Cationic Peptides Facilitate Iron-induced Mutagenesis in Bacteria

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Abstract

Pseudomonas aeruginosa is the causative agent of chronic respiratory infections and is an important pathogen of cystic fibrosis patients. Adaptive mutations play an essential role for antimicrobial resistance and persistence. The factors that contribute to bacterial mutagenesis in this environment are not clear. Recently it has been proposed that cationic antimicrobial peptides such as LL-37 could act as a mutagen in *P. aeruginosa*. Here we provide experimental evidence that mutagenesis is the product of a joint action of LL-37 and free iron. By estimating mutation rate, mutant frequencies and assessing mutational spectra in *P. aeruginosa* treated either with LL-37, iron or a combination of both we demonstrate that mutation rate and mutant frequency were increased only when free iron and LL-37 were present simultaneously. The addition of an iron chelator completely abolished this mutagenic effect, suggesting that LL-37 enables iron to enter the cells resulting in DNA damage by Fenton reactions. This was also supported by the observation that the mutational spectrum of the bacteria under LL-37-iron regime showed one of the characteristic Fenton reaction fingerprints: C to T transitions. Free iron concentration in nature and within hosts is kept at a very low level, but the situation in infected lungs of cystic fibrosis patients is different. Intermittent bleeding and damage to the epithelial cells in lungs may contribute to the release of free iron that in turn leads to generation of reactive oxygen species and deterioration of the respiratory tract, making it more susceptible to the infection.

Author Summary

Cationic antimicrobial peptides (cAMPs) are small proteins naturally produced by the immune system to limit bacterial growth mainly through pore formation in the membrane. It has recently been suggested that sub-inhibitory concentrations of cAMPs promote bacterial mutagenesis, similarly to antibiotics. However, we previously reported that cAMPs do not increase mutation rate and do not activate bacterial stress responses. Here we resolve this contradiction. We report that free iron in the culture medium increases mutagenesis in the presence of cAMPs. We show that sub-inhibitory concentrations of cAMPs facilitate entry of free iron into bacterial cells, where it interacts with hydrogen peroxide, thereby resulting in production of DNA-damaging reactive oxygen species and increased mutagenesis. Moreover, these results may have clinically-relevant implications: while very little free iron is normally present in healthy individuals, this is not the case in patients suffering from cystic fibrosis, where elevated bacterial mutagenesis promotes antibiotic resistance and contributes to persistence and severity of infection. Thus, an intervention aimed at reduction of free iron in the lungs could reduce the cAMPs-facilitation of iron-mediated mutagenesis; hence antibiotic resistance and pathoadaptation.

Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen involved in chronic respiratory and hospital-acquired infections [1]. In cystic fibrosis (CF) patients, one of the most common genetic diseases in humans, this bacterium causes chronic lung infections that result in significant morbidity and mortality [2]. *P. aeruginosa* infections are difficult to treat due the inherent resistance to many drug classes, its ability to acquire resistance, via mutations, to all relevant treatments and its high and increasing rates of resistance locally [3].

Mutagenesis plays a crucial role in adaptation of this pathogen for persistence and antibiotic resistance acquisition in CF [4], being found a high proportion of hypermutable bacteria among *P. aeruginosa* isolates [5]. Recently, Limoli *et al* [6] reported an increased mutant frequency after treatment of *P. aeruginosa* with the human cationic peptide LL-37. Based on this finding they proposed that cationic peptides elevated bacterial mutation rates. In a recent study, we reported mutation rates of *E. coli* in the presence of cationic antimicrobial peptides (cAMPs) and antibiotics. LL-37 was present in the panel of AMPs that we tested and we did not find any increase in mutation rates. We also used transcription reporter assays and qRT-PCR and showed that none of the AMPs elicited the main mutagenic stress pathways of bacteria SOS or *rpoS* [7].

Here, we aim to resolve this apparent contradiction based on the observation that the different studies used different media. Limoli *et al*. [6] used M63 for *P. aeruginosa* and LB for *E. coli*, while we used non-cation adjusted Mueller-Hinton Broth (MHB), commonly used for cAMP susceptibility testing . The most striking difference in culture

media between the two studies is that both, M63 [8] and LB are iron-rich [9,10], while MHB is not [11]. Fe^{2+} catalyses hydroxyl radical formation by reacting with hydrogen peroxide both intra- and extra-cellularly, the Fenton reaction [12]. Without free iron hydrogen peroxide reactivity is low at physiological pH and iron metabolism is strictly controlled to avoid DNA and other damage caused by oxygen radicals [13,14]. In most natural situations iron is in short supply, but in cystic fibrosis [15,16] due to accult bleeding of highly vascularised lung tissue and haemoptysis particularly during acute exacerbations, and damage of the respiratory tract epithelium where Fe^{2+} is present intermittently. Although ferrous iron is prone to oxidation, the anaerobic growth of at to high density of bacteria [17] may contribute to stabilise this metal in the reduced state.

Many cationic antimicrobial peptides change membrane permeability properties [18], and this led us to hypothesize that sub-inhibitory concentrations of LL-37 increase uncontrolled iron transport from the extracellular space to the cytoplasm without the intervention of the bacterial iron trafficking system. An intracellular surplus of iron should then result in DNA damage caused by the Fenton reaction.

Here, we (i) estimate the mutation rate of *P. aeruginosa* in the presence of LL-37, iron or both; (ii) we then investigate the hypothesis that ferrous iron (Fe^{+2}) is causal to an increase in mutation rate and that LL-37 facilitates this process, something that is true for colistin too; and finally (iii) we investigate the mutational spectra to find out if promoted mutations match with any of the molecular signatures of Fenton reactions.

Results and Discussion

Mutation rate is increased in the presence of ferrous Fe^{2+} and LL-37

First we determined the mutation rate of *P. aeruginosa* using a fluctuation assay, in the presence of either LL-37 (32 μg/ml), Fe^{2+} (40μM), or both (figure 1). Mutation rate was only increased when LL-37 and Fe^{2+} were added simultaneously, while addition of LL-37 or Fe^{2+} separately did not produce the same effect. To investigate the effect of different concentrations of LL-37 and Fe^{2+}, we estimated mutant frequencies. None of the LL-37 concentrations tested, ranging from 4 to 32 μg/ml, yielded any detectable changes in mutant frequency (figure S1). Fe^{2+} alone, at different concentrations, also did not increase the mutant frequencies in comparison with the control group (figure S2).

Figure 1. The joint action of LL-37 and ferrous ions induces an increase in the mutation rate of *P. aeruginosa.* No changes occur when LL-37 or iron are added to the culture separately. Error bars represent 95 % confidence intervals for mutation rates.

Subsequently, we assayed the MIC50 (32 μg/ml) of LL-37 with several iron concentrations and measured the impact on rifampicin mutant frequency of *P. aeruginosa* strain PAO1. We found that added concentrations of 10, 20, 40 and 80 μM of Fe^{2+}, increased the mutant frequency between three to five times (figure S3).

Limoli *et al.* [6] observed increased mutant frequencies in both, *P. aeruginosa* and *E. coli*, proposing that LL-37 induces mutagenesis in these bacteria. Taken at face value, this contrasts with our work, where we did not find such effect in *E. coli* [7]. *P. aeruginosa* in the Limoli *et al.* study however, was cultured in an iron-rich medium,

M63 [8], which contains 0.5 mg of iron sulphate per litre (Fe^{2+}). To confirm the results in a different bacterial model, the experiment was repeated with *E. coli* using LB as a culture medium. However, LB is also iron-rich (~16 µM of iron) [9,10]. In these experiments, just before the treatment with LL-37 in a saline solution, bacteria were washed. It has, however, been shown that Gram-negative bacteria can actively accumulate iron in the periplasmic space [19]. This led us to hypothesise that sub-lethal concentrations of cationic antimicrobial peptides can facilitate iron transport into the cells.

LL-37 facilitates mutagenesis but iron is causal

To confirm that iron is causing the observed increase in mutagenesis, we first repeated the assay in the presence of an iron chelator, 2-2′ bipyridyl. 2-2′ bipyridyl completely abolished the increase in mutant frequency in the tested concentration of LL-37+Fe^{2+} combination. The sole action of the chelator plus Fe^{2+} did not increase the mutant frequency (figure 2).

Figure 2. The mutagenesis of LL-37+Fe^{2+} combination is suppressed by the addition of an iron chelator. This supports the notion that iron is causal in increasing mutagenesis (A). In the same line, over-expression of Dps, a natural iron chelator in bacteria, also decreases the mutant frequency to rifampicin (B). Error bars represent 95 % confidence intervals for mutant frequencies.

Bacteria have a number of mechanisms to keep free iron as low as possible inside the cell. One of these is Dps, a natural iron chelating protein. To confirm the results obtained with 2-2′ bipyridyl, we used a Dps over-expressing strain by cloning the *dps*

gene into a multi-copy plasmid under a constitutive promoter. The mutagenesis was not completely reverted in comparison with non-treated controls, but there was a 1.8 fold-reduction (figure 2).

The iron uptake assay showed that there was a significant difference (P=0.00015) in total iron content in bacterial cells between LL-37+ Fe^{2+} (17.50 ± 5.10 nmoles/ml, mean ± standard deviation) and iron alone (5.65 ± 4.13 nmoles/ml, mean ± standard deviation) treatments after 30 minutes of incubation, indicating that LL-37 indeed promotes a non-physiological free iron entrance.

We also tested a further AMP: Colistin, which is of bacterial origin, and is one of the most effective agents against *P. aeruginosa* in CF infections [20]. We treated cultures of *P. aeruginosa* PAO1 with MIC50 of colistin (1.8 µg/ml) in high and low concentrations of iron in the media. The effects of colistin were very similar to the effects we found for LL-37 (figure 3). Although the mechanism of action of colistin is not fully understood [21], our results suggest pore forming mechanism or permeability changes in the cell as likely mechanisms. It has been demonstrated that colistin promotes the uptake of hydrophilic antibiotics, explaining their synergism with them [22].

Figure 3. Mutagenesis induced by colistin and Fe^{2+} combination. The mutation rate is only increased if both, iron and colistin, are present. Error bars represent 95 % confidence intervals for mutation rates.

Previous work has suggested that iron is involved in *Pseudomonas aeruginosa* pathoadaptation and antibiotic resistance acquisition [15]**.** All experiments were carried out with the more soluble Fe^{2+} because ferrous iron is abundant in the CF lung

(~39 µM on average for severely ill patients) and significantly correlates with disease severity [23]. Moreover, iron activates the two-component signal transduction system BqsRS in *P. aeruginosa,* which is transcriptionally active in CF sputum, and promotes tolerance to cationic stressors [24]. This increases the tolerance to both host peptides such as LL-37 and colistine, which is administered in CF therapy.

The new mechanism proposed here has almost certainly important implications. Under specific pathologies such as cystic fibrosis or other respiratory chronic diseases, iron that is usually in short supply, is abundant. Disturbances in iron metabolism have been shown to promote evolution of antibiotic resistance in *E. coli* [25]. Given that cationic antimicrobial peptides usually induce membrane permeability in all susceptible microbes and Fenton chemistry is universal, the mechanism we propose here is likely applicable to other AMPs. For example, beta-defensin-2 was shown to double the mucoid conversion rate, a mutagenic process, of *P. aeruginosa* [6]. Yet free iron is rarely available in most physiological situations but for a few pathologies such as cystic fibrosis

Additionally, it has been reported that some cationic antimicrobial peptides interact with bacterial membrane proteins and delocalise them [26]. It is conceivable then that LL-37 may interact and interfere with iron transport systems, which in turn may contribute to iron homeostasis disruption and enhance mutagenesis. This possibility requires additional investigation and will be the goal for future studies.

