Histidine biosynthesis, its regulation and biotechnological application in Corynebacterium glutamicum

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Summary
L-Histidine biosynthesis is an ancient metabolic pathway present in bacteria, archaea, lower eukaryotes, and plants. For decades L-histidine biosynthesis has been studied mainly in Escherichia coli and Salmonella typhimurium, revealing fundamental regulatory processes in bacteria. Furthermore, in the last 15 years this pathway has been also investigated intensively in the industrial amino acid-producing bacterium Corynebacterium glutamicum, revealing similarities to E. coli and S. typhimurium, as well as differences. This review summarizes the current knowledge of L-histidine biosynthesis in C. glutamicum. The genes involved and corresponding enzymes are described, in particular focusing on the imidazoleglycerol-phosphate synthase (HisFH) and the histidinol-phosphate phosphatase (HisN). The transcriptional organization of his genes in C. glutamicum is also reported, including the four histidine operons and their promoters. Knowledge of transcriptional regulation during stringent response and by histidine itself is summarized and a translational regulation mechanism is discussed, as well as clues about a histidine transport system. Finally, we discuss the potential of using this knowledge to create or improve C. glutamicum strains for the industrial L-histidine production.

Introduction
Corynebacterium glutamicum is a well-established microorganism for biotechnological applications. Although it has been engineered for the production of various fine chemicals like succinate (Litsanov et al., 2012) or isobutanol (Blombach et al., 2011), it is still mainly employed for the production of L-amino acids (Becker and Wittmann, 2012). The most important amino acids are L-glutamate (flavour enhancer) and L-lysine (feed additive) based on production scales (Becker and Wittmann, 2011). Furthermore, there are also efforts to create efficient producers for other amino acids like L-leucine, L-serine, and L-methionine. These efforts are supported by a detailed insight into the corresponding amino acid biosynthetic pathways and their regulation in C. glutamicum and have been summarized in several reviews or book chapters (Eggeling and Bott, 2005; Wendisch, 2007; Blombach and Seibold, 2010; Brinkrolf et al., 2010). However, to date there is no review available about L-histidine biosynthesis and its regulation in this amino acid-producing microorganism. Here, we intend to summarize the current knowledge on histidine biosynthesis, its regulation and attempts for application in C. glutamicum. The published data are discussed critically and compared with the knowledge of histidine biosynthesis in Escherichia coli and Salmonella enterica serovar Typhimurium (S. typhimurium), the reference organisms regarding this particular pathway.

Properties of L-histidine
L-Histidine is one of the 20 standard proteinogenic amino acids present in proteins of all living organisms. In the following, we will use the term histidine instead, meaning its biologically active isomer L-histidine. Its side-chain is an imidazole ring and therefore has aromatic properties. Histidine is the only amino acid whose side-chain can switch from an unprotonated to a protonated state under neutral pH conditions due to the pKa value of 6.0 of its side-chain (Nelson and Cox, 2009). This characteristic enables histidine residues to act as both, a proton acceptor or a proton donor, in many cellular enzymatic reactions (Rebek, 1990; Polgár, 2005).
The histidine biosynthesis pathway

Since the late 1950s, the histidine biosynthesis pathway has been studied intensively in different organisms like yeasts, *S. typhimurium*, and *E. coli*. Initially, Ames and Martin elucidated the complete histidine pathway by identifying all metabolic intermediates and the enzymes catalysing the corresponding reactions in *S. typhimurium* (Brenner and Ames, 1971; Martin et al., 1971). At that time, last uncertainties remained regarding the reaction steps and intermediates at the interconnection to the pathway of *de novo* purine biosynthesis. These issues were finally elucidated by Klem and Davisson revealing the final number of catalytic reactions and intermediates (Klem and Davisson, 1993). Based on this knowledge, histidine biosynthesis is an unbranched pathway with ten enzymatic reactions, starting with phosphoribosyl pyrophosphate (PRPP) and leading to L-histidine (Fig. 1) (Alifano et al., 1996; Stepansky and Leustek, 2006). It turned out early that the histidine pathways of *S. typhimurium* and *E. coli* are identical. Moreover, histidine biosynthesis seems to be conserved in all organisms including archaea (Lee et al., 2008), Gram-positive bacteria (Chapman and Nester, 1969), lower eukaryotes (Fink, 1964), and plants (Stepansky and Leustek, 2006). The general histidine pathway and its regulation has already been reviewed in great detail, mainly focusing on *E. coli*, *S. typhimurium*, and plants (Brenner and Ames, 1971; Martin et al., 1971; Alifano et al., 1996; Winkler, 1996; Stepansky and Leustek, 2006). This work focuses on the histidine biosynthesis, the involved enzymes and its regulation in *C. glutamicum*, since there are some interesting differences in comparison to other organisms.

*C. glutamicum* as an amino acid producer

*Corynebacterium glutamicum* is a Gram-positive, aerobic, rod shaped, and non-sporulating soil bacterium. It is a member of the genus *Corynebacterium*, family Corynebacteriaceae, order Corynebacteriales (also containing *Mycobacterium* spp.), class Actinobacteria (also containing *Streptomyces* spp. and other filamentous bacteria) (Gao and Gupta, 2012; Goodfellow et al., 2012). It was originally isolated in Japan in the late 1950s during a screening for glutamic acid-secreting bacteria (Kinosita et al., 1958). Already the unmodified type strain secretes up to 26 g l⁻¹ L-glutamate in minimal medium under biotin-limited conditions and strains improved by classical strain development accumulate more than 100 g l⁻¹ of this amino acid in the culture medium (Becker and Wittmann, 2012). Classical strain development played an important role in the beginnings of fermentative amino acid production. Since this technique has reached its limit to further increase productivity, nowadays metabolic engineering is used to further optimize L-glutamate production. At present these engineered strains do not reach the production titres of classical glutamate production strains (Sawada et al., 2010). However, there are promising results from metabolic engineering approaches with regard to the production of L-lysine. The implementation of 12 defined genome-based modifications enabled accumulation of 120 g l⁻¹ L-lysine in the culture supernatant (Becker et al., 2011). These production titres are even higher than those reached with strains created by classical strain development with consecutive rounds of mutagenesis and selection (Becker and Wittmann, 2012). The intensive investigations on L-glutamate and L-lysine biosynthesis pathways and the understanding of their regulation and interconnection to the central metabolism of *C. glutamicum* helped to further improve production strains. Today, about 2.5 million tons of L-glutamate and 1.5 million tons of L-lysine are produced annually by Corynebacteria with estimated growth rates of 6–8% per year (Becker and Wittmann, 2011). There are also several strains available for the production of other amino acids which were created either by classical strain development, by metabolic engineering, or by a combination of both techniques. This includes strains for the production of L-isoleucine, L-tryptophan, L-phenylalanine, L-valine, L-alanine, and L-serine (Becker and Wittmann, 2012).

*Corynebacterium glutamicum* strains suitable for the industrial production of L-histidine have been established by means of combining classical strain development and metabolic engineering. *Corynebacterium glutamicum* mutants resistant to histidine analogues were reported to secrete 6–8 g l⁻¹ L-histidine into the culture medium (Araki and Nakayama, 1971). The overexpression of a mutated ATP (adenosine triphosphate) phosphoribosyltransferase which is not inhibited by histidine analogues resulted in a *C. glutamicum* strain accumulating up to 23 g l⁻¹ histidine (Mizukami et al., 1994). These or similar strains are still used for industrial L-histidine fermentation today (Ikeda, 2003; Becker and Wittmann, 2012).

