1655[ΔyibH::KmR·PlacUV5·phoBDBD] strain was obtained. The strains MG1655-[ΔyibH::TcR·phoBDBD] and MG1655 [ΔyibH::KmR·PlacUV5·phoBDBD] were used as donors to individually transfer the constructs ΔyibH::TcR·phoBDBD and ΔyibH::KmR·PlacUV5·phoBDBD into strain KF37 by P1-duction.

Results
AICAR controls its own formation in the His biosynthetic pathway
The enzymatic activity of HisG is subject to (i) feedback inhibition by His as the final pathway product, (ii) competitive inhibition by PR-ATP as a reaction product [6], and (iii) competitive inhibition by ADP and AMP, compounds that are structurally similar to its ATP substrate. The synthesis of each His molecule is accompanied by the release of one molecule of AICAR, an intermediate of de novo purine biosynthesis. In turn, AICAR is a well-known natural analog of AMP and can substitute for it in a number of biochemical reactions [49, 50]. Considering the structural similarity between AMP and AICAR (Fig. 2), not only AMP but also AICAR might negatively affect HisG enzymatic activity.

To test this hypothesis, we measured the HisG activity of two purified His6-tagged enzymes, the wild type (HT-HisG) and a Fbr-mutant variant (HT-HisG E271K) that is resistant to feedback inhibition by His, in the presence of either AMP or AICAR (Fig. 2, Table 2). Figure 2 shows that the partial inhibition of HisG was detected for both tested compounds, but inhibition by AICAR was significantly more pronounced and was detected at low concentrations at which inhibition by AMP was nearly absent, that is in consistence with the literature data [51] (see Table 2). The dephosphorylated form of AICAR, (AICAR), does not affect HisG activity (data not shown). His biosynthesis is thus regulated not only by histidine itself as the terminal product of this pathway but also by AICAR as an AMP analog. Moreover, the biosynthesis of AICAR per se is under negative control via the initial step of His pathway, which is catalyzed by HisG.

AMP inhibits HisG activity due to competition with one of HisG’s native substrates, ATP [41]; AICAR could therefore be similar to AMP and thus exhibit an even stronger competitive inhibition of HisG.
Enhancement of AICAR conversion to ATP to intentionally overproduce His

The formation of AICAR by His biosynthetic enzymes is negatively controlled by AICAR inhibition of HisG. His production is thus directly regulated by this intermediate of purine biosynthesis, and a reduction in the AICAR pool due to its recycling to ATP may be one important way to overcome the bottleneck in His overproduction (Fig. 1). At a minimum, overexpressing \(\text{purH}\) and \(\text{purA}\) genes is necessary to enhance AICAR conversion to ATP (see Fig. 1). For \(\text{purH}\) overexpression, its native regulatory region in the chromosome of the MG1655 wild-type strain was replaced with the “strong” constitutive \(\lambda\)P\(_t\) promoter via \(\lambda\)Red mediated recombination, and the MG1655 \([\text{CmR-}\lambda\text{PL-purH}]\) strain was obtained (see Methods for details).

To increase \(\text{purA}\) gene expression, we specifically introduced an additional copy of the corresponding gene into the IS 5.11 locus of the MG1655 chromosome (as described in “Methods” section), followed by P1-duction of the \(\text{CmR-}\lambda\text{P}_{\text{lac}21}\)-\(\text{purA}\) cassette into several strains of interest. One of the obtained \(\text{CmR}\)-ductants, an MG1655-derivative that had earlier passed through several stages of selection for increased His overproduction, was used in the present study as the donor for P1-duction of the \(\text{purA}\)-carrier marked cassette. This cassette was P1-duced into the His-overproducing strain KF37 [13], followed by evaluation of His accumulation by the obtained \(\text{CmR}\)-ductants in test tube fermentations. Two groups of P1-ductants were selected according to their ability to overproduce His. The members of the first (main) group (more than 70% of the tested P1-ductants) increased His accumulation by approximately 20% over its levels in the initial KF37 strain. The members of the second (minor) group also exceeded KF37 in His overproduction, but the difference was approximately 6% (Table 3). Direct sequencing of the chromosomal regions flanking the bacterial locus IS5.11, the point of integration of the \(\text{purA}\)-carrier cassette in the P1-ductants obtained on the basis of KF37 and in the strain used as the donor for P1-duction, confirmed that the difference in His accumulation did not correspond to the structure