In the context of cystic fibrosis, the increase in salt concentration may lead to the reduced activity or complete inactivation of other antimicrobial peptides, as observed for human beta-defensin-1 [27], while other components could eventually contribute to mutagenesis in the same way that LL-37 does. Moreover, the PhoP-PhoQ and PmrA-

PmrB two-component regulatory systems of *P. aeruginosa* may play an important role in antimicrobial peptide tolerance. This resistance is reproduced *in vitro* when magnesium concentrations are low [28]. Our experimental conditions, where divalent cations are depleted or in low concentrations, seem comparable. In the light of our results this suggest that bacteria under certain conditions that elicit the expression of these two component systems alter the lipidA structure resulting in increased resistance to colistin [29]. The same systems up-regulate ferrous iron uptake which is mediated by feoAB operon [28,30]. This phenomenon could potentially contribute to saturate intracellular iron storage systems and to generate an excess of iron that eventually can participate in Fenton reaction operating by the mechanisms that we propose here.

How would the mechanism we propose here enhance the overall mutation rate of bacterial populations? In the context of cystic fibrosis, there is a high proportion of hypermutable bacteria due to the inactivation of their DNA miss-match repair (MMR) genes [31]. We may expect a synergistic effect of both types of mutagenesis as we proposed in the past for the mutagenic effect of cystic fibrosis lung environment and the intrinsic mutagenesis of *P. aeruginosa* [4]. It can be speculated that iron-mutagenesis can facilitate the rise of mutator bacteria by enhancing the inactivation of MMR genes. This could then weaken genetic constraints that impede the evolution of bacteria to resist antibiotics by multiple pathways as previously described [32].

Mutational spectra of iron-induced mutagenesis.

The results above strongly suggest that sub-lethal concentrations of cationic antimicrobial peptides facilitate the access of free iron to the cytoplasmic

compartment of *P. aeruginosa.* Given that the Fenton reaction results in DNA damage [33] and reactive oxygen species (ROS) damages to the DNA display specific molecular fingerprints, we investigated if there was evidence of the Fenton reaction effects on the mutational spectrum.

It would be very difficult to investigate the mutational spectrum in *rpoB* (sub-unit of RNA polymerase) that confers resistance to rifampicin in *P. aeruginosa* PAO1. This gene is essential in and detectable mutations are mostly point mutations that would constrain the analysis to a few types of changes. We therefore decided to use another strain, PA14, where mutations that confer resistance to fosfomycin are well characterized [34–36]. Resistance mutations to fosfomycin in *P. aeruginosa* PA14 depend on a single non-essential locus encoding the glycerol-3-phosphate transporter GlpT, making it a much better marker for studying the entire repertoire of mutations compared to *rpoB* [36,37] [38].

The treatment of *P. aeruginosa* P14, which shows a similar sensitivity to LL-37 as the PAO1 strain used above, with the mutagenic combination of LL-37+Fe^{2+} showed almost a four fold-increase in mutant frequency (figure S4).

To assess how addition of free iron and antimicrobial peptides affect the mutation spectrum of the *glpT* gene of PA14 strain we exposed bacteria to either free iron, antimicrobial peptide LL-37, or both. All of the resulting resistant mutants contained non-synonymous substitutions or deletions in *glpT* that potentially affect the stability of GlpT transporter and likely disrupt the function of the protein (figure 4, table S1, figures S5 and S6). LL-37+Fe^{2+}-treated bacteria displayed striking differences in mutational spectra when compared to the other treatments (figure 4 and table S1).

We used Monte Carlo hypergeometric test implemented in iMARS, a mutation analysis reporting software [39], to assess the overall differences between mutational spectra. The probability of the mutational spectra to be the same stood at 0.554706 (*P*-value confidence limits 0.531080 – 0.578) when the iron treatment was compared to LL-37, but was below 0.0000001 when either were compared with LL-37+Fe^{2+} treatment. Moreover, we found a mutation hotspot (R93 to W change, 10/20 clones) in the LL-37+Fe^{2+} treatment group that was significantly (*P*=0.0004, two-tailed Fisher's exact-test) different from the two other treatments (tables S2, S3 and S4). This mutation hotspot is a single nucleotide transition from C to T, which is one of the most frequent types of mutations caused by ROS [40]. We found that twelve out of twenty clones from the LL-37+Fe^{2+} condition had C to T transitions, whereas none were present in either iron or LL-37- treated bacteria (two-tailed Fisher's exact-test (*P*< 0.0001) (figure S7). It is striking that although several C to T mutations can potentially lead to *glpT* inactivation are in principle possible, that the majority are concentrated in a single point. Such mutation hotspots can be driven by a specific topology on the chromosome [41], or a particular sequence prone to double strand breaks resulting in mutations after repair [42]. In general, observable mutations are the results of mutation-repair balance and not all mutations are repaired with the same efficiency. A good example in *P. aeruginosa* is the mutational inactivation of the anti-sigma factor gene, *mucA*, with the mutated allele *mucA*22 most prevalent (25 to 40%). This inactivation seems to be spectrum dependent [43].

Figure 4. Mutation hotspots in *glpT*. Distribution of the mutations in the 1160 bp-long fragment of *glpT* in *P. aeruginosa* PA14 fosfomycin-resistant clones treated with iron, LL-37, or a combination of both. The number of mutations (nucleotide substitutions,

indels) is plotted against respective nucleotide positions within the gene fragment. Note the overlapping mutations at positions 220-225, 409 and 524 bp in iron and LL-37 treatments and absence of common mutations in LL-37+Fe^{2+} treatment. The figure was generated using the mutational spectrum analysis software iMARS [39].

Interestingly, C to T transitions were one of the most frequent types of single nucleotides changes in the genomes of *Salmonella typhimurium* evolved to increasing concentrations of LL-37 in modified LB medium short of sodium chloride and anions [44], which is fully consistent with our results.

A number of studies [45–48], which caused some controversy [49–51], suggested that hydroxyl radicals can be generated as a consequence of antibiotic treatments and this aggressive by-product may take part in the killing mechanism of bactericidal drugs or promote mutagenesis. Most of these studies were carried out in the iron-rich LB medium and whether processes as described here for the interaction between an AMP and iron apply to antibiotics remains to be explored.

Conclusions

Our results support the notion that under certain pathological situations, sub-inhibitory concentrations of cAMPs facilitate uncontrolled uptake of free iron by bacterial cells, which results in increased mutagenesis by Fenton reaction (figure 5). According to our results, this could be a general mechanism underlying mutagenesis by joint action of antimicrobial peptides and free iron in specific situations where iron is not limited.

Figure 5. A model for LL-37-mediated, iron–induced mutagenesis in *Pseudomonas aeruginosa*. The interactions of LL-37 with the membrane at sub-inhibitory concentrations lead to transient permeability changes in the membranes that promote iron movement in favour of the electrochemical gradient. Uncontrolled uptake of ferrous ions stimulates Fenton reaction that leads to hydroxyl radical formation and results in DNA damage and mutagenesis.

Free iron levels are kept as low as possible due to its toxic effect for all living beings but especially in bacteria that lack proper cell compartments. In fact, iron withdrawal is part of the natural innate immune response in inflammation that makes free iron even more scarce. During inflammation and infection a “hypoferremic response” (anaemia of inflammation) is observed [52,53]. Many chelating proteins such as transferrin and ferritin exhibit antibacterial activity simply by making iron availability incompatible with bacterial proliferation [54].

Despite showing that many cationic antimicrobial peptides are unable to increase mutation rates [7], the particular situation of iron-induced mutagenesis can be of great interest for certain types of infections where iron homoeostasis is compromised. In cystic fibrosis, bleeding happens frequently. Considering that *P. aeruginosa* is one of the most common pathogens that acquire all antibiotic resistance by mutations, the mechanism proposed here is likely very relevant for pathoadaptation.

Finally, our work has potential implications for the development of future treatment of chronic respiratory infections by *Pseudomonas aeruginosa.* For example, modulation of iron-chelating agent in CF therapy could potentially slow down the pathoadaptation and development of resistance in CF, and diminish lung damaging by ROS.

Materials and methods

Bacteria and growth conditions. The *P. aeruginosa* PAO1 wild-type strain was kindly provided by M. A. Jacobs. The *P. aeruginosa* PA14 was kindly provided by Nicole T. Liberati and Frederick Ausubel. All bacterial strains were cultured in Mueller-Hinton Broth, non-cation adjusted (Sigma), with total iron content 0.22 ± 0.02 μM, following recommendations of CLSI for cationic antimicrobial peptide susceptibility testing. The MHB pH was adjusted to 6 in all cases with acetic acid to enhance solubility of both, LL-37 and iron compound. All experiments were performed at 37°C, under agitation in liquid culture. For genetic manipulation, *Escherichia coli* DH5α strain was used and routinely cultured in Lysogeny Broth (LB medium), supplemented with antibiotic when appropriate.

Minimal inhibitory concentration (MIC). MICs were determined according to CLSI recommendations by a microdilution method with some modifications for antimicrobial peptides. The MIC was defined as the antimicrobial concentration that inhibited growth after 24 hours of incubation in liquid MHB medium at 37°C. Polypropylene non-binding multi-well plates (Th. Geyer, Germany) were used for all experiments.

Determination of MIC50. The MIC50s for all antimicrobials were determined by inoculating strains grown to mid-log phase into the wells of a 96-microwell plate. Approximately 10^2 cells from overnight cultures of PAO1 and PA14 strains were inoculated into 50 ml tubes containing 10 ml of MBH and incubated at 37°C with strong agitation until the mid-log phase of growth (approximately 108 cfu/ml). Then, 100 μl of 2 $\times 10^8$ cells from these cultures were inoculated in each well of

polypropylene non-binding 96-multiwell plates containing 100 µl of fresh Mueller-Hinton medium with growing concentration of serially diluted LL-37. The plates were incubated at 37°C during four hours with continuous agitation in a plate reader (Synergy HT, BioTeK). Four replicates per concentration were prepared and the experiments were repeated twice. MIC50s at 4 hours were defined as the concentrations at which 50% of growth reduction in comparison to the control at OD_{600} were observed.

Estimation of mutant frequencies and mutation rates. For spontaneous-mutation rate measurements of PAO1 strain, 1/100 dilutions of overnight cultures were inoculated into four tubes per group, each containing two ml of MHB medium. The cultures were incubated at 37°C with strong agitation to reach ~10^8 cfu/ml. At this point, appropriate concentration of LL-37, colistin, iron sulphate ($FeSO_4$) or combinations of antimicrobial peptides and iron, were added to the cultures. The tubes were allowed to continue their normal growth overnight until saturation. In the experiments with colistin, the bacterial suspensions were washed twice with saline solution 0.9 % NaCl before plating. The cultures were appropriately diluted and plated on MHB agar plates with or without rifampicin (100 µg/ml). The mutant frequency was estimated by the number of colonies growing on rifampicin divided by the number of total cfu/ml. To confirm the results, relevant concentrations were also assayed with ten replicates to see the influence of the treatment on the population mutation rates (the number of mutations per cell per generation). Mutation rates were calculated by maximum verisimilitude method and data were processed using the on-line web-tool Falcor (http://www.mitochondria.org/protocols/FALCOR.html) as recommended [55,56]. Falcor software was used to estimate the mutant frequency too. The mutation rates

for the strain PA14 under the selected treatments were determined in the same way as described for PAO1, but fosfomycin (128 μg/ml) was used instead of rifampicin.