Enzymes involved in histidine biosynthesis

Histidine biosynthesis genes in *C. glutamicum*

*Corynebacterium glutamicum* strain AS019, a derivative of *C. glutamicum* ATCC 13059, was used for the first genetic studies on histidine biosynthesis. The genes *hisA*, encoding the 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide (5′ProFAR) isomerase, and *hisF*, encoding one subunit of the imidazole glycerol phosphate synthase, were identified by complementation of corresponding histidine auxotrophic *E. coli* mutants (Jung et al., 1998). The gene *hisH*, coding for the second subunit of imidazole glycerol phosphate synthase (Kim and Lee, 2001), and the genes *hisG* and *hisE*, coding for the ATP
Fig. 1. Histidine biosynthetic pathway in C. glutamicum. PRPP, phosphoribosyl pyrophosphate; ATP; adenosine triphosphate; PPi, pyrophosphate; PR-ATP, phosphoribosyl-ATP; PR-AMP, phosphoribosyl-AMP; 5′ProFAR, 1-(5-phosphoribosyl)-5-[[5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide; PRFAR, 5-[[5-phospho-1-deoxyribulos-1-ylamino)methylideneamino]1-(5-phosphoribosyl)imidazole-4-carboxamide; IGP, imidazole-glycerol phosphate; AIACAR, 1-(5-phosphoribosyl)-5-amino-4-imidazolecarboxamide; IAP, imidazole-acetol phosphate; Hol-P, L-histidinol phosphate; P, phosphate; NAD+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; HisG, ATP phosphoribosyltransferase; HisE, phosphoribosyl-ATP pyrophosphotase; HisI, phosphoribosyl-AMP cyclohydrolase; HisA, 5′ProFAR isomerase; HisF, synthase subunit of IGP synthase; HisH, glutaminase subunit of IGP synthase; HisB, imidazoleglycerol-phosphate dehydratase; HisC, histidinol-phosphate aminotransferase; HisN, histidinol-phosphate phosphatase; HisD, histidinol dehydrogenase.
phosphoribosyltransferase and phosphoribosyl-ATP pyrophosphatase, respectively, were identified by applying the same method (Kwon et al., 2000). The release of the complete genome sequence of the type strain C. glutamicum ATCC 13032 in 2003 (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) provided the opportunity for the reconstruction of various metabolic pathways, including histidine biosynthesis. The annotation of the genome led to the identification of genes coding for nine of the 10 enzymatic activities needed for histidine biosynthesis. In addition to the genes hisAEFGH, already known from C. glutamicum AS019, these were the genes hisl, encoding phosphoribosyl-AMP cyclohydrolase, hisB, coding for imidazoleglycerol-phosphate dehydratase, hisC, coding for histidinol-phosphate aminotransferase, and hisD, encoding histidinol dehydrogenase, which catalyse the final two steps of histidine biosynthesis in C. glutamicum. However, a gene encoding an enzyme with histidinol-phosphate phosphatase activity has neither been identified by automatic annotation of the genome sequence, nor by heterologous complementation of E. coli mutants. In 2006 a random mutagenesis approach using an IS6100-based transposon vector finally identified the gene encoding histidinol-phosphate phosphatase (Mormann et al., 2006). The gene was designated hisN, since the enzymatic activity is located on the N-terminal part of a bifunctional hisB gene product in S. typhimurium and E. coli (Houston, 1973a; Carlomagno et al., 1988). Additionally, the random transposon mutagenesis approach confirmed the involvement of the genes hisABDEFGI in histidine biosynthesis. Transposon insertion into either one of these genes resulted in histidine auxotrophy of the corresponding mutants (Mormann et al., 2006). Furthermore, participation of the genes hisBCD in histidine biosynthesis was again confirmed in complementation experiments with auxotrophic E. coli mutants (Jung et al., 2009). To sum up, C. glutamicum possesses ten histidine biosynthesis genes coding for nine enzymes which catalyse ten enzymatic reactions. This includes one bifunctional enzyme, the histidinol dehydrogenase (hisD), and one enzyme consisting of two subunits, the imidazoleglycerol-phosphate synthase (hisF and hisH). As a part of our own studies, each histidine gene has been deleted individually in C. glutamicum (Table 1). As for the transposon mutants, each single in frame deletion of one of the eight genes hisABCDEFGI resulted in histidine auxotrophy (R.K. Kulis-Horn, unpubl. obs.), confirming the essentiality of these genes. Interestingly, clear auxotrophies were not found for the deletions of hisH and hisN (discussed below).

**ATP phosphoribosyltransferase (HisG)**

ATP phosphoribosyltransferase (ATP-PRT) catalyses the first step of histidine biosynthesis, the condensation of ATP and PRPP to phosphoribosyl-ATP (PR-ATP) and pyrophosphate (PP) (Allfano et al., 1996). ATP phosphoribosyltransferases can be divided into two subfamilies, the long and the short ATP-PRTs. Enzymes of the long subfamily are 280–310 amino acids in length and are present in lower eukaryotes and bacteria, like E. coli, S. typhimurium, or Mycobacterium tuberculosis (Zhang et al., 2012). The short forms of ATP-PRTs are lacking about 80 amino acids at their C-terminus. They are present in some bacteria, such as Bacillus subtilis, Lactococcus lactis, and Pseudomonas aeruginosa (Bond and Franclyn, 2000). These short ATP-PRTs require the presence of the hisZ gene product for their catalytic activity (Sissler et al., 2013).

### Table 1. Histidine biosynthesis genes in C. glutamicum ATCC 13032 and the effect of gene disruption on histidine-dependent growth in minimal medium.

| Gene | Function | Effect of gene disruption | References |
|------|----------|--------------------------|------------|
| hisG | ATP phosphoribosyltransferase | auxotrophy | (1), (2), (3) |
| hisE | phosphoribosyl-ATP pyrophosphatase | auxotrophy | (1), (3) |
| hisl | phosphoribosyl-AMP cyclohydrolase | auxotrophy | (1), (3) |
| hisA | 5’ProFAR isomerase | auxotrophy | (1), (3) |
| hisF | synthase subunit of IGP synthase | auxotrophy | (1), (3) |
| hisH | glutaminase subunit of IGP synthase | no effect | (3) |
| hisB | imidazoleglycerol-phosphate dehydratase | auxotrophy | (1), (3) |
| hisC | histidinol-phosphate aminotransferase | auxotrophy | (3) |
| hisN | histidinol-phosphate phosphatase | auxotrophy | (1), (3)* |
| hisD | histidinol dehydrogenase | auxotrophy | (1), (3) |
| cg0911 | putative inositol monophosphatase | no effect | (3) |
| impA | putative inositol monophosphatase | no effect | (3) |
| cg2301 | putative antibiotic efflux permease, MFS-type | no effect | (3) |
| cg1305 | L-phenylalanine transporter | no effect | (3), (4) |

* a. Growth strongly reduced but still measurable (this study).

* b. No growth of a Δcg1305ΔhisG double mutant in histidine supplemented medium (this study).

* (1) Mormann et al. (2006): transposon insertion.

* (2) Zhang et al. (2012): substitution of hisG 3’ end with a chloramphenicol resistance gene.

* (3) This study: in frame gene deletion.

* (4) Zhao et al. (2011): in frame gene deletion.
The HisZ protein has no sequence homology to the C-terminus of long ATP-PRTs, but is a paralogue of histidyl-tRNA synthetase (Sissler et al., 1999). With a length of 281 amino acids, ATP-PRT from *C. glutamicum* (HisG<sub>Cg</sub>) belongs to the long form of ATP-PRTs. Therefore, it is not surprising that the *C. glutamicum* genome lacks a paralogue of the hisZ gene. Kinetic parameters of HisG<sub>Cg</sub> have been determined recently. The enzyme has a specific activity of 2.19 ± 0.09 μmol min<sup>−1</sup> mg<sup>−1</sup>, a K<sub>m</sub> value for PRPP of 0.08 ± 0.01 mM, a K<sub>m</sub> value for ATP of 0.22 ± 0.02, and a k<sub>cat</sub> value of 1.91 ± 0.14 s<sup>−1</sup> (Zhang et al., 2012). Comparison of crystal structures and structure-based multiple alignments of ATP-PRTs from bacteria, archaea, and baker’s yeast revealed a common 3D structure of ATP-PRTs (Zhang et al., 2012). ATP-PRTs have no structural and sequence similarities to other phosphoribosyltransferases, besides the PRPP binding site. Therefore, ATP-PRT is considered a member of the new type IV class of phosphoribosyltransferases (Lohkamp et al., 2004; Zhang et al., 2012). The crystal structure of HisG<sub>Cg</sub> is not available yet. However, a homology model based on the 3D structure of ATP-PRT from *M. tuberculosis* (HisG<sub>mt</sub>) (62% sequence identity and 89% sequence similarity) revealed an almost identical structure to HisG<sub>Cg</sub> (Zhang et al., 2012). Knowledge about the 3D structure of HisG<sub>mt</sub> is therefore most likely also true for HisG<sub>Cg</sub>. According to the predicted structure model, HisG<sub>Cg</sub> is a L-shaped monomer composed of three distinct domains (Zhang et al., 2012). The first two domains form the catalytic core. The active site is located in a cleft between these two domains. The third domain is able to bind histidine and is therefore regarded as the regulatory domain (Cho et al., 2003; Zhang et al., 2012). The native HisG-enzyme from *E. coli* and *S. typhimurium* is in equilibrium between a dimeric and hexameric form (Winkler, 1996). Gel filtration experiments with purified HisG<sub>Cg</sub> confirmed this quaternary structure in *C. glutamicum* (Zhang et al., 2012). ATP-PRT is subject to feedback inhibition and its activity is also influenced by additional factors such as enzyme concentration or the energy status of the cell (Araki and Nakayama, 1974; Zhang et al., 2012). Since, the regulation of ATP-PRT is of great importance it will be discussed in more detail below.

**Phosphoribosyl-ATP pyrophosphatase (HisE) and phosphoribosyl-AMP cyclohydrolase (HisI)**

Phosphoribosyl-ATP pyrophosphatase catalyses the irreversible hydrolysis of PR-ATP to phosphoribosyl-AMP (PR-AMP) in the second step of histidine biosynthesis. Subsequently, in the third step PR-AMP cyclohydrolase opens the purine ring of PR-ATP releasing 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylene-imidazole-4-carboxamide (5′ProFAR) (Alifano et al., 1996). Both enzymatic activities are carried out by a single polypeptide chain in *E. coli* and *S. typhimurium* (Carlamagno et al., 1988). In *C. glutamicum*, the two activities are encoded by separate genes (Kalinowski et al., 2003). Bifunctional His(IE) enzymes exist in all eukaryotes and in several unrelated taxonomic bacterial lineages, but are absent in all Actinobacteria (Fani et al., 2007). Most likely, bifunctional His(IE) proteins in bacteria are the result of several independent fusion events and horizontal gene transfer (Fani et al., 2007). The native bifunctional His(IE) enzymes from *E. coli* and *S. typhimurium* act as dimers (Winkler, 1996). The crystal structure of phosphoribosyl-ATP pyrophosphatase from *M. tuberculosis* (HisE<sub>wo</sub>) was solved and revealed that it also forms a dimer (Javid-Majd et al., 2008). The amino acid sequences of HisE<sub>wo</sub> and HisE<sub>co</sub> share 62% identity and 90% similarity, assuming a very similar structure for both proteins. Based on this deduced 3D structure, native HisE<sub>co</sub> most likely acts as a dimer, too.