![Fig. 2 Lineweaver–Burk plots. a Inhibition of HT-His\(^{\text{GWT}}\) by AMP. b Inhibition of HT-His\(^{\text{GWT}}\) by AICAR. Chemical structures of AMP (a) and AICAR (b).](image-url)

| Enzyme          | ATP-phosphoribosyltransferase activity, \(\mu\text{mol/min/mg}\) | Inhibition, % |
|-----------------|---------------------------------------------------------------|---------------|
|                 | – AMP \(\mu\text{mol/min/mg}\) | 1 mM | 20 mM | 0.5 mM | 1 mM | – AMP | 1 mM | 20 mM | 0.5 mM | 1 mM |
| HT-HisG         | 184±9                                                        | 195±1         | 120±15 | 151±9  | 120±5 | 0      | 35   | 18    | 35    |
| HT-HisG\(^{\text{E271K}}\) | 120±1                                                   | 129±12        | nm     | 67±4   | 58±8  | 0      | nm   | 44    | 52    |

Average data of 3 independent experiments are represented
\(\text{nm}\) not measured

Table 2 Specific activity of purified His\(_6\)-tagged wild-type and feedback-resistant ATP-phosphoribosyltransferases and their inhibition by AMP and AICAR
of the cassettes, which were the same, in full agreement with the proposed design. The difference could be explained by the nonsense mutation that inactivated the pitA gene being closely coupled with the point of purA integration in the initial donor and in the tested P1-ductants that produced more His. This mutation was absent in the tested chromosomes of the P1-ductants from the second group. Moreover, the absence or presence of the mutation leading to pitA inactivation was confirmed by allele-specific PCR for the different members of the second and the first groups of P1-ductants, respectively (see Additional file 1: Table S1 for details, primers 1–3). One of the variants from the first group of P1-ductants that produced more His was cured of the CmR-marker due to Xis/Int-mediated recombination; the obtained marker-less strain was assigned as EA79 and used for the following improvement process.

Figure 3 shows that the overexpressed purH-carrier cassette was P1-ducted from the strain MG1655 [CmR-PL-purH] into EA79 using CmR as a selective marker, and the marker was then excised to yield the EA83 strain, which accumulated 10% more His than EA79. In full accordance with the earlier supposition, the possible decrease in the intracellular AICAR pool due to the overexpression of purA and purH genes thus actually increased the level of His accumulation. At the same time, the occasional co-transduction of the mutation-inactivated pitA gene closely located to a P1-ducted cassette had a much greater positive effect on His production than the overexpression of purA itself (see Table 3).

### PitA deficiency positively affects L-His production

To analyze the individual effect of PitA transporter elimination on His production, we deleted the corresponding gene in strain KF37, and the positive influence of PitA deficiency was confirmed (Table 4). In opposite, enhancement PitA transporter by introduction of the CmR-PL-pitA expression cassette, harboring over-expressed pitA gene under the strong λP lac promoter control, into the His-producing strain KF37 lead to the drastically reduction of growth rate and His accumulation. The inactivation of pitB, which encodes a minor metal phosphate/H⁺ symporter, alone or in addition to

| Strain                                      | His, % | His, g/L |
|---------------------------------------------|--------|---------|
| KF37                                        | 100    | 3.3 ± 0.5 |
| KF37 [ΔpitA::KmR]                          | 112    | 3.8 ± 0.3 |
| KF37 [CmR-PL-pitA derog]                   | 41     | 1.4 ± 0.2 |

**Table 4 Influence of PitA deficiency and enhancement on His production**

Average data from 6 independent experiments are represented.

### Table 3 Effect of PitA inactivation on His production by the strain KF37 [IS5.11::CmR-PL-purA pitA⁻]

| Strain                                      | pitA allele | OD₅₄₀     | His, g/L | His, %  |
|---------------------------------------------|-------------|-----------|----------|---------|
| KF37                                        | pitA⁺       | 14.9 ± 0.4| 3.3 ± 0.1| 100     |
| KF37 [IS5.11::CmR⁺-purA⁺, PitA⁺, P_tac²₁⁻-numR] | pitA⁺       | 16.1 ± 0.1| 3.5 ± 0.1| 106     |
| KF37 [IS5.11::CmR⁺-P_tac²₁⁻-purA, pitA⁻]     | pitA⁻       | 15.6 ± 0.4| 4.0 ± 0.1| 121     |

**Table 3**

**Effect of PitA inactivation on His production by the strain KF37 [IS5.11::CmR-PL-purA pitA⁻]**

Average data from 8 independent experiments are represented.