Sequencing of fosfomycin resistant mutants (Fos-R) and sequence analysis. To assess the effects of iron and antimicrobial peptides and their combination on the mutation spectrum of *P. aeruginosa* strain PA14, the *glpT* gene of twenty randomly selected Fos-R clones from independent cultures for each treatment group (MHB supplemented either with 40 μM Fe^{2+}, LL-37 (32 μg/ml) or a combination of LL-37+Fe^{2+} at same concentrations for both), was amplified by colony PCR using *glpT*-P14-F1 (5-AGCGGAGCTCGCGATGTTC-3) and g*lpT*-P14-R1 (5-TCAGCCGGCTTGCTGCGG-3) primers [36] and Kapa2G Fast ReadyMix PCR with dye kit (KAPA Biosystems, Boston, US). Cycling conditions were as follows: 95°C 7'/(95°C 15''/60°C 15''/72°C 40'')x 35/72°C 7'/4°C hold. The PCR products were purified and sequenced at Macrogen Europe using the forward and reverse primers described above. Sequences were assembled using SeqTrace software. Assembled sequences were imported into CLC Sequence Viewer 6 and aligned using default settings. Low quality flanking sequences were removed and the alignment was trimmed to the 1160 bp fragment (the 52-1212 bp region relative to the A in the start ATG codon of the 1347bp-long *glpT* ORF). A tridimensional homology model of GlpT was generated using Cn3D software by performing a BLASTP search using PA14 strain GlpT protein sequence as a query and mapping the resulting alignment against the experimentally determined *Escherichia coli* K-12 GlpT protein structure. To evaluate the potential effects of amino acid substitution on protein stability, the online tool I-Mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) was used. TMHMM server v. 2.0 (**http://www.cbs.dtu.dk/services/TMHMM/**) was used for prediction of

transmembrane helices in *glpT* protein sequence. Mutational spectrum differences were analysed using the software iMARS [39].

Influence of 2-2' bipyridyl on LL-37+Fe^{2+} mutagenesis. The effect of 2-2' bipyridyl, an iron chelating agent, on LL-37+Fe^{2+} mutagenesis was determined by measuring its influence on mutant frequency on a selected concentration of LL-37+Fe^{2+} combination (32 µg/ml and 40 µM of Fe^{2+}respectively), where mutagenesis was observed. The experiment consisted of adding a titrating concentration of 2-2' bipyridyl (114 µM) to chelate 95 % of the added iron of treated cultures, in order to make the treatment compatible with bacterial growth. Cultures with the described LL-37+Fe^{2+} combination with no addition of 2-2' bipyridyl were used as a control. The mutant frequencies of both groups were determined as described elsewhere in this section. LL-37, iron and 2-2' bipyridyl were simultaneously added to the exponentially growing cultures.

Cloning of *dps* gene and mutagenesis experiment. DNA fragment containing the PAO1 *dps* gen from genomic DNAs was amplified by PCR using the oligonucleotides PA-DPS-F1 (5'-ATGGAAATCAATATCGGAATCG-3') and PA-DPS-R1 (5'-CTACTCAAATCAAGCGGTTGGC-3') as forward and reverse primers, respectively. The fragment contains the ATG codon and 50 nucleotides downstream of the stop codon. The PCR product was directly cloned into the *Sma*I-digested and T-tailed pUCP24 plasmid vector (replicative in both *P. aeruginosa* and *E. coli*), which harbours Gentamicin resistance markers [57]. *E. coli* DH5α was used following standard protocols for genetic manipulations. The resulting plasmids, termed pUCP24-DPS, were introduced by electroporation into PAO1 wild type strain. The cloning vector was also transformed into the same strain as control. An experiment similar to the one designed for 2-2' bipyridyl was carried out. A mutagenic combination of LL-37+Fe^{2+} was

assayed in the strains carrying pUCP24-DPS plasmid or the empty vector pUCP24 and mutant frequencies were determined for both groups.

Quantification of iron concentrations. Total iron quantification was carried our as previously described with minor modifications [58,59]. Cultures of *P. aeruginosa* PAO1 were grown to an OD_{600} of approximately 0.5 at 37°C with agitation in a volume of two ml in MHB. The cultures were centrifuged at 4000 *g* during ten minutes at 20°C. The pellets were re-suspended in fresh MHB and three different groups were prepared. The treatments consisted of LL-37 (32 µg/ml) (I), iron sulphate to a final concentration of 40 µM (II), a combination iron sulphate and LL-37 (III), both of them at the same concentrations of their respective group I and II, and a control group (IV) to which the proportional amounts of LL-37 and iron sulphate solvent were added (sterile dH_20 and dH_20, pH=5 respectively). The cultures were incubated for up to 30 minutes and harvested by centrifugation as before but at 4°C. The cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS) and re-suspended in 1 ml of TE buffer containing 5 mg/ml of egg lysozyme (Sigma) and incubated during 10 minutes at room temperature. To quantify total iron, the lysate (one ml) was mixed with one ml of HCl 10 mM and 1 ml of iron-releasing reagent containing HCl 1.4 M + 4.5% (weight/volume) aqueous solution of $KMnO_4$; 1/1 and incubated at 60°C for two hours. After cooling, 0.06 ml iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, 1 M ascorbic acid in water) was added and the sample absorbance was read at 550 nm in a plate reader Synergy HT (Biotek). The iron concentrations were determined based on a standard curve obtained with increasing concentrations of ferric chloride and normalized to protein concentration of the lysates. Each group consisted of five cultures. We determined the content of total iron

in our cultures media MHB and LB, using the same procedure describe above, starting by the addition of 1 ml of HCl 10 mM and 1 ml of the iron-releasing reagent. Under the suspicion that MHB had lower iron content, the samples of this medium were prepared ten-fold concentrated.

Statistical analyses. An unpaired Student's t test or Mann-Whitney U test was used where appropriate for statistical analysis, according to the nature of the data (parametric or nonparametric adjustment). Two-tailed Fisher's exact test or Monte Carlo hypergeometric test were used to calculate statistical significance of differences in mutation frequencies at each codon site of the alignment between treatments in the mutational spectrum analysis. P values less than 0.05 were considered statistically significant. All tests were performed with statistic software R except for mutational spectrum analysis where iMARS [39] was used instead.

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Supplement figure and table legends

Figure S1. Different concentrations of LL-37 (from 4 to 32 µg/ml) have no impact on mutant frequencies to rifampicin of *P. aeruginosa* PAO1 in MHB. Error bars represent 95 % confidence intervals for mutant frequencies.

Figure S2. Different concentrations of Fe^{2+} have no impact on mutant frequencies to rifampicin of *P. aeruginosa* PAO1. Error bars represent 95 % confidence intervals for mutant frequencies.

Figure S3. Different concentrations of Fe^{2+} increase mutant frequencies to rifampicin of *P. aeruginosa* PAO1 if LL-37 is present. The experiment was carried out at MIC50 for LL-37 (32 µg/ml). Error bars represent 95 % confidence intervals for mutant frequencies.

Figure S4. The combination of Fe^{2+} (40 µM) and LL-37 (32 µg/ml) increases the mutant frequency of *P. aeruginosa* PA14 to fosfomycin. Error bars represent 95 % confidence intervals for mutant frequencies.

Figure S5. 3D-structural model of GlpT. A homology model of GlpT was generated using Cn3D software by performing a BLASTP search using PA14 GlpT protein sequence as a query and mapping the resulting alignment against the experimentally determined *Escherichia coli* K-12 GlpT protein structure. Substitutions found in Fos-R mutants in all treatments are highlighted in yellow.

Figure S6. DNA alignment of the 1160 bp fragment of *glpT* from twenty Fos-R *P. aeruginosa* PA14 clones treated with iron, LL-37 and LL-37+Fe^{2+}. The 52-1212 bp region relative to the A in the start ATG codon of the 1347bp-long *glpT* ORF.

Figure S7. Mutation types in *glpT*. Prevalence of certain mutation types in iron, LL-37 and LL-37+Fe^{2+} treatments. C to T transitions was the most common type of nucleotide substitutions in LL-37+Fe^{2+} treatment, but not in two other treatments.

Table S1. Mutations in *glpT* of Fos-R *P. aeruginosa* PA14 clones treated with iron, LL-37 and LL-37+Fe^{2+}.

Table S2. Two-tailed Fisher's exact-test of probability of having the same mutation in both LL-37 and iron treatments. H0 is rejected if $P<0.05$.

Table S3. Two-tailed Fisher's exact-test of probability of having the same mutation in both LL-37 and LL-37+Fe^{2+} treatments. H0 is rejected if $P<0.05$. Mutation hotspots that significantly different are highlighted.

Table S4. Two-tailed Fisher's exact-test of probability of having the same mutation in both iron and LL-37+Fe^{2+} treatments. H0 is rejected if $P<0.05$. Mutation hotspots that significantly different are highlighted.

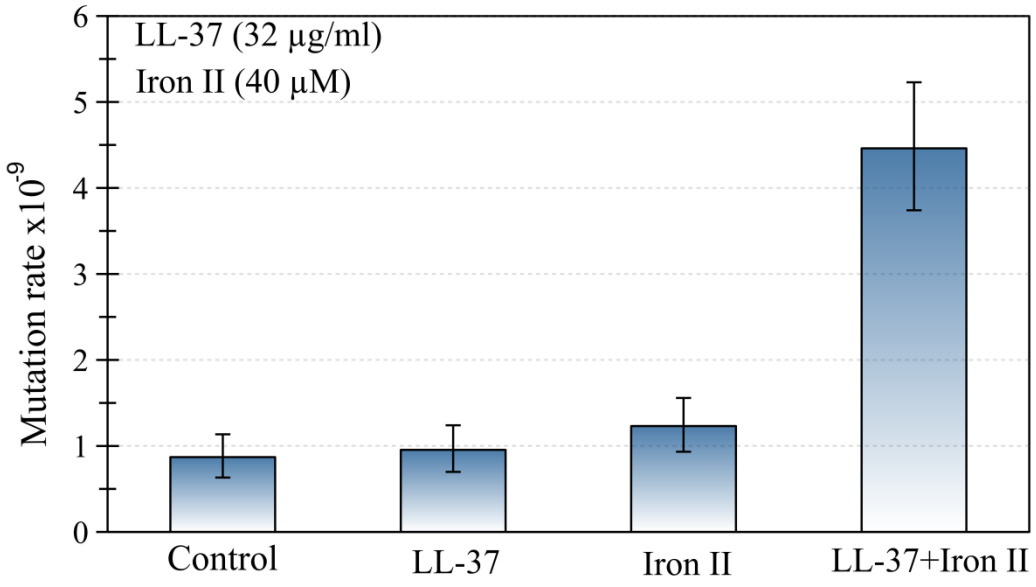


Figure 1.