**5′ProFAR isomerase (HisA)**

The fourth step of histidine biosynthesis is performed by 5′ProFAR isomerase. This enzyme catalyses an internal redox reaction converting 5′ProFAR to 5-((5-phospho-1-deoxyribulos-1-ylamino)methylideneamino)-1-(5-phosphoribosyl)imidazole-4-carboxamide (PRFAR) (Alifano et al., 1996). The native enzymes from *E. coli* and *S. typhimurium* act as monomers (Winkler, 1996). The crystal structure of 5′ProFAR isomerase from *M. tuberculosis* (PriA<sub>wo</sub>) encoded by the priA gene was solved recently (Due et al., 2011). Interestingly, PriA<sub>wo</sub> is also involved in tryptophan biosynthesis due to its phosphoribosylanthranilate isomerase activity. So far it cannot be excluded that 5′ProFAR isomerase from *C. glutamicum* (HisA<sub>co</sub>) is also involved in tryptophan biosynthesis. However, deletion of hisA resulted in histidine auxotrophy only (R.K. Kulis-Horn, unpublished observation), indicating that *C. glutamicum* must at least possess one additional gene coding for a phosphoribosylanthranilate isomerase. This enzyme activity is most likely exerted by the *trp CF* gene product, already annotated as a bifunctional phosphoribosylanthranilate isomerase/indoleglycerolphosphate synthase in *C. glutamicum* (Kalinowski et al., 2003). Nevertheless, the 3D structure of the bifunctional PriA<sub>wo</sub> enzyme, exhibiting 61% identity and 89% similarity on amino acid level, allows a deeper insight into the structure of 5′ProFAR isomerase from *C. glutamicum* (HisA<sub>co</sub>). Based on these data, native HisA<sub>co</sub> most likely acts as a monomer with an αβ<sub>6</sub> barrel fold. [Corrections added on 09 October 2013, after first online publication: In the paragraph above, occurrences of the gene name “pirA” are now amended to “priA.”]
Imidazoleglycerol-phosphate synthase (HisFH)

The fifth step of histidine biosynthesis is the conversion of PRFAR to the next histidine intermediate imidazole-glycerol phosphate (IGP) and the byproduct 1-(5′-phosphoribosyl)-5-amino-4-imidazolecarboxamide (AICAR), an intermediate of de novo purine biosynthesis (Alifano et al., 1996). Glutamine is used as nitrogen donor in this amination step releasing glutamate (Smith and Ames, 1964). Mutations in either hisH or hisF result in histidine auxotrophy of S. typhimurium (Jartman et al., 1960). These genes were later linked to the fifth step of histidine biosynthesis, although both were initially assumed to code for independent enzymes catalysing different steps in the conversion of PRFAR to IGP and AICAR (Smith and Ames, 1964). The exact role of hisF and hisH gene products remained elusive for many years. It was finally demonstrated for hisF and hisH of E. coli that the two gene products act as a stable 1:1 dimeric complex which constitutes the IGP synthase holoenzyme (Klem and Davisson, 1993).

Corynebacterium glutamicum also possesses hisF and hisH genes. They exhibit 44% and 38% identity on amino acid level compared with enzymes from E. coli respectively. A genomic DNA fragment containing both genes from C. glutamicum AS019 was able to complement histidine auxotrophic hisF and hisH E. coli mutants, demonstrating that these two gene products have the same catalytic activities in both organisms (Jung et al., 1998; Kim and Lee, 2001). In accordance with these results, the deletion of hisF resulted in histidine auxotrophy in C. glutamicum. The deletion of hisH, however, did not have any effect on the growth behaviour of the mutant grown in minimal medium (R.K. Kulis-Horn, unpubl. result). This finding is also accordant with the results from the transposon mutagenesis approach where a transposon insertion in hisH was not observed in any of the histidine auxotrophic mutants (Mormann et al., 2006). There are different possible explanations for this surprising growth behaviour of the ΔhisH mutant on minimal medium. (1) The hisH gene in C. glutamicum might be wrongly annotated and another gene has the true hisH gene function. (2) There is a hisH paralogue which complements the gene function. (3) Unlike in E. coli and S. typhimurium, hisH is not essential for histidine biosynthesis in C. glutamicum.

Concerning hypotheses (1) and (2): There are no further genes within the genome of C. glutamicum encoding proteins with considerable sequence similarities to HisH (glutaminase subunit of IGP synthase). The two best BLAST hits are with pabAB (cg1134) and trpG (cg3360). The pabAB gene encodes a paraaminobenzoate synthase, an enzyme involved in folic acid biosynthesis (Stolz et al., 2007), and trpG, encoding the second subunit of anthranilate synthase, is involved in tryptophan biosynthesis (Heery and Duncan, 1993). It is known from studies with other organisms that these enzymes exhibit glutamine amidotransferase activity, which is also the reaction performed by HisH (Crawford and Eberly, 1986; Viswanathan et al., 1995). In theory, these two enzymes could take over the enzymatic activity of HisH. But this scenario seems rather unlikely, since it was demonstrated for IGP-synthase from E. coli that two perfectly matching HisF (synthase subunit of IGP synthase) and HisH monomers are needed for glutaminase activity of HisH and channelling of ammonia to the catalytic centre of HisF (Klem et al., 2001; Amaro et al., 2005).

Concerning hypothesis (3): E. coli HisF is able to perform the fifth step of histidine biosynthesis without HisH activity in vitro in the presence of unphysiologically high ammonia concentrations and pH > 8 (Smith and Ames, 1964; Klem and Davisson, 1993). The HisH activity is only needed if glutamine is the only nitrogen donor in the in vitro reaction, since this subunit of the IGP synthase exhibits a glutamine amidotransferase activity (Klem and Davisson, 1993). However, glutamine seems to be the true nitrogen donor in vivo. Mutations in hisH result in histidine auxotrophy of S. typhimurium and E. coli despite the presence of ammonia in the minimal medium (Hartman et al., 1960). On the contrary, a C. glutamicum ΔhisH mutant still grows in ammonia containing minimal medium (R.K. Kulis-Horn, unpubl. obs.). The IGP synthase from C. glutamicum seems to have different properties than the enzymes from S. typhimurium, E. coli, and other species reported. The most probable explanation for this phenomenon is an ammonia-dependent substrate amination activity of HisF_{Cg} in vivo (Fig. 1). Our findings support this theory, since hisF_{Cg} is able to complement both, a hisF and a hisH deletion, in E. coli (R.K. Kulis-Horn and P. Humbert, unpubl. obs.). The other possibility, a glutamine amidotransferase activity already present in the HisF protein like observed in the monomeric IGP synthase HIS7 from Saccharomyces cerevisiae (Kuenzler et al., 1993), seems unlikely. HisF_{Cg} is only of the size of HisF_{E}, and does not exhibit any sequence similarities to known amidotransferases. The overexpression of hisH_{Cg} is able to complement a hisH deletion in E. coli, demonstrating that the hisH_{Cg} gene product is functional though not needed in C. glutamicum (Jung et al., 1998). So far, no other IGP synthase has been reported being able to catalyse the fifth step of histidine biosynthesis without glutamine amidotransferase activity in vivo. These findings are very interesting especially in the view of the biotechnological application of C. glutamicum as histidine producer, since histidine production in this organism seems to be independent of glutamine biosynthesis.

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Histidine in C. glutamicum

Imidazolglycerol-phosphate dehydratase (HisB)

The imidazolglycerol-phosphate dehydratase catalyses the sixth step of histidine biosynthesis. The enzyme dehydrates IGP and the resulting enol is then ketonized non-enzymatically to imidazole-acetol phosphate (IAP) (Alifano et al., 1996). In S. typhimurium and E. coli this step is catalysed by a bifunctional enzyme comprising both, the imidazolglycerol-phosphate dehydratase activity and the histidinol-phosphate phosphatase activity, catalysing the eighth step of biosynthesis (Loper, 1961; Houston, 1973a). In these two organisms the bifunctional enzyme is encoded by the his(NB) gene, comprising phosphatase activity at the N-terminus of the encoded protein and dehydratase activity at the C-terminus (Houston, 1973b; Rangarajan et al., 2006). There is evidence that this bifunctional his(NB) gene results from a rather recent gene fusion event in the γ-proteobacterial lineage (Brilli and Fani, 2004). In eukaryotes, archaea and most bacteria the two activities are encoded by separate genes (Fink, 1964; Le Coq et al., 1999; Lee et al., 2008). This is also true for C. glutamicum, with IGP dehydratase being encoded by hisB and histidinol-phosphate phosphatase by hisN (Mormann et al., 2006; Jung et al., 2009).