**Fig. 3** Genealogy of His-producing strains with enhanced AICAR conversion into ATP. me marker elimination. Average data from 10 independent experiments are represented.
the elimination of PitA did not influence the level of His accumulation (data not shown).

The nature of this phenomenon is unclear, although several hypotheses concerning the positive influence of ΔpitA on His production were generated on the basis of the known properties of the PitA Me²⁺-Pi transporter. We supposed that the deletion of pitA can decrease import of Pi and Zn²⁺. The decreased import of Zn²⁺ may increase availability of Mg²⁺ for the efficient PPhase-mediated PP₃ hydrolysis [24, 52] and result in the promotion of PP₃-generating reactions, including those of the His biosynthetic pathway (Fig. 4, see “Discussion” for details). In turn, the His biosynthetic proteins HisG and HisL are PP₃-releasing enzymes, and a metabolic significance of their function could thus be coupling with PPase activity to increase the intracellular Pi pool.

Influence of ΔpitA on Pho regulon induction and Pi uptake
To test the supposition concerning the decrease in inorganic phosphate import and probable alterations of the Pi pool in the absence of PitA, we evaluated the possible influence of PitA inactivation on Pho regulon gene activation. The activity of the bacterial alkaline phosphatase (EC 3.1.3.1, PhoA, the product of the phoA gene that belongs to the E. coli Pho regulon [53–57]) was measured for this purpose. Growth in the MOPS-based minimal medium and Pi consumption were initially investigated for the wild-type strain MG1655, the His-producing strain KF37 and their respective pitA-deficient variants (see Additional file 3: Figure S2A and B). PhoA enzymatic activity was analyzed 24 h after Pi exhaustion. PitA transporter inactivation did not significantly change the growth efficiency and capability of Pi uptake of either MG1655 or KF37 under the tested conditions (see Additional file 3: Figure S2A), indicating that the total Pi uptake could be only slightly dependent on activity of PitA transporter. The insignificant effect of PitA transporter inactivation on capability of Pi uptake confirmed the knowledge concerning the main formation of intracellular Pi pools in E. coli. The variety of recent data show that the Pst system, but not PitA system, serves as the primary Pi transporter when Pi is in excess [22].

Interestingly, PhoA activity after Pi depletion was practically absent in the His-producing strain KF37, regardless of the pitA allele status and in contrast to the wild-type strain (Fig. 5). In the framework of the existing model of the control of Pho regulon [22, 58, 59], the hypothesis of possible AICAR negative influence on Pho regulon, in particular, PhoR kinase activity, was proposed. As known, E. coli PhoR histidine kinase has catalytic ATP-binding CA domain [58]. Pho regulon repression in case of His-producing strains could be explained by a reduction of PhoR’s ability to ATP-dependently autophosphorylate, which is necessary for the subsequent manifestation of its kinase activity in relation to PhoB when the Pho regulon activates (see Fig. 6, adapted from Hsieh and Wanner [22]). This reduction is probably based on an inhibition of ATP-dependent PhoR

![Fig. 4 Schematic view of the putative role of PitA deficiency in the cellular Pi/PPi balance and histidine biosynthetic pathway.](image)

![Fig. 5 AP enzymatic activity under conditions of Pi limitation in His-producing and non-producing E. coli strains with different pitA alleles.](image)
by AMP structural analog, AICAR, whose pool might be increased in His-producing strains.

**Table 5** Alkaline phosphatase (PhoA) activity under conditions of AICAR overproduction

| Strain                          | AICAr, mg/L | PhoA activity, µmol/min/mg |
|--------------------------------|------------|---------------------------|
| MG1655 (wild-type)             | < 0.1      | 410 ± 7                   |
| MG1655ΔpurR::CmR ΔpurH::KmR    | 7.6        | 261 ± 1                   |

PhoA enzymatic activity was analyzed 2 h after Pi exhaustion. Average data of PhoA activity from 3 independent experiments are represented.