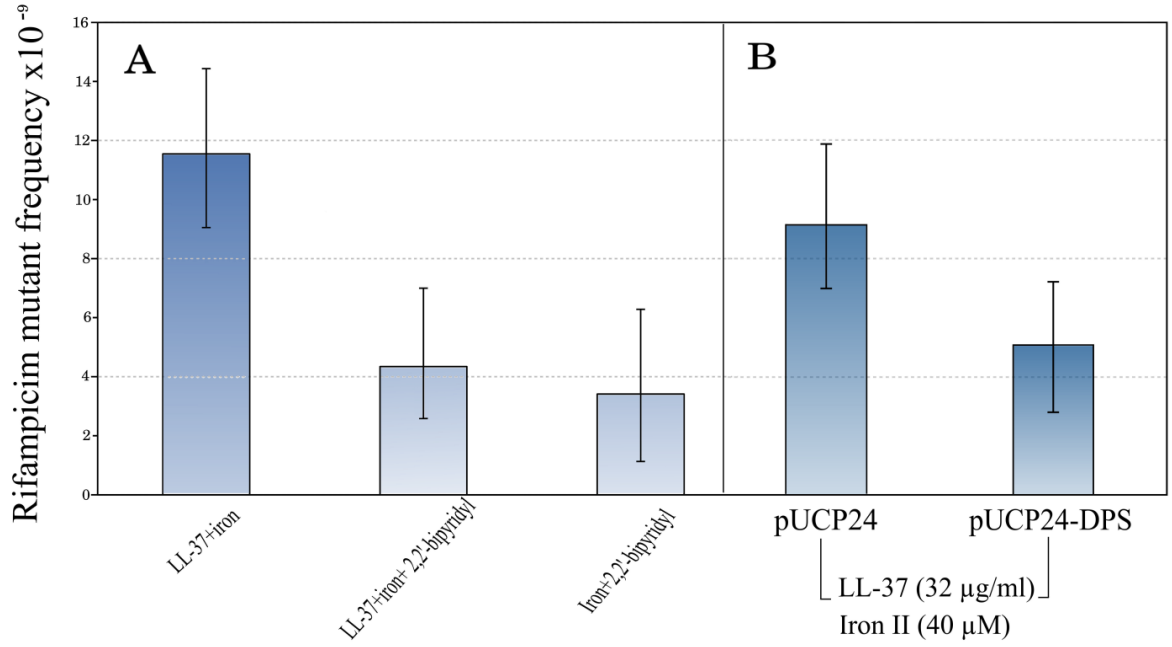


Figure 2.

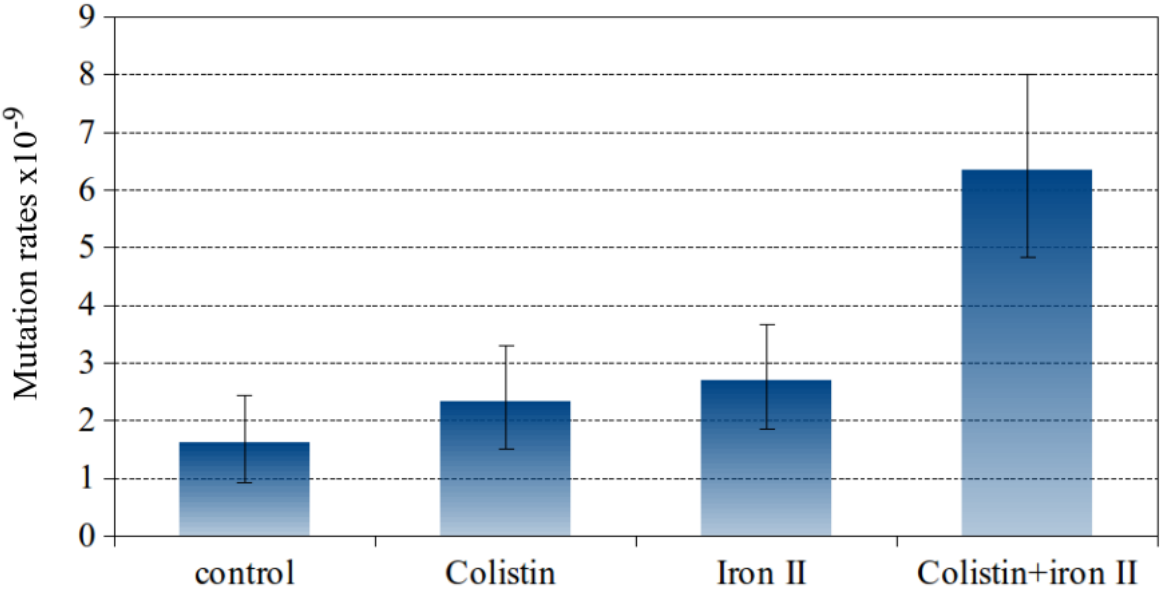


Figure 3.

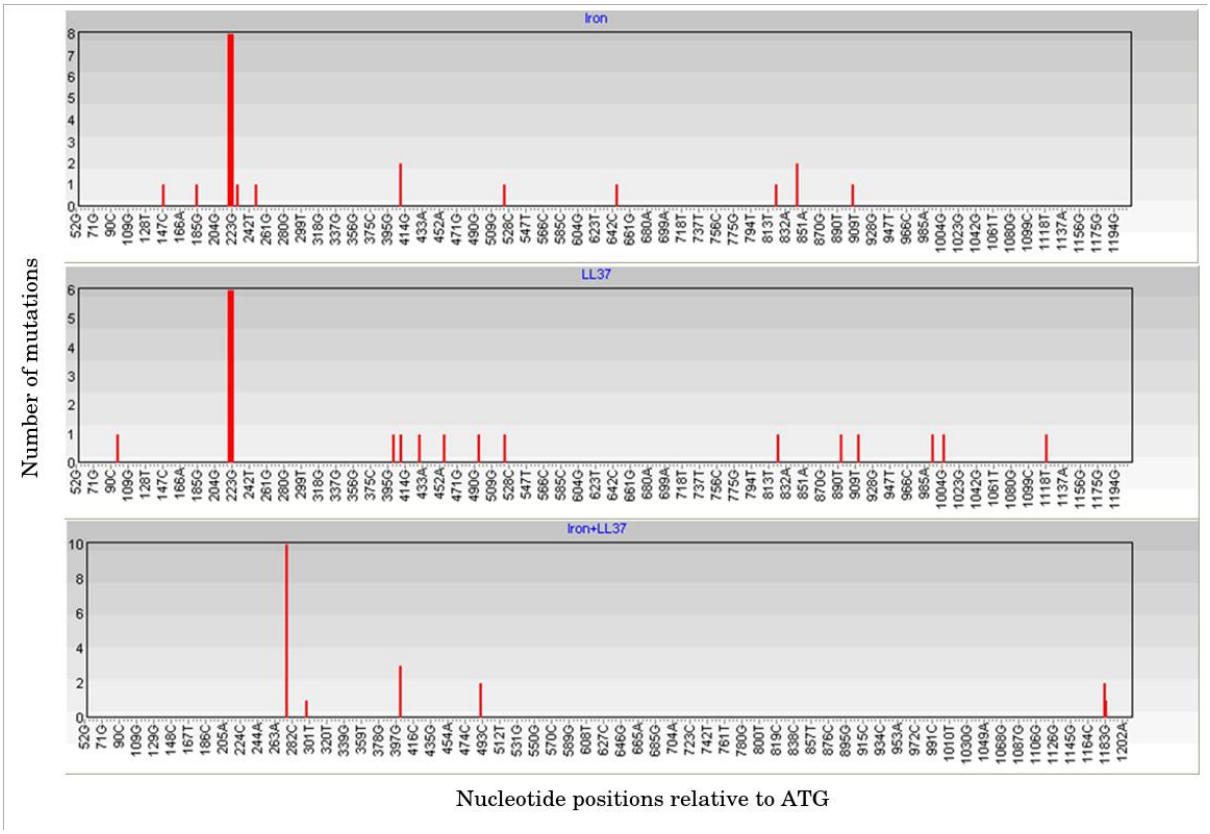


Figure 4.

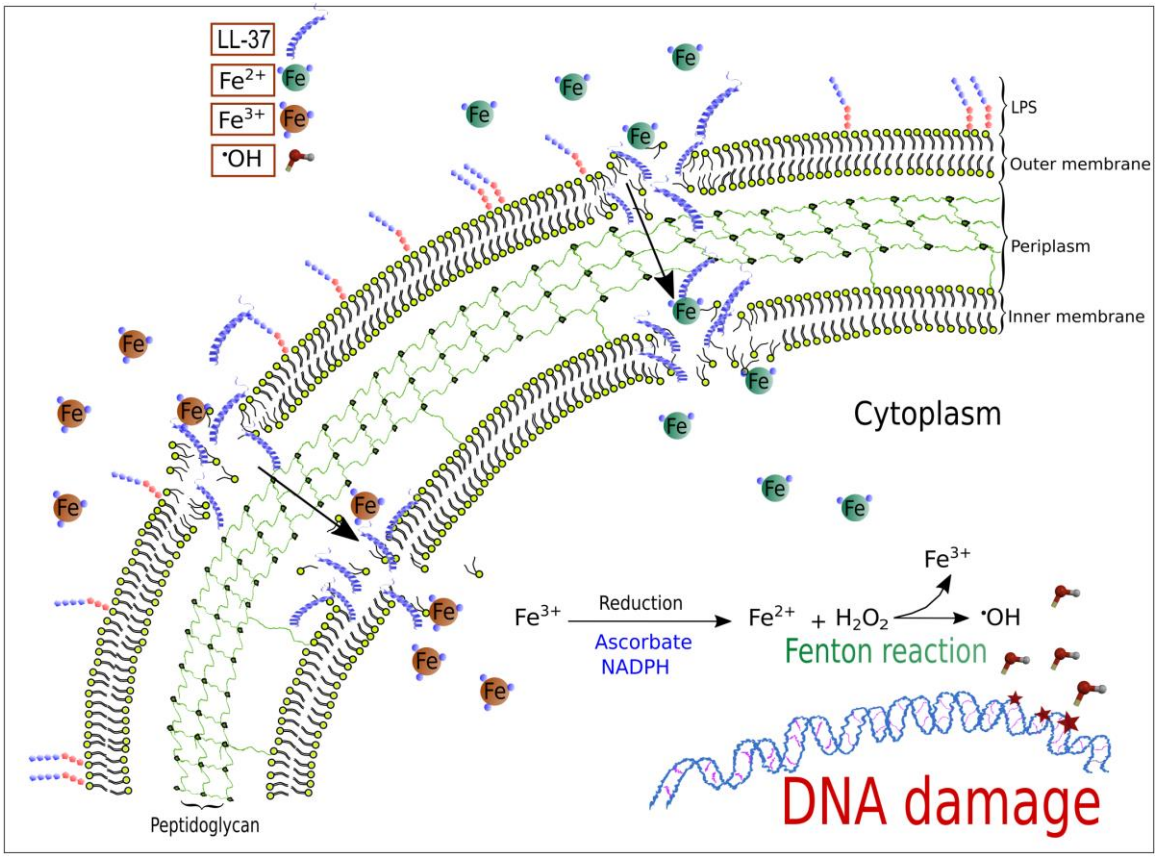


Figure 5.