Histidinol-phosphate aminotransferase (HisC)

The seventh step of histidine biosynthesis is the transamination of IAP to L-histidinol phosphate (Hol-P) using glutamate as amino group donor (Alifano et al., 1996). This step is catalysed by the pyridoxal 5′-phosphate (PLP) dependent histidinol-phosphate aminotransferase in C. glutamicum (Marienhagen et al., 2008). Like HisC from E. coli and S. typhimurium (Winkler, 1996), native HisC<sub>G</sub> acts as a dimer (Marienhagen et al., 2008). Kinetic parameters of HisC<sub>G</sub> were determined only for the back-reaction converting Hol-P and α-ketoglutarate into IAP and L-glutamate. The enzyme exhibits a K<sub>m</sub> value for Hol-P of 0.89 ± 0.1 mM, a k<sub>cat</sub> value of 1.18 ± 0.1 s<sup>−1</sup> and a specific activity of 2.8 μmol min<sup>−1</sup> mg<sup>−1</sup> (Marienhagen et al., 2008). Interestingly, HisC<sub>G</sub> shows also activity with the precursors of leucine and aromatic amino acids in <i>in vitro</i> assays, but the K<sub>m</sub> values are two orders of magnitude higher compared with those observed with the histidine precursor and HisC<sub>G</sub> does not contribute to aromatic amino acid synthesis <i>in vivo</i> (Marienhagen et al., 2005; 2008). The crystal structure of HisC<sub>G</sub> has been solved revealing a three-domain structure of the monomer, with a N-terminal arm, a large PLP binding domain, and a small C-terminal domain (Marienhagen et al., 2008). HisC<sub>G</sub> dimerization occurs via extensive hydrophobic interactions and 24 intersubunit hydrogen bonds with the N-terminal arm contributing significantly to the intersubunit interface (Marienhagen et al., 2008). The active sites are made up almost exclusively of residues within one subunit, but the tight packing of the dimer shields the active sites from the solvent (Marienhagen et al., 2008). Site-directed mutagenesis experiments highlighted the importance of the conserved residue Tyr21 for Hol-P substrate specificity and Asn99 for the orientation of the cofactor PLP inside the active centre (Marienhagen et al., 2008). Recently, the structure of histidinol-phosphate aminotransferase from <i>M. tuberculosis</i> (HisC<sub>Mt</sub>) has also been published (Nasir et al., 2012). Interestingly, in <i>M. tuberculosis</i> two genes (hisC1 and hisC2) are annotated encoding Hol-P aminotransferases (Camus et al., 2002). The first gene is clustered together with other histidine biosynthesis genes in the same order as in <i>C. glutamicum</i>. The second gene, however, is monocistronic and located in the genome distant from other his genes. The deduced amino acid sequence of hisC2 from <i>M. tuberculosis</i> is most similar to the aromatic amino acid aminotransferase encoded by <i>aroT</i> (cg0267) in <i>C. glutamicum</i>. HisC<sub>G</sub> and AroT<sub>G</sub> both exhibit high sequence similarity to Hol-P aminotransferases (McHardy et al., 2003). Whereas HisC<sub>G</sub> is most similar to aminotransferases being exclusively involved in histidine biosynthesis, AroT<sub>G</sub> is more similar to aminotransferases with a broader substrate spectrum being involved in histidine but also aromatic amino acid biosynthesis (McHardy et al., 2003). Enzyme assays with purified AroT<sub>G</sub> demonstrated its involvement in synthesis of the aromatic amino acids tyrosine and phenylalanine (Marienhagen et al., 2005). Its function in histidine biosynthesis was not assayed. However, since the presence of the <i>aroT</i> gene is not able to prevent histidine auxotrophy in a hisC deletion mutant of <i>C. glutamicum</i> (R.K. Kulis-Horn, unpubl. obs.) it is very likely that <i>aroT</i> does not encode a Hol-P aminotransferase. The structure of the protein crystallized by Nasir and colleagues (2012) therefore does not give deeper insight into the 3D structure of Hol-P aminotransferase from <i>C. glutamicum</i>, but rather into the structure of AroT<sub>G</sub>.

Histidinol-phosphate phosphatase (HisN)

During the eighth step of histidine biosynthesis Hol-P is dephosphorylated to L-histidinol. In E. coli and S. typhimurium this reaction is catalysed by a bifunctional enzyme comprising both, the Hol-P phosphatase activity and the IGP dehydratase activity catalysing the sixth step of the biosynthesis (see above). In <i>C. glutamicum</i> both activities are encoded by two genes, hisB encoding IGP phosphatase (Jung et al., 2009) and hisN encoding Hol-P phosphatase (Mormann et al., 2006). IGP phosphatases seem to be derived from a common ancestor in all organisms. But there is a difference in the origin of the Hol-P phosphatases being part of a bifunctional enzyme and those being encoded by a separate gene (Brilli and Fani,
genes exhibiting high sequence homology to trophic in and identification of all to this, Jung and colleagues (2009) reported the cloning of inositol monophosphatases (Mormann (cg0967 it is hard to decide on the basis of the sequence alone if Frankiales, Micrococcales and Streptomycetales (data families Corynebacteriaceae and Mycobacteriaceae, Actinomycetales, Corynebacteriales, with the important Almost all taxonomical orders of the class Actinobacteria already present in the genome of C. glutamicum (1977). We observed a strongly impaired growth of a strain (Struhl and Davis, 1988). This concentration of all histidine biosynthesis genes at one locus seems not to be the rule but rather an exception and restricted to the enterobacteria, since in other bacteria his genes are more scattered throughout the genome (Alifano et al., 1996).

Transcriptional organization of histidine biosynthesis genes

The histidine gene cluster of S. typhimurium and E. coli was one of the model gene clusters leading to the development and approval of the operon theory (Alifano et al., 1996). In these two organisms all eight histidine biosynthesis genes are part of one operon and therefore transcribed and regulated as a single unit (Martin, 1963b; Fink and Martin, 1967; Carlonamagni et al., 1988). This concentration of all histidine biosynthesis genes at one locus seems not to be the rule but rather an exception and restricted to the enterobacteria, since in other bacteria his genes are more scattered throughout the genome (Alifano et al., 1996).

Histidinol dehydrogenase (HisD)

The last two steps of histidine biosynthesis are catalysed by a single enzyme. L-Histidinol is first oxidized by histidinol dehydrogenase to L-histidinal, which is further oxidized to L-histidine (Alifano et al., 1996). Both steps are catalysed by the same enzyme to prevent the decomposition of the unstable L-histidinal intermediate (Görisch and Hölke, 1985) and two molecules NAD\(^+\) (oxidized nicotinamide adenine dinucleotide) are reduced during the reaction (Adams, 1954). The native HisD enzyme from S. typhimurium (HisD\(_\text{S}\)) acts as a homodimer and both subunits are linked by disulfide bridges (Eccleston et al., 1979). HisD\(_\text{S}\) is Zn\(^{2+}\) dependent (Grubmeyer et al., 1989). Native histidinol dehydrogenase from M. tuberculosis (62% identity, 83% similarity to HisD from C. glutamicum) also acts as a homodimer and is metal dependent (Nunes et al., 2011). However, it remains uncertain if Zn\(^{2+}\) or rather Mn\(^{2+}\) is the preferred metal ion. Nunes et al. also performed molecular homology modelling of HisD\(_\text{S}\) employing the crystal structure of histidinol dehydrogenase from E. coli (Barbosa et al., 2002) as template. Enzymes from both organisms have a very similar structure. Each homodimer comprises two identical active sites located at the interface of both subunits. Residues from both subunits form the binding sites for L-histidinol and the metal ion, whereas NAD\(^+\) binds only to residues from one subunit (Barbosa et al., 2002; Nunes et al., 2011). A Bi-Uni Uni-Bi ping-pong reaction mechanism was proposed for HisD\(_\text{S}\). L-Histidinol binds first, followed by NAD\(^{+}\). NADH\(^+\) is released while L-histidinal stays enzyme-bound. Then the second NAD\(^{+}\) binds and is reduced, again releasing NADH\(^+\)H\(^+\) and finally L-histidine (Nunes et al., 2011). This reaction mechanism most probably also reflects the HisD\(_\text{Cg}\) reaction mechanism.

Transcriptional organization of the histidine biosynthesis genes

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Histidinol dehydrogenase (HisD)

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The genes orf1 and orf2 correspond to genes cg2302 and cg2301 in *C. glutamicum* ATCC 13032 respectively. The release of the complete genome sequence of *C. glutamicum* (Kalinowski et al., 2003) revealed that the hisN, hisGE, and hisDCB-cg2302-cg2301-hisHA-impA-hisFI loci are each separated by several hundred kilobase pairs forming independent transcriptional units (Fig. 2). A closer look is needed to verify the operon structure of the hisDCB-cg2302-cg2301-hisHA-impA-hisFI locus. The conclusion that the genes hisDCB-orf1-orf2-hisHA-impA-hisFI form one transcriptional unit in *C. glutamicum* AS019 is based on results from RT-PCR analysis (Jung et al., 2009). In *C. glutamicum* ATCC 13032 the genes cg2301 and hisH are separated by a 1044 bp non-coding region. In contrast to the results from strain AS019, this large intergenic region implies two independent transcriptional units for hisDCB-cg2302-cg2301-hisHA-impA-hisFI. Strand specific cDNA sequencing (RNA-Seq) was performed for the whole transcriptome of *C. glutamicum* ATCC 13032 in our group (K. Pfeifer-Sancar, A. Mentz, C. Rückert, and J. Kalinowski, manuscript in preparation). This study enabled for the first time the analysis of transcripts with a single nucleotide resolution for whole pathways in one experiment. The RNA-Seq data revealed transcription of all 10 histidine biosynthesis genes, but it did not give any evidence for transcripts spanning the non-coding region between cg2301 and hisH in *C. glutamicum* ATCC 13032 under all tested conditions (complex and minimal media, various stresses; R.K. Kulis-Horn, unpubl. data). Furthermore, the data showed transcription start sites in front of the hisD and the hisH gene. It appears that hisDCB-cg2302-cg2301 and hisHA-impA-hisFI represent two unlinked transcriptional units in *C. glutamicum* ATCC 13032 (Fig. 2). The genes cg2294, cg2301 and cg2302 are of unknown function. The deduced protein sequence of cg2301 shows characteristics of a permease of the major facilitator superfamily (BLASTP).