**Fig. 6** Proposed model of Pho regulon expression control. a) Activation of the Pho regulon during Pi limitation. b) Partial deactivation of the Pho regulon during Pi limitation in the presence of AICAR. PhoR histidine kinase, PhoB response regulator, PstSCAB phosphate-specific ABC transporter, PhoU negative regulator of PhoR, AICAR 5-aminoimidazole-4-carboxamide ribonucleotide, Pi inorganic phosphate.

**Changes in the activity of PhoA in response to AICAR oversynthesis**

To study whether the Pho regulon is repressed by AICAR, we constructed a strain with elevated intracellular levels of this metabolite and tested the activation of the Pho regulon in this strain under Pi-limiting conditions. Overproduction of AICAR was stimulated by inactivation of the PurR, a repressor of purine biosynthetic genes, and AICAR metabolism was simultaneously prevented by purH gene deletion. The AICAR pool was increased in the resulting double mutant strain MG1655 [ΔpurR::CmR ΔpurH::KmR], and this increase was detected by the appearance of AICAr, the dephosphorylated form of AICAR, in the culture broth (Table 5).

We cultivated the E. coli wild-type strain and the strain overproducing AICAR under Pi-limiting conditions (their growth and Pi exhaustion kinetics are shown in Additional file 4: Figure S3). PhoA enzymatic activity was analyzed 2 h after Pi exhaustion. As expected, the level of PhoA alkaline phosphatase activity was appreciably less in the AICAR-overproducing strain than the wild-type strain (Table 5) but significantly higher than the KF37-based His-producing strains (compared in Fig. 5). These results collectively supported the hypothesis that an increased AICAR pool (in AICAR- and His-overproducing strains) was the main reason for the decreased expression of the Pho regulon genes and were likely due to AICAR inhibiting PhoR autophosphorylation, which in turn restricted the phosphorylation of PhoB.

**Pi-independent activation of phoA gene expression in the His-producing strain KF37**

The expression of a truncated PhoB regulator containing only the DNA-binding C-terminal domain (PhoBDD [48, 57, 60]) was examined to confirm the normal functionality of the phoA gene in the KF37 His-producing strain. The expression of PhoBDD provides a Pi-independent activation of the Pho regulon [48]. PhoA synthesis in the growing cells was easily visualized as blue-colored colonies on plates containing medium supplemented with the specific chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Derivatives of the His-producing strain KF37 that also harbored the cassette containing the PhoBDD gene under the control of the IPTG-inducible promoter P_{lacUV5} synthesized PhoA after induction, as did derivatives of the wild-type strain MG1655 harboring the same cassette (Fig. 7). This finding supports the hypothesis that His overproduction affects the function of the two-component signal transduction system PhoBR rather than the Pho regulon genes themselves.

**Discussion**

AICAR is formed during His biosynthesis and in the purine biosynthetic pathway [14, 29]. The negative effects of intracellular AICAR accumulation on different aspects
of bacterial metabolism, such as adenosine homeostasis, gluconeogenesis, and thiamine synthesis, were investigated in S. enterica [61]. Moreover, several studies suggested that AICAR accumulation represses not only purine biosynthesis [62] but also one or more steps of the His pathway in S. cerevisiae [63]; however, the mechanism underlying such effects remains unclear. On the other hand, AICAR acts as a natural analog of AMP and can substitute for it in some enzymatic reactions. In turn, AMP inhibits enzymes involved in de novo purine and His biosynthetic pathways in which AICAR is formed as an intermediate [41]. We therefore hypothesized that AICAR controls its own formation, particularly in the His biosynthetic pathway. This supposition was investigated in this study by an examination of the possible influence of AICAR on the conversion of PRPP and ATP to PR-ATP, which is accomplished by HisG. The inhibitory effect of AICAR on HisG activity in vitro was demonstrated for the first time. The inhibition of HisG by high concentrations of AMP was also confirmed. Both tested substances compete with ATP in HisG-catalyzed reactions, suggesting that similar mechanisms are present for enzyme-ligand interactions. Figure 2 shows that AICAR was an even more effective inhibitor than AMP. AICAR thus controls its own formation with regard to the His biosynthetic pathway. Similar negative control may occur during AICAR formation via de novo purine biosynthesis; in this case, AICAR might control PurF enzyme activity, which is known to be negatively affected by AMP. In addition, other possibilities for His biosynthesis regulation with participation of AICAR could be investigated. It is known that AICAR is identified as an alarmone that senses e.g. 10-formyl-tetrahydrofolate deficiency in bacteria and activates a conserved riboswitch to regulate the expression of tetrahydrofolate genes involved in one-carbon metabolism [64, 65]. Therefore, it would be particularly interesting to study further the other aspects of the influence of AICAR on His biosynthesis, especially, considering close relationship of this biosynthetic pathway with one-carbon metabolism.