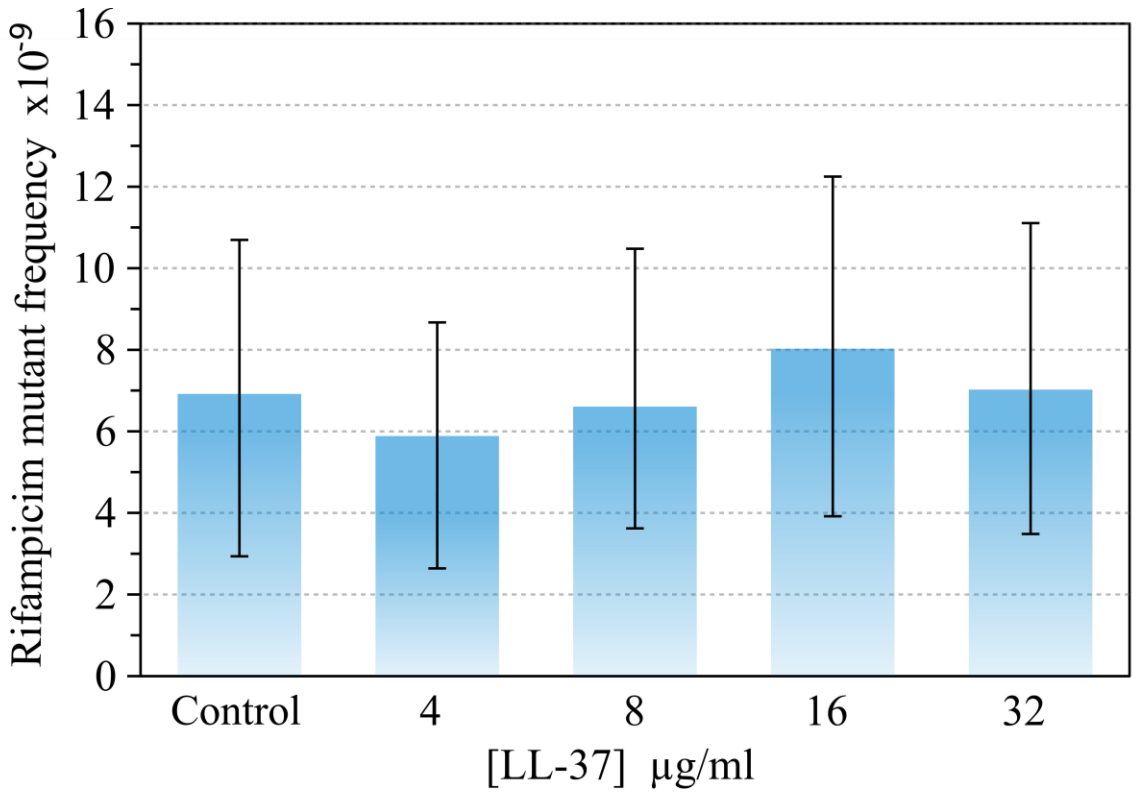


Figure S1.

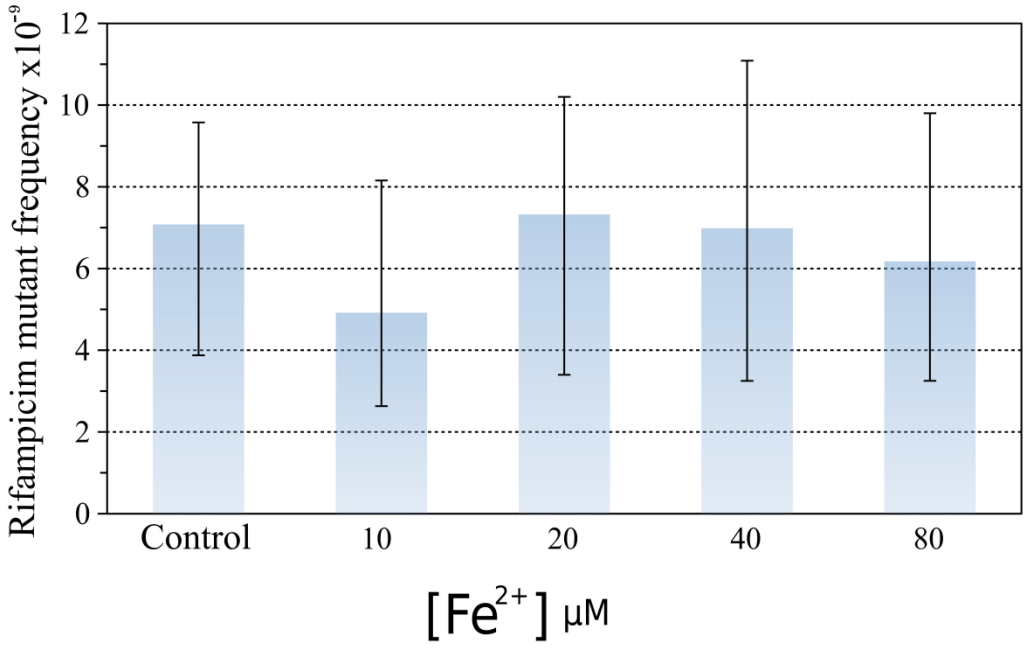


Figure S2.

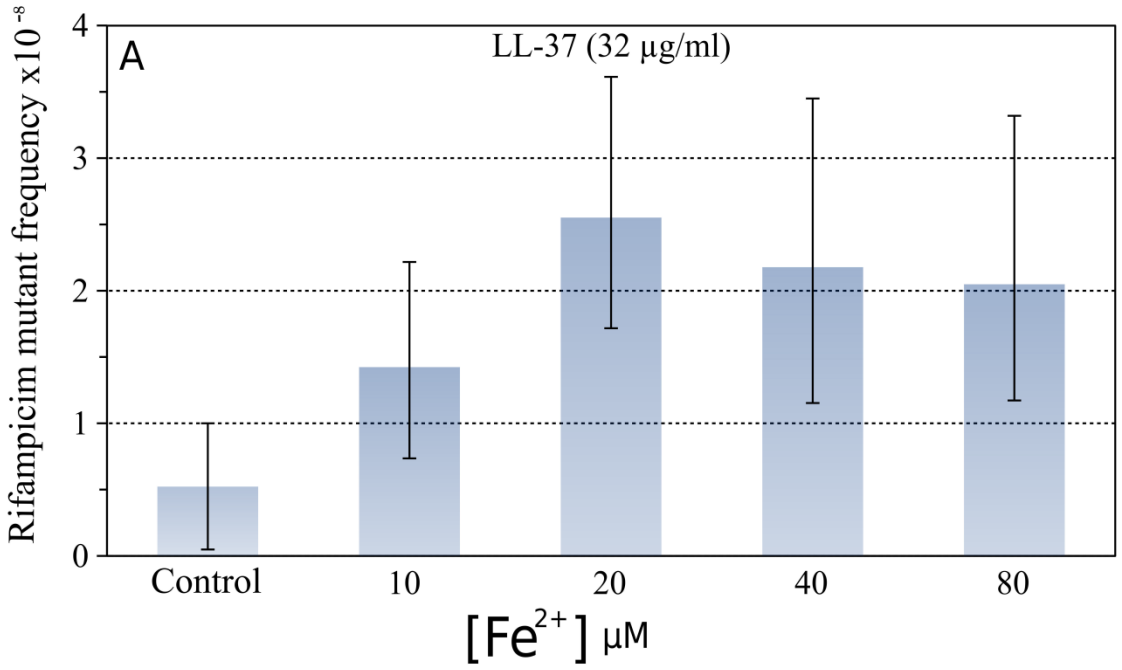


Figure S3.

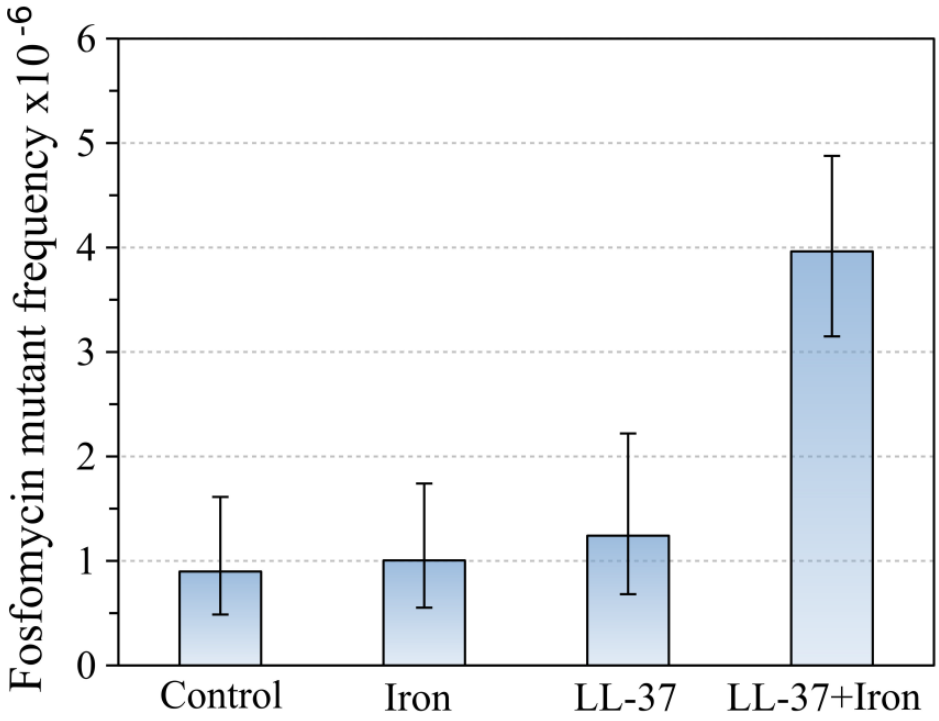


Figure S4.

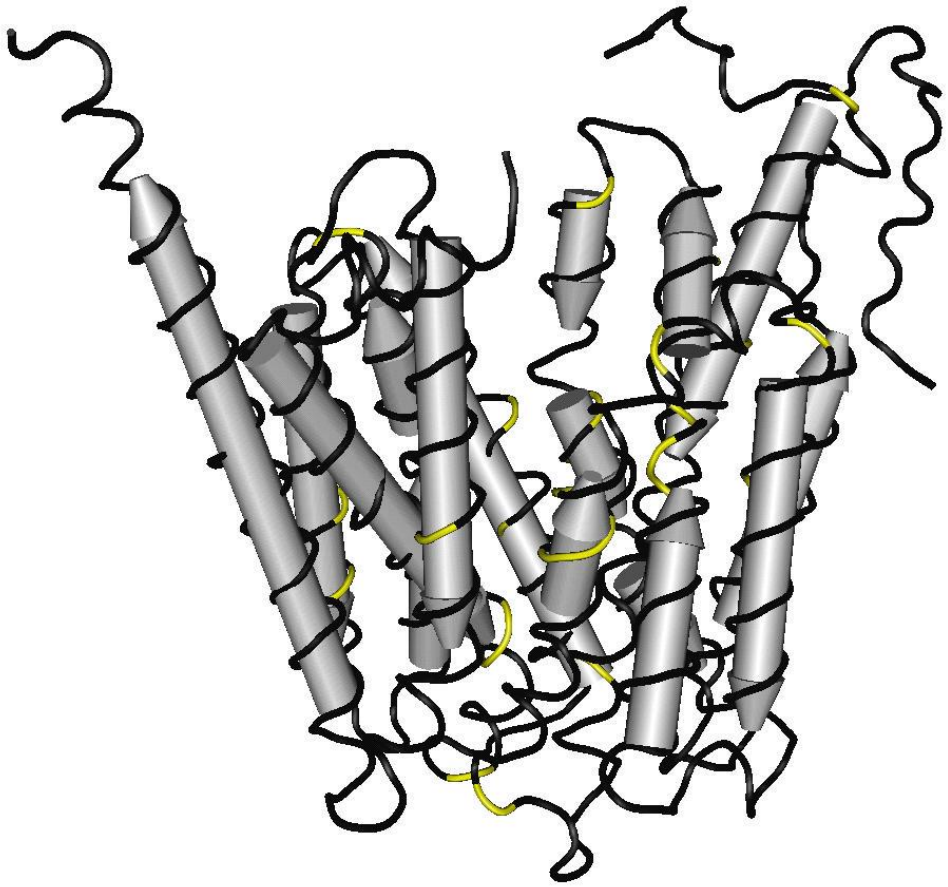


Figure S5.