Furthermore, the identification of transcription start sites by RNA-Seq revealed that the operons cg0911-hisN, hisEG, and hisHA-impA-hisFI-cg2294 are transcribed as leaderless mRNAs, meaning that the start of transcription is identical with the translational start site of the first gene (R.K. Kulis-Horn, unpubl. data). Leaderless transcripts are rarely found in Firmicutes and Proteobacteria, but are common in Actinobacteria where they represent on average 20% of all transcripts (Zheng et al., 2011). The hisDCB-cg2302-cg2301 operon on the other hand comprises a 5′ untranslated region (5′ UTR) with a classical Shine–Dalgarno (SD) sequence. The length of this 5′ UTR was determined by means of primer extension experiments to be 196 nucleotides in *C. glutamicum* AS019 (Jung et al., 2009). The RNA-Seq data for *C. glutamicum*
ATCC 13032 revealed a shorter 5′ UTR comprising only 93 nucleotides (R.K. Kulis-Horn, unpubl. data). Although the DNA sequence of both C. glutamicum strains is identical in this particular region, there is no evidence for a transcription start site in C. glutamicum ATCC 13032 corresponding to the position mapped in C. glutamicum AS019 (data not shown).

**Promoters**

The putative promoter in front of hisD in C. glutamicum AS019 identified by primer extension experiments (Jung et al., 2009), so far was the only known his promoter determined in C. glutamicum. The RNA-Seq technique modified for the detection of transcription start sites in C. glutamicum ATCC 13032 enabled the search for further his promoter sequences (K. Pfeifer-Sancar, A. Mentz, C. Rücker, and J. Kalinowski, manuscript in preparation). Four primary promoters were identified in front of the four his operons (\(P_{\text{cg0911}}, P_{\text{hisE}}, P_{\text{hisH}}, P_{\text{hisD}}\)). Additionally four internal promoters were observed (\(P_{\text{hisN}}, P_{\text{hisA}}, P_{\text{hisF}}, P_{\text{hisB}}\)).

A −10 box hexamer fitting well to the consensus sequence TAN\(aTN\) of sigma factor A (\(\sigma^A\)) dependent promoters in C. glutamicum (Pátek and Nešvera, 2011) was determined for all four primary promoters (Fig. 3). The spacing between the transcription start site and the −10 box is 7 ± 1 nucleotides. Only the −10 box of the hisE promoter exhibits the TGn extension which seems to enhance promoter strength (Vasicová et al., 1999). The −35 boxes located 17 ± 1 nucleotides upstream of the −10 box exhibit only low similarities to the −35 consensus sequence. However, −35 boxes are generally poorly conserved in C. glutamicum (Pátek and Nešvera, 2011).

RNA-Seq data indicated additional internal promoters within the four histidine operons (R.K. Kulis-Horn, unpubl. data). A start of transcription was observed in front of the hisN gene within the hisN-cg0911 operon. The data revealed a transcription start for a leaderless mRNA for hisN from the internal promoter \(P_{\text{hisN}}\). Furthermore, internal promoters were found in front the hisA \((P_{\text{hisA}})\) and hisF \((P_{\text{hisF}})\) genes within the hisHA-impA-hisFl-cg2294 operon. The hisA gene was shown to be transcribed leaderless and hisF with a 5′ UTR (61 nt). A fourth internal promoter \((P_{\text{hisB}})\) was observed in front of hisB within the hisDCB-cg2302-cg2301 operon, resulting in a 5′ UTR (77 nt) in front of hisB. For all internal promoters a −10 box well-fitting the TAN\(aTN\) consensus sequence is located 7 ± 1 nucleotides upstream of the transcription start site. The −35 boxes related to these promoters hardly fitted to any consensus sequence. Up to now, no data regarding the biological relevance of these internal promoters are available. However, they could play a role in regulation of

![Fig. 3. Putative promoter sequences of histidine biosynthesis genes.](image-url)

**Consensus**

- **all**: akgact artAsaTag
- **primary**: ttcaca NRTcAaTRK
- **internal**: agggMT acTAggaTTR
- **\(\sigma^A\)**: tgtica gNTaNNTg

(Pátek and Nešvera, 2011)
histidine biosynthesis. In *S. typhimurium* and *E. coli* two internal promoters, *hisp2* and *hisp3*, were identified within the histidine operon (Grisolia *et al.*, 1983; Schmid and Roth, 1983; Carlomagno *et al.*, 1988). Promoter *hisp2* enables the additional transcription of the genes *his*(NB), *hisH*, *hisA*, *hisF* and *his*(IE), whereas *hisp3* enables transcription only of the *his*(IE) gene. Alifano and colleagues (1996) speculated that such internal promoters might reinforce the expression of distal genes in large operons to counteract the effects of natural polarity. Another function might be to temporally allow expression of some of the genes organized in an operon under specific growth conditions (Schmid and Roth, 1983). Transcription starting from *hisp2* is occluded by transcription from the primary promoter *hisp1* under histidine limited conditions, but it increases if transcription from *hisp1* does not occur at sufficient histidine supply (Alifano *et al.*, 1992). In *E. coli* and *S. typhimurium* transcription from promoter *hisp1* is known to be regulated by an attenuation mechanism in response to the availability of charged histidyl- tRNAs (Kasai, 1974; di Nocera *et al.*, 1978; Johnston *et al.*, 1980). As transcription from the internal promoters *hisp2* and *hisp3* is not affected by this attenuation mechanism, transcription of genes from these promoters may occur even in the presence of high levels of charged histidyl-tRNA. The biological function of such a transcriptional regulation, however, still remains unexplained.

Regulation of histidine gene expression

Regulation of biosynthetic pathways is of great importance for organisms to prevent wasting energy for the production of metabolites which are not needed under certain growth conditions. On the other hand, the regulation must also prevent the complete drainage of metabolites needed for survival and growth by temporarily activating the biosynthesis. Such an accurate regulation is especially needed for the biosynthesis of amino acids as they are the building blocks of proteins and therefore needed for any enzymatic activity.

The biosynthesis of histidine is associated with high energy costs for the cell. Brenner and Ames (1971) calculated a demand of 41 ATP equivalents for the synthesis of one histidine molecule in *S. typhimurium*. Unregulated histidine biosynthesis would waste about 2.5% of the bacterial cells metabolic energy (Brenner and Ames, 1971). Based on a genome-scale stoichiometric model of the *C. glutamicum* metabolism, the ATP demand for histidine biosynthesis was calculated to be 9.4 molATP molHis⁻¹ (E. Zelle *et al.*, pers. comm.). Since this ATP demand is the third highest for all proteinogenic amino acids exceeded only by arginine (12.0 molATP molArg⁻¹) and tryptophan (13.0 molATP molTrp⁻¹), the cellular demand for a strict regulation of histidine biosynthesis is obvious.

There are three general levels of regulation of a metabolic pathway: transcriptional or translational repression, and enzyme inhibition. All three possibilities will be discussed in the following chapters.

Transcriptional regulation

The transcriptional regulation is the first level in a regulatory cascade for metabolic pathways. Various studies concerning *E. coli* and *S. typhimurium* revealed changing mRNA levels of histidine genes with varying culture conditions (Winkler, 1996). This indicates regulation on transcriptional level, which has been also reported for *C. glutamicum* (Brockmann-Gretza and Kalinowski, 2006; Jung *et al.*, 2009; 2010). The most common way of transcriptional regulation is the action of a regulatory protein binding to the operator region of a gene and thereby repressing or activating transcription (Huffman and Brennan, 2002). However, such regulatory proteins have not been identified in *S. typhimurium* or *E. coli* (Johnston *et al.*, 1980). There is also no report of such a regulator in any other prokaryote, including *C. glutamicum*.

The transcription of histidine genes is under positive stringent control

Even though no regulatory protein is involved in transcription regulation of histidine biosynthesis genes, it is addressed by the stringent response in *E. coli* and *S. typhimurium* (Winkler, 1996). The stringent response is the answer to amino acid starvation in bacteria. The effector molecules of the stringent response, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), accumulate under starvation conditions (Chatterji and Ojha, 2001). On the one hand transcription of stable RNA species like tRNAs and rRNAs is repressed during Stringent Response, thereby downregulating protein synthesis. On the other hand transcription of amino acid biosynthesis genes is mostly upregulated (Chatterji and Ojha, 2001). The effector molecule (p)ppGpp is synthesized by the relA gene product, which catalyses phosphorylation of GDP or GTP using ATP as phosphate donor (Cashel, 1975). The spoT gene product was later identified to also participate in (p)ppGpp synthesis, most likely in the hydrolysis of (p)ppGpp (Laffler and Gallant, 1974; Jain *et al.*, 2006). It was demonstrated for *S. typhimurium* that expression of the *his* genes is stimulated 10-fold by addition of ppGpp in a relA deficient strain (Stephens *et al.*, 1975). This stimulation is a result of enhanced transcription and not dependent on the regulatory elements needed for transcriptional attenuation (Stephens *et al.*, 1975).