His overproduction cannot be engineered solely by enabling the resistance of the first biosynthetic enzyme, HisG, to feedback inhibition (see e.g., [13]). The effective metabolism of AICAR to ATP is also required to prevent the undesirable accumulation of AICAR, which is a HisG inhibitor, and to enhance the supply of ATP, which is an essential component for the synthesis of this amino acid. The effectiveness of such an approach was tested, yielding the E. coli His-overproducing strain EA83, which enhances AICAR regeneration into ATP (Fig. 3).

Furthermore, we found that a deficiency in PitA of the Me$_{2}^{+}$-Pi transport system considerably increased His accumulation (Table 3). A spontaneous mutation that inactivated the pitA gene was accidentally selected during the process of constructing the His-overproducing strains. The mechanism underlying this effect is not clear. Nevertheless, effective Zn$_{2}^{2+}$-Pi, import via the PitA transporter under conditions of excess extracellular phosphate and Zn$_{2}^{2+}$ ions may restrict the total PP$_{i}$ hydrolysis efficacy, thereby inhibiting the His biosynthetic pathway reactions (Fig. 4). In E. coli, the hydrolysis of pyrophosphate (PP$_{i}$) to P$_{i}$ is catalyzed by inorganic pyrophosphatase (PPase, EC 3.6.1.1, the ppa gene product) in the presence of different divalent metal ions (e.g., Mg$_{2}^{2+}$, Zn$_{2}^{2+}$, Mn$_{2}^{2+}$, and Co$_{2}^{2+}$), but only using Mg$_{2}^{2+}$ as physiological activator does PPase significantly increase the efficiency and specificity of PP$_{i}$ hydrolysis [52, 66, 67]. However, this specificity is lost when transition metal ions such as Zn$_{2}^{2+}$, Mn$_{2}^{2+}$, and Co$_{2}^{2+}$ are used as cofactors, leading to markedly increased activity against polyphosphates [66] and the ability to hydrolyze organic tri- and diphosphates such as ATP and
ADP [68, 69]. To test this hypothesis, we examine the effect of PitA deficiency on capability of Pi. Deletion of the pitA gene did not significantly decrease the import of Pr in the mutant strains from its levels in the PitA+ E. coli strains (Pr consumption values for both types of strains were within experimental uncertainty, see Additional file 3: Figure S2). These results correlate well with main formation of intracellular Pi pools by a basal level of Pst transporter activity in conditions of excess extracellular Pr [22]. The intracellular Pi pool was thus expected to be only slightly lower in pitA mutants than wild type. However, the deletion of pitA could additionally decrease import of Zn2+ ions and perhaps significantly increase the portion of PPase in complex with Mg2+, which improves enzyme–substrate specificity [70]. Decreasing the import of both Pr and Zn2+ could thus promote PPi-releasing reactions (in the His biosynthetic pathway, in particular) due to subsequent thermodynamically defensible hydrolysis of PPi to Pr.

To elucidate the roles of the other known Zn2+ transport systems during His production, we tested variants of a His-producing strain with a deleted znuA gene, which encodes the substrate-binding component of the ZnuABC high-affinity zinc uptake system [71], for His accumulation; the znuA deletion did not affect cell growth and had no positive effect on the rate of His accumulation (data not shown). These findings support the supposition that the effect of PitA deficiency on His production could not be explained solely by a decrease in Zn2+ uptake.

The last new feature that was detected for the His-producing strains in the present study was the significantly decreased Pho regulon activation in the growing cells after the depletion of the P (that had been initially added in the cultural medium. This effect may have been based on the two-component PhoBR system and was likely to depend on AICAR-dependent inhibition of the PhoR autophosphorylation process. Indeed, the level of PhoA activation in the specially constructed AICAR-overproducing strain was lower than that of the wild-type strain, but this effect was not so pronounced as in His-producing cells.