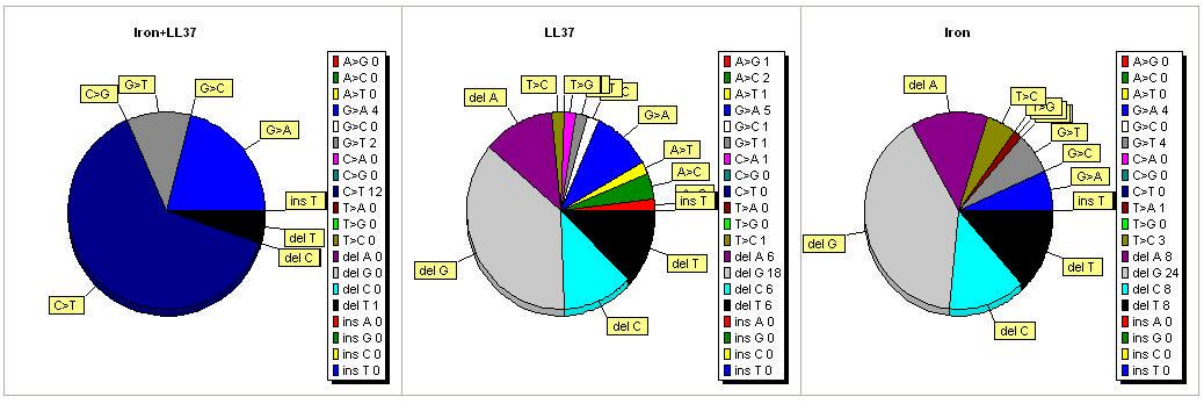


Figure S7.

Table S1. Mutations in *glpT* of Fos-R *P. aeruginosa* PA14 clones treated with iron, LL-37 and LL-37+Fe^{2+}

Treatment	Mutant number	Mutation position[1]	Amino acid change[2]	Protein stability ddG Value[3]	Protein domain[4]
Iron	1	G230 to A transition	G77 to D	-1.09 Large Decrease of Stability	TMhelix
	2	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	3	A846 to T transversion	E282 to V	0.08 Increase of Stability	outside
	4	G185 to A transition	R62 to H	-1.33 Large Decrease of Stability	outside
	5	A846 to T transversion	E282 to V	0.08 Increase of Stability	outside
	6	G250 to T transversion	G84 to C	-1.38Large Decrease of Stability	TMhelix
	7	G409 to A	G137 to S	-1.01 Large Decrease of Stability	TMhelix
	8	T908 to C transition	I303 to T	-2.05 Large Decrease of Stability	TMhelix
	9	T823 to A transversion	W275 to R	-1.09 Large Decrease of Stability	outside
	10	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	11	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	12	T149 to C transition	L50 to P	-1.91 Large Decrease of Stability	outside
	13	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	14	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	15	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	16	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	17	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	18	G409 to T	G137 to C	-0.84 Large Decrease of Stability	TMhelix
	19	T524 to C transition	L175 to P	-1.06 Large Decrease of Stability	TMhelix
	20	G647 to A transition	G216 to D	-1.03 Large Decrease of Stability	inside
LL-37	1	A496 to G transition	N166 to D	-0.08 Decrease of Stability	TMhelix
	2	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	3	A1006 to C transversion	T336 to P	-0.70 Large Decrease of Stability	TMhelix
	4	A401 to T transversion	Q134 to L	-0.10 Decrease of Stability	TMhelix
	5	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	6	T524 to C transition	L175 to P	-1.06 Large Decrease of Stability	TMhelix
	7	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	8	C894 to A transversion, stop codon	Y298 to stop , truncation	NA	NA
	9	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix

	10	G98 to A transition	G33 to D	-0.95,Large Decrease of Stability	TMhelix
	11	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	12	G994 to C transversion	V332 to L	-1.30 Large Decrease of Stability	TMhelix
	13	G457 to A transition	E153 to K	-0.70 Large Decrease of Stability	inside
	14	G409 to T	G137 to C	-0.84 Large Decrease of Stability	TMhelix
	15	A430 to C transversion	T144 to P	-0.39 Decrease of Stability	inside
	16	G1120 to A transition	E374 to K	-0.49 Decrease of Stability	Inside
	17	G824 to A transition, stop codon	W275 to stop , truncation	NA	outside
	18	A220TCGCC225, deletion 6 bp	deletion of I74- A75	NA	TMhelix
	19	G913 to A transition	G305 to S	-1.10 Large Decrease of Stability	TMhelix
	20	G229 to A transition	G77 to S	-1.23 Large Decrease of Stability	TMhelix
LL-37+Fe^{2+}	1	G403 to A transition	G135 to S	-1.05 Large Decrease of Stability	TMhelix
	2	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	2	G403 to T transversion	G135 to C	-0.90 Large Decrease of Stability	TMhelix
	3	C822 to G transversion	D274 to E	-0.08 Decrease of Stability	outside
	4	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	5	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	6	G1183 to A transition	G395 to S	-1.20 Large Decrease of Stability	TMhelix
	7	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	8	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	9	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	10	G1184 to A transition	G395 to D	-0.85 Large Decrease of Stability	TMhelix
	11	C491 to T transition	A164 to V	0.01 Increase of Stability	TMhelix
	12	C491 to T transition	A164 to V	0.01 Increase of Stability	TMhelix
	13	C822 to G transversion	D274 to E	-0.08 Decrease of Stability	outside
	14	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	15	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	16	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	17	C277 to T transition	R93 to W	-0.57 Large Decrease of Stability	inside
	18	T299, deletion 1bp, frameshift	frameshift	NA	NA

19	G1183 to A transition	G395	to S	-1.20 Large Decrease of Stability	TMhelix
20	G403 to T transition	G135	to C	-0.90 Large Decrease of Stability	TMhelix

[1] relative to the A in the start ATG codon of the 1347bp-long *glpT* ORF

[2] relative to the first amino acid of the GlpT protein

[3] ddG values are calculated using I-Mutant3.0 software for prediction of the protein stability change upon mutation, available from http://gpcr.biocomp.unibo.it/~emidio/I-Mutant3.0/I-MutantDDG_Help.html

[4] location of transmembrane helices in proteins was predicted using TMHMM Server v. 2.0, available from http://www.cbs.dtu.dk/services/TMHMM-2.0/

Table S2.

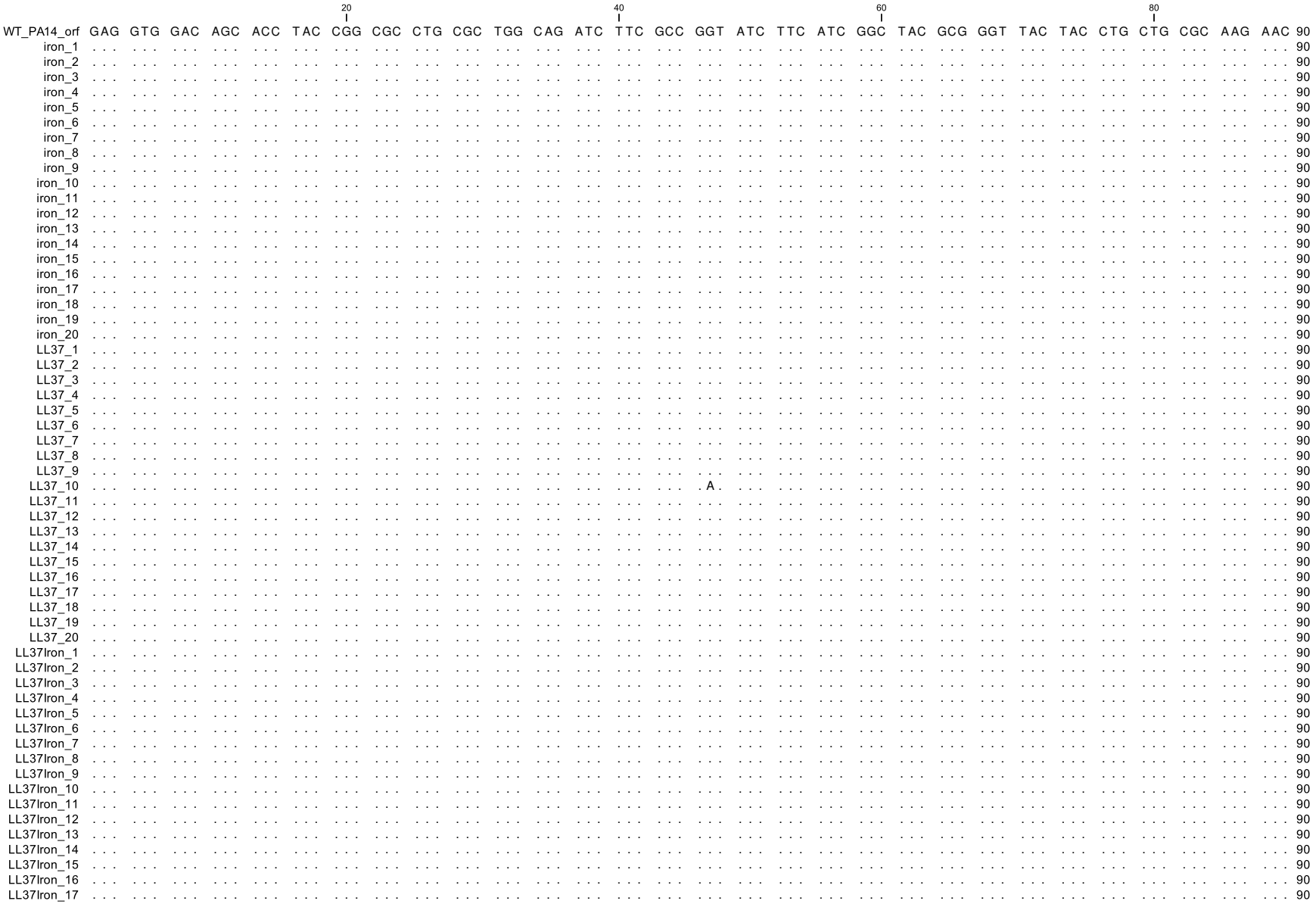
Mutation site	Number of mutants per treatment		Two-tailed *P*-value	Interpretation
	LL-37	Iron		
G33 to D	1	0	> 0.9999	The groups are not significantly different.
L50 to P	0	1	> 0.9999	The groups are not significantly different.
R62 to H	0	1	> 0.9999	The groups are not significantly different.
deletion of 6 bp I74 - A75	6	8	0.7411	The groups are not significantly different.
G77 to D	1	1	> 0.9999	The groups are not significantly different.
G84 to C	0	1	> 0.9999	The groups are not significantly different.
R93 to W	0	0	> 0.9999	The groups are not significantly different.
Q134 to L	1	0	> 0.9999	The groups are not significantly different.
G135 to S	0	0	> 0.9999	The groups are not significantly different.
G137 to S	1	2	> 0.9999	The groups are not significantly different.
T299 frameshift	0	0	> 0.9999	The groups are not significantly different.
T144 to P	1	0	> 0.9999	The groups are not significantly different.
E153 to K	1	0	> 0.9999	The groups are not significantly different.
N166 to D	1	0	> 0.9999	The groups are not significantly different.
A164 to V	0	0	> 0.9999	The groups are not significantly different.
L175 to P	1	1	> 0.9999	The groups are not significantly different.
G216 to D	0	1	> 0.9999	The groups are not significantly different.
D274 to E	0	0	> 0.9999	The groups are not significantly different.
W275 to R	1	1	> 0.9999	The groups are not significantly different.
E282 to V	0	2	0.4872	The groups are not significantly different.
Y298 to stop, truncation	1	0	> 0.9999	The groups are not significantly different.
I303 to T	0	1	> 0.9999	The groups are not significantly different.
G305 to S	1	0	> 0.9999	The groups are not significantly different.
V332 to L	1	0	> 0.9999	The groups are not significantly different.
T336 to P	1	0	> 0.9999	The groups are not significantly different.
E374 to K	1	0	> 0.9999	The groups are not significantly different.
G395 to S	0	0	> 0.9999	The groups are not significantly different.