*Corynebacterium glutamicum* and other Actinobacteria possess a bifunctional Rel protein comprising both gene
functions encoded by relA and spoT (Wehmeier et al., 1998; Avarbock et al., 1999). Analysis of a C. glutamicum Δrel mutant, not able to synthesize (p)ppGpp, revealed a growth requirement for histidine and serine. This result suggested that transcription of histidine and serine biosynthesis genes might be positively controlled by (p)ppGpp (Tauch et al., 2001). The stringent response can be induced artificially by addition of the serine analogue DL-serine hydroxamate (SHX) which inhibits the serine-tRNA synthase (Tosa and Pizer, 1971). Real-time RT-PCR analysis revealed elevated transcript levels of all histidine genes in C. glutamicum organized in the three operons hisEG, hisHA-impA-HisFl-cg2294, and hisDCB-cg2302-cg2301 after treatment with SHX compared with untreated samples (Brockmann-Gretza and Kalinowski, 2006). The mRNA levels of his genes increased two to threefold 10 min after induction of the stringent response (Brockmann-Gretza and Kalinowski, 2006). These results clearly demonstrate that transcription of histidine biosynthesis genes is under positive stringent control in C. glutamicum. The cg0911-hisN operon was not identified to the time the study by Brockmann-Gretza and Kalinowski was conducted. It remains therefore unclear if this operon is also subject to positive stringent control in C. glutamicum.

Transcription of histidine biosynthesis genes in C. glutamicum is regulated by an attenuation mechanism

Next to the global transcriptional regulation of amino acid biosynthesis genes during stringent response, transcription of histidine genes in particular is regulated by an additional mechanism in S. typhimurium and E. coli. Research on the regulation of this pathway, in addition to tryptophan biosynthesis, led to the discovery of the transcriptional attenuation mechanism (Winkler, 1996). Escherichia coli and S. typhimurium possess a leader sequence between the hisp1 promoter and the first structural gene of the operon (Carlomagno et al., 1988). This leader sequence contains an open reading frame (ORF) coding for a short peptide (18 amino acids) with seven consecutive histidine residues. Transcription of the whole histidine operon is coupled to the translation of this leader peptide. During translation of the leader peptide the ribosome senses the availability of charged histidyl-tRNAs thereby influencing two possible alternative secondary structures of the nascent mRNA (Johnston et al., 1980). In brief, if enough charged histidyl-tRNAs are available to allow fast translation of the leader peptide, transcription of the operon is stopped due to the formation of a rho-independent terminator. On the other hand, a delay in translation due to lack of charged histidyl-tRNA promotes the formation of an anti-terminator allowing transcription of the whole operon (Johnston et al., 1980).

Jung and colleagues (2009) suggested a histidine-dependent transcription regulation of the hisDCB-orf1-orf2(-hisHA-impA-hisFl) operon in C. glutamicum AS019, since the corresponding mRNA was only detectable by RT-PCR if cells were grown in histidine free medium. Later, a 196 nt leader sequence in front of hisD was identified (Jung et al., 2010). Since no ORF coding for a short peptide containing several histidine residues is present in this leader sequence, a translation-coupled transcription attenuation mechanism like in E. coli and S. typhimurium can be excluded. Instead, a T-box mediated attenuation mechanism controlling the transcription of the hisDCB-orf1-orf2(-hisHA-impA-hisFl) operon has been proposed (Jung et al., 2010). Computational folding analysis of the 196 nt 5′ UTR from C. glutamicum AS019 revealed two possible stem-loop structures. In the first structure, the terminator structure, the SD sequence (−10 to −17 nt; numbering relative to hisD translation start site) is sequestered by formation of a hairpin structure. In the second structure, the anti-terminator structure, the SD sequence is available to ribosomes. Additionally, a histidine specifier CAU (−92 to −94 nt) and the binding site for uncharged tRNA 3′ ends UGGA (−58 to −61 nt) were identified. All these components are characteristics of T-box RNA regulatory elements. T-box RNAs are members of riboswitch RNAs commonly modulating the expression of genes involved in amino acid metabolism in Gram-positive bacteria (Gutierrez-Preciado et al., 2009). They were first discovered in B. subtilis regulating the expression of aminoacyl-tRNA synthases (Henkin, 1994). Uncharged tRNAs are able to concurrently bind to the specifier sequence and the UGGN-sequence of the T-box RNA via the tRNAs anti-codon loop and free CCA-3′ end, respectively, thereby influencing the secondary structure of the mRNA (Vitreschak et al., 2008). The T-box mechanism results in premature transcription termination due to the formation of a rho-independent transcription terminator hairpin structure in the absence of uncharged tRNAs (Henkin, 1994). Jung and colleagues (2010) showed that chloramphenicol acetyltransferase (CAT) activity decreases in response to histidine in the medium if the 196 nt 5′ UTR in front of hisD is transcriptionally fused to the chloramphenicol acetyltransferase (cat) gene, demonstrating its transcription termination ability. Additionally, the replacement of the UGGA sequence (−58 to −61 nt) reduced specific CAT activity even in the absence of histidine, strongly supporting the involvement of uncharged tRNAs in the regulatory mechanism (Jung et al., 2010).

To test the effect of histidine on the transcription of the remaining his operons we conducted real-time RT-PCR analysis of C. glutamicum ATCC 13032 grown on minimal medium without and with 1 mM histidine supplementation.
Surprisingly, these experiments did not reveal any differences in transcription of his genes, neither organized in the hisDCB-cg2302-cg2301 operon nor in the three remaining operons (data not shown). The same holds true for a histidine dipeptide addition experiment analysed via subsequent real-time RT-PCR and micro-array analysis. No differences in transcription of his genes were observed after addition of the dipeptide, suggesting that, in contrast to C. glutamicum AS019, histidine does not affect transcription of the his genes in C. glutamicum ATCC 13032 (data not shown). The inconsistent results for the two C. glutamicum strains cannot be explained so far. Although the DNA-sequence upstream of the hisD seems to be identical in both C. glutamicum strains, it cannot be excluded completely that transcription of his genes is regulated differently in these two strains in respect to the effect of histidine. Strain-specific differences are already obvious in the different transcription start sites in front of the hisD genes in both organisms resulting in 5’ UTRs of different length. The hisD leader sequence in C. glutamicum ATCC 13032 is much shorter and consists of only 93 nt (R.K. Kulis-Horn, unpubl. obs.). Whereas the longer hisD 5’ UTR from C. glutamicum AS019 is clearly involved in transcriptional regulation, we suggest a translational control mechanism for the shorter hisD 5’ UTR from C. glutamicum ATCC 13032, which will be discussed in detail below.

Translational regulation

The translation process is part of the attenuation mechanism regulating transcription of his genes in E. coli and S. typhimurium (see above). Besides this, there is no report that translation of the his operon is regulated once the complete mRNA is transcribed in these two organisms. However, there is evidence for a translational regulation mechanism in C. glutamicum.

Jung and colleagues (2010) identified a 196 nt long 5’ UTR in front of the hisDCB-orf1-orf2 operon in C. glutamicum AS019 which can fold into two alternative secondary structures, one of them sequestering the SD sequence in a hairpin (see above). Although Jung and colleagues (2010) demonstrated that this T-box like RNA-element affects transcription of the hisDCB-orf1-orf2 operon, the possibility of an additional translational control by this process remains. T-box RNAs are usually known to regulate transcription only (Gutierrez-Preciado et al., 2009). However, a comparative analysis of RNA regulatory elements of amino acid metabolism genes from various bacteria revealed that some T-boxes from Actinobacteria might be involved in regulation of translation initiation instead (Seliverstov et al., 2005). The T-box RNA identified in front of the ilcS gene of C. glutamicum for example sequesters the SD sequence in the alternative hairpin instead of forming a transcriptional terminator (Seliverstov et al., 2005). Yet, the bioinformatics analysis performed by Seliverstov et al. did not identify similar regulatory RNA elements in front of the his genes in C. glutamicum or other Actinobacteria. This might be attributed to the fact that translation-regulating T-boxes of Actinobacteria have a slightly different structure as compared with classical T-boxes. They are shorter and the specifier codon is located in the loop of the specifier hairpin and not in the bulge (Vitreschak et al., 2008). The RNA element in front of the hisDCB-orf1-orf2 proposed by Jung and colleagues (2010) is shorter as compared with classical T-boxes, since two of the conserved stems (stem II and III) are missing. For this reason, however, it is even much shorter than other known T-box RNAs from Actinobacteria. Moreover, the ‘T-box-sequence’, the 14 most highly conserved residues in T-box RNAs (Gutierrez-Preciado et al., 2009), is only moderately conserved and the histidine specifier is present in a bulge of the specifier hairpin and not in its loop. Consequently, the structure of this regulatory RNA element does not fit the characteristics of neither classical nor short T-box RNAs. It remains ambiguous if this RNA element really represents a T-box or another type of riboswitch.