Conclusions

Both the native HisG and feedback resistant HisG^{E271K} from E. coli were shown to be sensitive to inhibition by AICAR. Accordingly, the production of L-histidine was improved in E. coli through the construction of strain EA83. In this strain, the AICAR regeneration pathway was enhanced via purA and purH gene overexpression and PitA-dependent phosphate/metal transport system was inactivated. Moreover, PhoA enzymatic activity was shown to be almost absent in the His-producing strain after Pr depletion in the medium, leading to the hypothesis that Pho regulon depression is associated with the inhibitory effect of AICAR, which is produced during the excessive production of His and acts as a regulatory molecule.

Additional files

Additional file 1: Table S1. PCR primers used in this study.

Additional file 2: Figure S1. Expression and purification of HT-HisG{\textit{WT}} (wild-type) and mutant HT-HisG^{E271K}. (a) SDS-PAGE of total protein containing HT-HisG^{WT} and HT-HisG^{E271K} before purification. M, protein molecular weight standards; HisG^{WT}, crude cell lysate of BL21(DE3)/pET15s-HisG{\textit{WT}} after IPTG induction; HisG^{E271K}, crude cell lysate of BL21(DE3)/pET15s-HisG^{E271K} after induction. (b) SDS-PAGE of the two purified HT-HisG{\textit{WT}} proteins. M, protein molecular weight standards; HisG^{WT}, HT-HisG{\textit{WT}} after purification; HisG^{E271K}, HT-HisG^{E271K} after purification.

Additional file 3: Figure S2. Effect of PitA deficiency on growth (a) and P_{i} uptake (b) during P_{i} starvation. (a) Growth of wild-type MG1655, MG1655 ΔpitA, MG1655 [ΔpitA::Km\textsuperscript{\textit{R}}], KF37, KF37 ΔpitA, KF37 [ΔpitA::Km\textsuperscript{\textit{R}}] strains. Growth of the KF37 His-producing strains was better during the initial period in MOPS medium, but the final optical density was the same as or lower than that of wild-type. The better initial growth of the KF37 strain can be explained by its effective sugar consumption, which was also monitored (data not shown) and was found to be exhausted at 17 h for the His-producing strain compared to 22 h for the wild-type strain (data not shown). These results confirm the measured kinetics of P_{i} uptake from the medium, as expected, the rate of P_{i} uptake was higher for the His-producing strains under such conditions. Error bars show the standard deviation (SD).

Additional file 4: Figure S3. Effect of AICAR on growth (a) and P_{i} uptake (b) during P_{i} starvation of wild-type MG1655, MG1655 ΔpurR, ΔpurH, MG1655 [ΔpurR::Cm\textsuperscript{\textit{R}}] ΔpurH::Km\textsuperscript{\textit{R}} strains. The vertical arrow indicates the sampling time for the measurement of AP enzymatic activity. Error bars show the standard deviation (SD).

Abbreviations

His: L-histidine; Fbr: feedback resistance; PRPP: phosphoribosyl pyrophosphate; AS: adenylosuccinate; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, phosphorylated form; ZMP: 5′-monophosphate, dephosphorylated form; AICAR: 5′-phosphoribosyl-5-formamido-4-imidazole carboxamide; Pi: inorganic phosphate; PPi: inorganic pyrophosphate; Pst: PstSCAB, phosphate specific transport; Pr: phospho inorganic transport; Me\textsuperscript{3+}P: metal phosphate (MeHPO\textsubscript{4}) complexes; TLC: thin layer chromatography; HT-HisG: His\textsuperscript{\textit{R}}-Tag-HisG; Da: Dalton; OD: optical density; SD: standard deviation; PCR: polymerase chain reaction; OE-PCR: overlap extension PCR, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; pNPP: p-nitrophenyl phosphate; AP: alkaline phosphatase; Pho: regulon; D: diphosphate; IPTG: isopropyl β-D-1-thiogalactopyranoside; BCIP: 5-bromo-4-chloro-3-indolyl phosphate disodium salt.

Authors’ contributions

EAM carried out the molecular genetic studies, constructed and analyzed the strain with improved His production ability, coordinated the work, and drafted the manuscript. IAB designed and performed the enzymatic study of HisG.
and participated in drafting the manuscript. AAK designed and performed the expression analysis of P2-independent phoA gene activation. ABR participated in the strains and plasmids construction. SWM coordinated the work and helped to draft the manuscript. NVS designed and supervised the study and coordinated the writing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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