Table S3.

Mutation site	Number of mutants per treatment		Two-tailed *P*-value	Interpretation
	LL-37	LL-37+Fe^{2+}		
G33 to D	1	0	> 0.9999	The groups are not significantly different.
L50 to P	0	0	> 0.9999	The groups are not significantly different.
R62 to H	0	0	> 0.9999	The groups are not significantly different.
deletion of 6 bp I74 - A75	6	0	0.0202	The groups are significantly different.
G77 to D	1	0	> 0.9999	The groups are not significantly different.
G84 to C	0	0	> 0.9999	The groups are not significantly different.
R93 to W	0	10	0.0004	The groups are significantly different.
Q134 to L	1	0	> 0.9999	The groups are not significantly different.
G135 to S	0	3	0.2308	The groups are not significantly different.
G137 to S	1	0	> 0.9999	The groups are not significantly different.
T299 frameshift	0	1	> 0.9999	The groups are not significantly different.
T144 to P	1	0	> 0.9999	The groups are not significantly different.
E153 to K	1	0	> 0.9999	The groups are not significantly different.
N166 to D	1	0	> 0.9999	The groups are not significantly different.
A164 to V	0	2	0.4872	The groups are not significantly different.
L175 to P	1	0	> 0.9999	The groups are not significantly different.
G216 to D	0	0	> 0.9999	The groups are not significantly different.
D274 to E	0	2	0.4872	The groups are not significantly different.
W275 to R	1	0	> 0.9999	The groups are not significantly different.
E282 to V	0	0	> 0.9999	The groups are not significantly different.
Y298 to stop, truncation	1	0	> 0.9999	The groups are not significantly different.
I303 to T	0	0	> 0.9999	The groups are not significantly different.
G305 to S	1	0	> 0.9999	The groups are not significantly different.
V332 to L	1	0	> 0.9999	The groups are not significantly different.
T336 to P	1	0	> 0.9999	The groups are not significantly different.
E374 to K	1	0	> 0.9999	The groups are not significantly different.
G395 to S	0	3	0.2308	The groups are not significantly different.

Table S4.

Mutation site	Number of mutants per treatment		Two-tailed *P*-value	Interpretation
	Iron	LL-37+Fe^{2+}		
G33 to D	0	0	> 0.9999	The groups are not significantly different.
L50 to P	1	0	> 0.9999	The groups are not significantly different.
R62 to H	1	0	> 0.9999	The groups are not significantly different.
deletion of 6 bp I74 - A75	8	0	0.0033	The groups are significantly different.
G77 to D	1	0	> 0.9999	The groups are not significantly different.
G84 to C	1	0	> 0.9999	The groups are not significantly different.
R93 to W	0	10	0.0004	The groups are significantly different.
Q134 to L	0	0	> 0.9999	The groups are not significantly different.
G135 to S	0	3	0.2308	The groups are not significantly different.
G137 to S	2	0	0.4872	The groups are not significantly different.
T299 frameshift	0	1	> 0.9999	The groups are not significantly different.
T144 to P	0	0	> 0.9999	The groups are not significantly different.
E153 to K	0	0	> 0.9999	The groups are not significantly different.
N166 to D	0	0	> 0.9999	The groups are not significantly different.
A164 to V	0	2	0.4872	The groups are not significantly different.
L175 to P	1	0	> 0.9999	The groups are not significantly different.
G216 to D	1	0	> 0.9999	The groups are not significantly different.
D274 to E	0	2	0.4872	The groups are not significantly different.
W275 to R	1	0	> 0.9999	The groups are not significantly different.
E282 to V	2	0	> 0.9999	The groups are not significantly different.
Y298 to stop, truncation	0	0	> 0.9999	The groups are not significantly different.
I303 to T	1	0	> 0.9999	The groups are not significantly different.
G305 to S	0	0	> 0.9999	The groups are not significantly different.
V332 to L	0	0	> 0.9999	The groups are not significantly different.
T336 to P	0	0	> 0.9999	The groups are not significantly different.
E374 to K	0	0	> 0.9999	The groups are not significantly different.
G395 to S	0	3	0.2308	The groups are not significantly different.


20
40
60
80
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iron_1 90
iron_2 90
iron_3 90
iron_4 90
iron_5 90
iron_6 90
iron_7 90
iron_8 90
iron_9 90
iron_10 90
iron_11 90
iron_12 90
iron_13 90
iron_14 90
iron_15 90
iron_16 90
iron_17 90
iron_18 90
iron_19 90
iron_20 90
LL37_1 90
LL37_2 90
LL37_3 90
LL37_4 90
LL37_5 90
LL37_6 90
LL37_7 90
LL37_8 90
LL37_9 90
LL37_10 . . . . . A 90
LL37_11 90
LL37_12 90
LL37_13 90
LL37_14 90
LL37_15 90
LL37_16 90
LL37_17 90
LL37_18 90
LL37_19 90
LL37_20 90
LL37Iron_1 90
LL37Iron_2 90
LL37Iron_3 90
LL37Iron_4 90
LL37Iron_5 90
LL37Iron_6 90
LL37Iron_7 90
LL37Iron_8 90
LL37Iron_9 90
LL37Iron_10 90
LL37Iron_11 90
LL37Iron_12 90
LL37Iron_13 90
LL37Iron_14 90
LL37Iron_15 90
LL37Iron_16 90
LL37Iron_17 90

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LL37Iron_18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 90
LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 90
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 90
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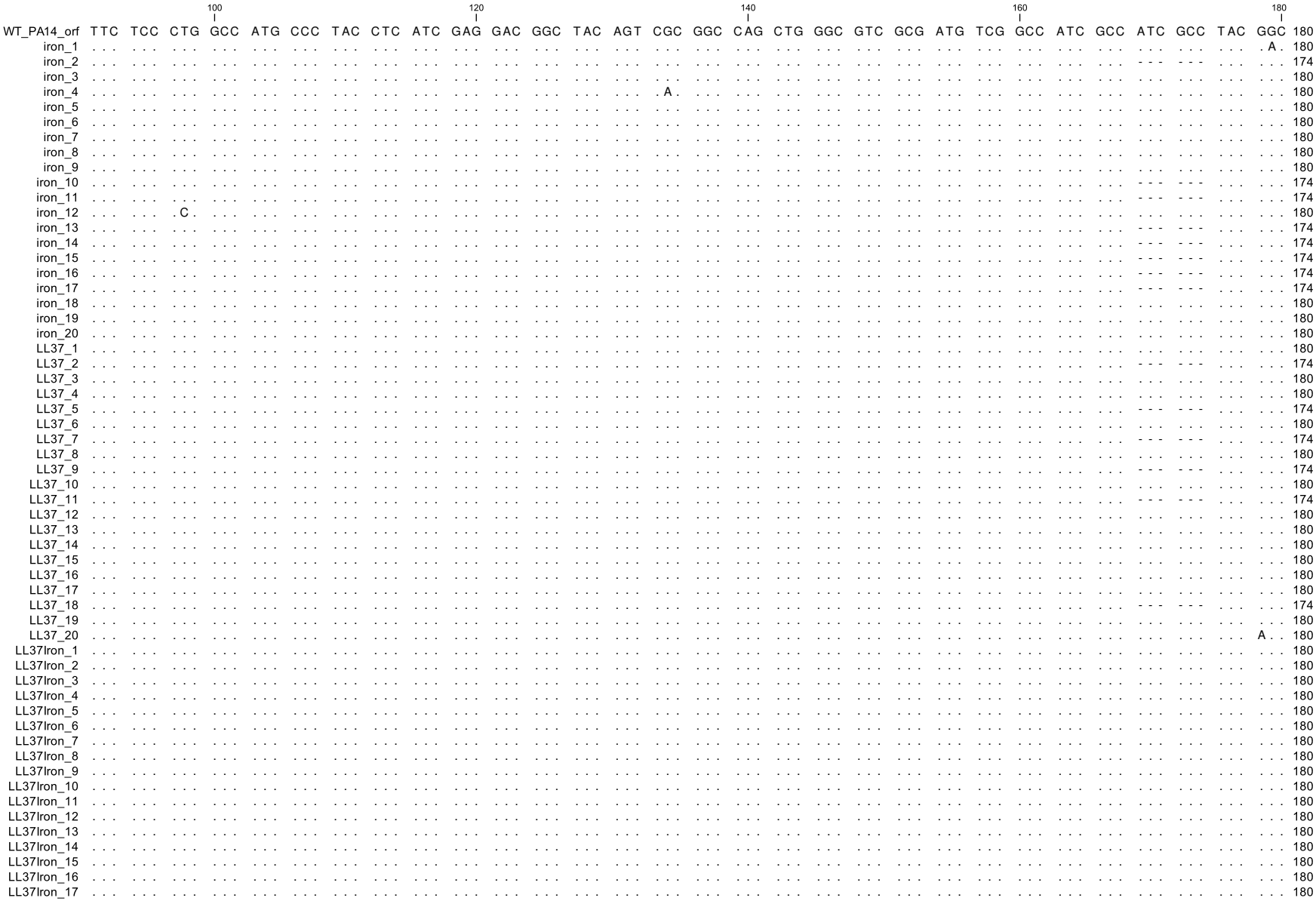
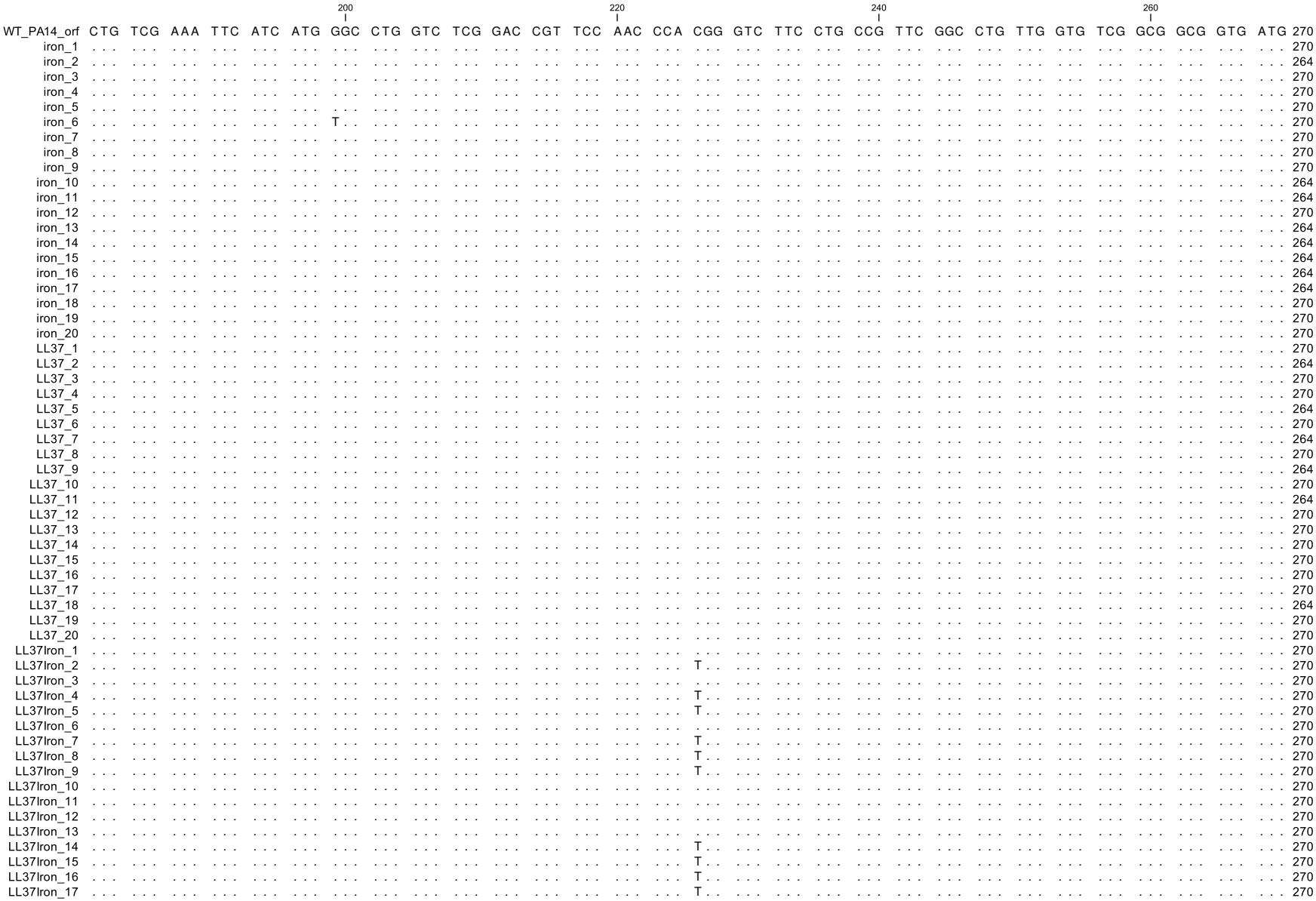