We identified an even shorter 5’ UTR in front of the hisDCB-cg2302-cg2301 operon in C. glutamicum ATCC 13032 by means of RNA-Seq (see above). This 5’ UTR is only 93 nt in length. Like the 196 nt 5’ UTR identified in C. glutamicum AS019 (Jung et al., 2010), this short 5’ UTR is able to fold into two alternative secondary structures, one of them sequestering the SD sequence (Fig. 4). The binding site for uncharged tRNA 3’ ends UGGA (~58 to ~61 nt; numbering relative to hisD translation start site) is present in this structure, too. However, the histidine specifier CAU described by Jung and colleagues (2010) is already located upstream of the transcribed region in C. glutamicum ATCC 13032 and is therefore not part of its 5’ UTR. But there is another histidine specifier CAC (~49 to ~51, numbering relative to hisD translation start site) present in this short 5’ UTR. This CAC histidine specifier seems to be even better suited for interaction with histidyl-tRNAs as it is exactly complementary to the GUG anticodon of the single histidyl-tRNA in C. glutamicum (Kalinsonski et al., 2003). There is also a strong bias towards the presence of a C in the third position of the specifier sequence of most T-box RNAs (Gutierrez-Preciado et al., 2009). Nevertheless, a T-box regulatory mechanism seems to be even more unlikely than in the case of C. glutamicum AS019. The histidine specifier and the binding site for uncharged tRNA 3’ ends are separated by only seven nucleotides. The interaction of both binding sites with one histidyl-tRNA molecule at the same time seems to be improbable, if not impossible. Moreover, in all known T-box RNAs the specifier is always located upstream of the binding site for uncharged tRNA.

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to the SD anti-sequester structure. 

The SD sequence is sequestered in a hairpin in one of these secondary structures, the CCA binding site might be involved in the stabilization of the SD sequence within a hairpin in one of these structures, together with the observation that histidine does not affect the transcription of his genes (see above), suggests a translational regulatory role of the 5′ UTR in front of hisDCB-cg2302-cg2301 operon in *C. glutamicum* ATCC 13032. Such a translational control would primary affect the expression of the *hisD* gene itself, since SD sequences are present in front of all genes in the operon. As long as these SD sequences are not sequestered by additional secondary mRNA structures, de novo initiation of translation of all downstream genes should still be possible even if *hisD* is not translated (Yoo and RajBhandary, 2008). Since HisD catalyses the final steps of histidine biosynthesis, the translational regulation of only this particular enzyme would enable a very rapid recovery of biosynthesis if histidine becomes limiting.

### Fig. 4

Secondary structure model of the 5′ UTR of the *hisDCB-cg2302-cg2301* mRNA from *C. glutamicum* ATCC 13032. Nucleotides shown in orange and yellow represent the SD sequence and the *hisD* start codon respectively. The histidine specifier (CAC) is shown in red and the putative CCA binding site for uncharged tRNA 3′ ends (UGGA) is shown in blue. Both sequences might be involved in a histidyl-tRNA dependent riboswitch mechanism.

A. SD sequester structure. The SD sequence is sequestered in a hairpin and not available to ribosomes. Translation of the *hisD* gene is blocked.

B. SD anti-sequester structure. The formation of the anti-sequester hairpin prevents the formation of the sequester hairpin. The SD sequence is available to ribosomes and *hisD* is translated.

Uncharged histidyl-tRNA interacting with the histidine specifier and the CCA binding site might be involved in the stabilization of the anti-sequester hairpin, resulting in a switch from the SD sequester to the SD anti-sequester structure.

### Enzymatic regulation

The gene product of *hisG*, the ATP phosphoribosyltransferase (HisG), is the most important enzyme being regulated on enzymatic level in histidine biosynthesis. This enzyme catalyses the first step of histidine biosynthesis, the condensation of ATP and PRPP to PR-ATP. The regulation of this particular enzyme is of remarkable importance, as it prevents waste of ATP and also of PRPP. The latter is not only the substrate for the biosynthesis of histidine, but also used for the *de novo* synthesis of purines (Zhang et al., 2008) and pyrimidines (Garavaglia et al., 2012), the tryptophan biosynthesis (Sprenger, 2007), and for the synthesis of arabinogalactan, an important component of the corynebacterial cell wall (Alderwick et al., 2006).

*HisG is affected by feedback inhibition in *C. glutamicum*

It has been demonstrated very early that HisG from *S. typhimurium* (*HisG**)**) is subject to histidine-mediated feedback inhibition in a non-competitive manner (Martin, 1963a) and the same holds true for HisG from *E. coli* (*HisG*) (Winkler, 1996). It has been suggested that ATP-PRT from *C. glutamicum* (*HisG*) is subject to histidine-mediated feedback inhibition, too, since the histidine analogues 2-thiazolyl-DL-alanine (2-TA) and 1,2,4-triazolyl-3-alanine (TRA) inhibit growth of *C. glutamicum* (Araki and Nakayama, 1971). These two analogues are known to be non-competitive inhibitors of ATP-PRT in *S. typhimurium* (Martin, 1963a). Analogue-resistant *C. glutamicum* mutants isolated by Araki and Nakayama (1971) accumulate histidine in the supernatant, indicating that these mutants are deregulated in histidine biosynthesis most likely due to loss of feedback inhibition. Later, by performing enzyme assays with cell-free extracts it was demonstrated that HisG is indeed inhibited by L-histidine (Araki and Nakayama, 1974), and recently, Zhang and colleagues (2012) confirmed the inhibition by histidine on the purified HisG enzyme. Histidine acts as non-competitive inhibitor of HisG having a *K* value of 0.11 ± 0.02 mM (Zhang et al., 2012). The enzyme is
inhibited stronger by histidine than the corresponding ATP-PRTs from *Thermotoga maritima*, but less than those from *S. typhimurium* and *L. lactis* (Zhang et al., 2012). It was also demonstrated that, like in *S. typhimurium* (Martin, 1963a; Morton and Parsons, 1977a), AMP and ADP are competitive inhibitors with respect to ATP with Kᵢ values of 1.29 ± 0.42 mM and 0.88 ± 0.35 mM respectively (Zhang et al., 2012). The inhibitory effect of these two substances with respect to PRPP was not tested. The inhibition of ATP-PRT by AMP and ADP enables to stop the highly energy-demanding histidine biosynthesis if the cells overall energy status is low.

D-Histidine and the histidine intermediates IGP, IAP, Hol-P, L-histidol, and L-histidinal show no inhibitory effect on HisGₛₑ (Martin, 1963a), indicating that HisG inhibition is very specific. L-Histidine itself inhibits both, HisGₛₑ and HisGₑₛ, only as dipolar ion with a positively charged α-amino group, since the inhibitory effect is abolished under alkaline pH conditions (Martin, 1963a; Zhang et al., 2012). It is known from studies with *S. typhimurium* that ppGpp enhances the inhibitory effect of histidine, resulting in complete inhibition of enzyme activity already at moderate histidine concentrations (Morton and Parsons, 1977b). The alarmone ppGpp accumulates during general amino acid starvation and positively effects his operon transcription (see above). Therefore, the synergetic inhibition of HisGₛₑ by ppGpp and histidine prevents unneeded histidine biosynthesis during stringent response induced by an amino acid different from histidine (Winkler, 1996). Since transcription of his genes in *C. glutamicum* is induced during stringent response, a synergetic inhibitory effect of ppGpp and L-histidine on HisGₑₛ might exist, too, but has never been tested.

Gel filtration experiments with HisGₑₛ demonstrated that it exists in a dimeric and a hexameric form (Zhang et al., 2012). It is already known for the highly similar HisGₑₛ that it exists as homodimer in the absence of histidine and at low enzyme concentrations, but it forms hexamers or higher oligomers in the presence of histidine (Cho et al., 2003). This is in accordance with data obtained with HisGₑₛ, whose dimer represents the active form of the enzyme whereas higher oligomers are inactive (Tébar et al., 1973). Due to the high structural similarity (Zhang et al., 2012) it is very likely that HisGₑₛ acts in the same way, i.e. active in its dimeric form and inactive in a histidine-induced hexamer form. The histidine-induced change in quaternary structure from a dimeric to a hexameric form of HisGₑₛ can be reversed by addition of the substrate PRPP (Tébar et al., 1973). This might also be true for HisGₑₛ since the inhibitory effect of histidine is reduced by excess of PRPP (Araki and Nakayama, 1974). According to a predicted structure model, HisGₑₛ monomers are L-shaped and composed of three distinct domains (Zhang et al., 2012). The first two domains are the catalytic domains and the third domain is able to bind histidine and therefore is regarded to be the regulatory domain (Cho et al., 2003; Zhang et al., 2012). It is known from the highly similar HisGₑₛ that one histidine molecule interacts with residues of two different domains III at the same time. This led to the suggestion that direct interaction with histidine is responsible for aggregation of three active HisG dimers to one inactive hexamer (Cho et al., 2003). Binding of AMP additionally establishes intersubunit interactions that stabilize the histidine-bound HisGₑₛ hexamer (Cho et al., 2003). This effect of AMP on HisG quaternary structure is also known for *E. coli*, where the same hexamer-stabilizing effect is observed additionally with the product PR-ATP and high enzyme concentrations (Klungsöyr and Kryvi, 1971). The crystal structure of HisGₑₛ in complex with AMP demonstrated that AMP is not binding to the ATP binding site. Instead, the monophosphate and ribose moieties of AMP are binding to the PRPP binding site and only the adenine ring occupies the ATP binding site (Lohkamp et al., 2004), which explains the surprising observation that AMP acts as a competitive inhibitor with respect to both substrates ATP and PRPP (Morton and Parsons, 1977a).

The crystal structure of HisGₑₛ in complex with histidine enabled the identification of amino acid residues interacting with histidine (Cho et al., 2003). These residues correspond to Gln215, Leu231, Thr235, Met250, and Ala270 in HisGₑₛ. The role of the residues Gln215, Leu231, Thr235, and Ala270 in feedback inhibition of HisGₑₛ has been confirmed by mutation studies, recently (Zhang et al., 2012). A mutated enzyme with three simultaneous substitutions (N215K/L231F/T235A) turned out to be least inhibited by histidine in this study. The mutated enzyme showed a Kᵢ of 4.15 ± 0.21 mM without decreasing the specific enzyme activity (Zhang et al., 2012).

To conclude, the HisG enzyme performing the first step in histidine biosynthesis is the only known enzyme in the biosynthesis that is controlled by a feedback inhibition mechanism involving the end-product of the biosynthesis, L-histidine. Therefore, it constitutes the most interesting target in the development of histidine-producing *C. glutamicum* strains.

### L-Histidine uptake

Uptake systems for L-histidine are known from several microorganisms. *Escherichia coli* and *S. typhimurium* possess two main uptake systems, the high affinity HisJQMP ABC transport system specific for histidine and the low affinity AroP system transporting all aromatic amino acids (Winkler, 1996). The Kᵢ values for histidine transport in *S. typhimurium* are 8 × 10⁻⁸ M and 10⁻⁸ M for the HisJQMP permease and the AroP-system respectively (Ames and Roth, 1968). In *B. subtilis* the histidine-
A specific transporter is encoded by \textit{hutM} as a part of the histidine utilization operon (Yoshida \textit{et al.}, 1995).

**L-Histidine uptake system in \textit{C. glutamicum} is encoded by cg1305**

\textit{Corynebacterium glutamicum} possesses a general uptake system for aromatic amino acids encoded by the gene \textit{aroP} (Wehrmann \textit{et al.}, 1995). The transporter is able to import the three aromatic amino acids tyrosine, tryptophan, and phenylalanine. But, unlike the corresponding transporter from \textit{E. coli} and \textit{S. typhimurium} (Ames and Roth, 1968), it is unable to import histidine (Wehrmann \textit{et al.}, 1995). Therefore, a histidine uptake system in \textit{C. glutamicum} still remains unidentified. Since the gene \textit{cg2301} exhibits characteristics of a transporter and is part of the \textit{hisDCB-cg2302-cg2301} operon, it can be regarded as a candidate to encode a \textit{L}-histidine uptake system. However, the deletion of \textit{cg2301} did not affect growth of a histidine-auxotrophic $\Delta$\textit{hisG} mutant in minimal medium supplemented with histidine, demonstrating still functional histidine uptake (R.K. Kulis-Horn, unpubl. obs.). Further candidates for encoding the unknown \textit{L}-histidine uptake system in \textit{C. glutamicum} are the genes \textit{cg1305}, \textit{cg0555}, and \textit{aroP}, since the amino acid sequence of the histidine transporter \textit{HutM} of \textit{B. subtilis} shows the highest similarity to their deduced amino acid sequences. The gene \textit{cg1305} has been recently reported to encode the \textit{L}-phenylalanine-specific transporter (Zhao \textit{et al.}, 2011) and the gene product of \textit{cg0555} has been characterized as $\gamma$-aminobutyric acid uptake system (Zhao \textit{et al.}, 2012). Since deletion of \textit{aroP} did not affect growth of a histidine auxotrophic $\Delta$\textit{hisG} mutant on minimal medium supplemented with histidine (R.K. Kulis-Horn, unpubl. obs.), the gene product of \textit{aroP}, confirming the results of Wehrmann and colleagues (1995), does not encode the histidine uptake system in \textit{C. glutamicum}. The same holds true for \textit{cg0555}, since a deletion had no effect on growth of the $\Delta$\textit{hisG} mutant (R.K. Kulis-Horn, unpubl. obs.). The deletion of \textit{cg1305}, however, resulted in a strongly reduced growth rate of the histidine auxotrophic mutant already on complex medium and growth of this mutant was almost completely inhibited on minimal medium supplemented with histidine (R.K. Kulis-Horn, unpubl. obs.). These results strongly suggest that \textit{cg1305} encodes a histidine uptake system, and probably that it is the only histidine importer in \textit{C. glutamicum}. Recently, $^{14}$C-labelling experiments demonstrated that the transporter encoded by \textit{cg1305} is able to import \textit{L}-phenylalanine (Zhao \textit{et al.}, 2011). Additionally, the uptake of labelled \textit{L}-tyrosine, \textit{L}-tryptophan, and \textit{L}-proline was tested in this study, but does not occur via this transporter. The ability of importing labelled \textit{L}-histidine was not tested, but strikingly unlabelled \textit{L}-histidine does not compete with the uptake of labelled \textit{L}-phenylalanine (Zhao \textit{et al.}, 2011). This surprising result is somehow inconsistent with our finding that \textit{cg1305} encodes the only histidine uptake system in \textit{C. glutamicum}, since one would expect that unlabelled histidine slows down the uptake of labelled phenylalanine. A possible explanation is the existence of several uptake systems for \textit{L}-phenylalanine in \textit{C. glutamicum} (\textit{Cg1305}, \textit{AroP}, and at least one additional unknown) (Zhao \textit{et al.}, 2011). Although Zhao and colleagues (2011) used a $\Delta$\textit{aroP} strain in their study, the unknown third \textit{L}-phenylalanine transporter might counteract the reduced phenylalanine uptake via \textit{Cg1305} in the presence of histidine, assuming that the unknown transporter does not additionally import histidine. Since our results with the \textit{C. glutamicum} $\Delta$\textit{hisG} $\Delta$\textit{cg1305} did not indicate additional \textit{L}-histidine uptake systems beside \textit{Cg1305}, our observation and the results from Zhao \textit{et al.} might still be consistent. However, the uptake of labelled \textit{L}-histidine should be tested to undoubtedly confirm that \textit{cg1305} encodes the \textit{L}-histidine uptake system in \textit{C. glutamicum}.

**L-Histidine export**

To our knowledge no histidine export system has been described in any organism. Exporters for other amino acids, however, are well known in \textit{E. coli} and \textit{C. glutamicum}, including efflux systems for \textit{L}-lysine, \textit{L}-arginine, \textit{L}-threonine, \textit{L}-cysteine, \textit{L}-leucine, \textit{L}-isoleucine, and \textit{L}-valine (Eggeling and Sahm, 2003). Hashimoto \textit{et al.} recently showed that \textit{L}-glutamate, \textit{L}-aspartate and \textit{L}-phenylalanine are secreted via a mechanosensitive channel by passive diffusion in \textit{C. glutamicum} (Hashimoto \textit{et al.}, 2012). In the past, the export of amino acids by bacteria was believed to be an artificial result of industrial overdosage and to have no biological relevance. But, next to regulation of the biosynthesis of an amino acid and degradation, the corresponding export might be an important possibility to maintain amino acid homeostasis, especially in peptide-rich environments (Eggeling and Sahm, 2003). Genes for histidine utilization, which are present in several pathogenic \textit{Corynebacterium} species, are missing in \textit{C. glutamicum} (Schröder \textit{et al.}, 2012). However, Bellmann and colleagues (2001) demonstrated the ability of \textit{C. glutamicum} to export histidine, which may allow to maintain histidine homeostasis in an environment rich in histidine-containing peptides. Addition of 2 mM His-Ala dipeptide to a \textit{C. glutamicum} culture resulted in a steady increase of external histidine concentration (Bellmann \textit{et al.}, 2001). The export, however, seems to be rather inefficient as internal histidine concentration rises from zero to 200 mM after addition of the dipeptide (Bellmann \textit{et al.}, 2001). Since \textit{C. glutamicum} does not secrete any peptidases (Erdmann \textit{et al.}, 1993), the only explanation for the rising external histidine...
concentration is export of histidine that was cleaved of from the dipeptide intracellularly. However, no candidate gene encoding the exporter has been proposed so far. Interestingly, histidine acts as a co-inducers of lysE transcription, a gene encoding the L-lysine and L-arginine efflux system in C. glutamicum, although histidine is not exported by LysE (Bellmann et al., 2001). There is no explanation, why histidine acts as co-inducer of the exporter, which is unable to export L-histidine. In fact, this might cause a disadvantageous situation for the cell as high histidine concentrations might trigger efflux of L-lysine and L-arginine although their concentrations are low. This negative effect, however, might somehow be counteracted by the high $K_m$ value of 20 mM for L-lysine export (Bröer and Krämer, 1991).

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Conflict of interest

None declared.

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