100
120
140
160
180
WT_PA14_orf TTC TCC CTG GCC ATG CCC TAC CTC ATC GAG GAC GGC TAC AGT CGC GGC CAG CTG GGC GTC GCG ATG TCG GCC ATC GCC ATC GCC TAC GGC 180
iron_1 . . . . . A . 180
iron_2 - - - - - - 174
iron_3 180
iron_4 . . . . . A 180
iron_5 180
iron_6 180
iron_7 180
iron_8 180
iron_9 180
iron_10 - - - - - - 174
iron_11 - - - - - - 174
iron_12 . . . C 180
iron_13 - - - - - - 174
iron_14 - - - - - - 174
iron_15 - - - - - - 174
iron_16 - - - - - - 174
iron_17 - - - - - - 174
iron_18 180
iron_19 180
iron_20 180
LL37_1 180
LL37_2 - - - - - - 174
LL37_3 180
LL37_4 180
LL37_5 - - - - - - 174
LL37_6 180
LL37_7 - - - - - - 174
LL37_8 180
LL37_9 - - - - - - 174
LL37_10 180
LL37_11 - - - - - - 174
LL37_12 180
LL37_13 180
LL37_14 180
LL37_15 180
LL37_16 180
LL37_17 180
LL37_18 - - - - - - 174
LL37_19 180
LL37_20 . . . . . A . . 180
LL37Iron_1 180
LL37Iron_2 180
LL37Iron_3 180
LL37Iron_4 180
LL37Iron_5 180
LL37Iron_6 180
LL37Iron_7 180
LL37Iron_8 180
LL37Iron_9 180
LL37Iron_10 180
LL37Iron_11 180
LL37Iron_12 180
LL37Iron_13 180
LL37Iron_14 180
LL37Iron_15 180
LL37Iron_16 180
LL37Iron_17 180

LL37Iron_18	. . . .	180
LL37Iron_19	. . . .	180
LL37Iron_20	. . . .	180


200
220
240
260
WT_PA14_orf CTG TCG AAA TTC ATC ATG GGC CTG GTC TCG GAC CGT TCC AAC CCA CGG GTC TTC CTG CCG TTC GGC CTG TTG GTG TCG GCG GCG GTG ATG 270
iron_1 270
iron_2 264
iron_3 270
iron_4 270
iron_5 270
iron_6 T 270
iron_7 270
iron_8 270
iron_9 270
iron_10 264
iron_11 264
iron_12 270
iron_13 264
iron_14 264
iron_15 264
iron_16 264
iron_17 264
iron_18 270
iron_19 270
iron_20 270
LL37_1 270
LL37_2 264
LL37_3 270
LL37_4 270
LL37_5 264
LL37_6 270
LL37_7 264
LL37_8 270
LL37_9 264
LL37_10 270
LL37_11 264
LL37_12 270
LL37_13 270
LL37_14 270
LL37_15 270
LL37_16 270
LL37_17 270
LL37_18 264
LL37_19 270
LL37_20 270
LL37Iron_1 270
LL37Iron_2 T 270
LL37Iron_3 270
LL37Iron_4 T 270
LL37Iron_5 T 270
LL37Iron_6 270
LL37Iron_7 T 270
LL37Iron_8 T 270
LL37Iron_9 T 270
LL37Iron_10 270
LL37Iron_11 270
LL37Iron_12 270
LL37Iron_13 270
LL37Iron_14 T 270
LL37Iron_15 T 270
LL37Iron_16 T 270
LL37Iron_17 T 270

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LL37Iron_18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .-. ... ... ... ... ... ... .... 269
LL37Iron_19 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .... 270
LL37Iron_20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .... 270
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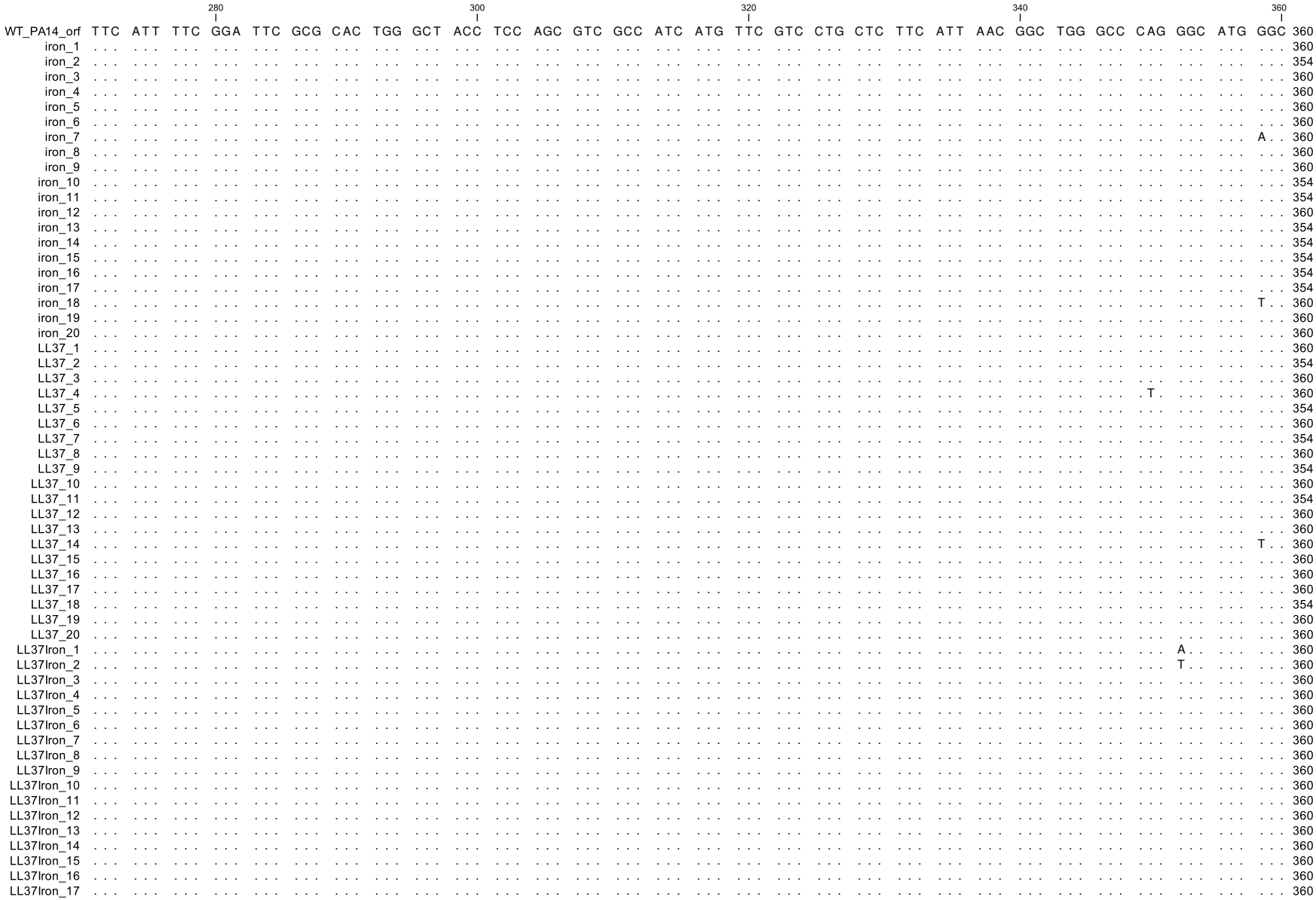
280
300
320
340
360
WT_PA14_orf TTC ATT TTC GGA TTC GCG CAC TGG GCT ACC TCC AGC GTC GCC ATC ATG TTC GTC CTG CTC TTC ATT AAC GGC TGG GCC CAG GGC ATG GGC 360
iron_1 360
iron_2 354
iron_3 360
iron_4 360
iron_5 360
iron_6 360
iron_7 . . . . . A . . 360
iron_8 360
iron_9 360
iron_10 354
iron_11 354
iron_12 360
iron_13 354
iron_14 354
iron_15 354
iron_16 354
iron_17 354
iron_18 . . . . . T . . 360
iron_19 360
iron_20 360
LL37_1 360
LL37_2 354
LL37_3 360
LL37_4 . . . . . T 360
LL37_5 354
LL37_6 360
LL37_7 354
LL37_8 360
LL37_9 354
LL37_10 360
LL37_11 354
LL37_12 360
LL37_13 360
LL37_14 . . . . . T . . 360
LL37_15 360
LL37_16 360
LL37_17 360
LL37_18 354
LL37_19 360
LL37_20 360
LL37Iron_1 . . . . . A 360
LL37Iron_2 . . . . . T 360
LL37Iron_3 360
LL37Iron_4 360
LL37Iron_5 360
LL37Iron_6 360
LL37Iron_7 360
LL37Iron_8 360
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LL37Iron_10 360
LL37Iron_11 360
LL37Iron_12 360
LL37Iron_13 360
LL37Iron_14 360
LL37Iron_15 360
LL37Iron_16 360
LL37Iron_17 360

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LL37Iron_20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ... 360
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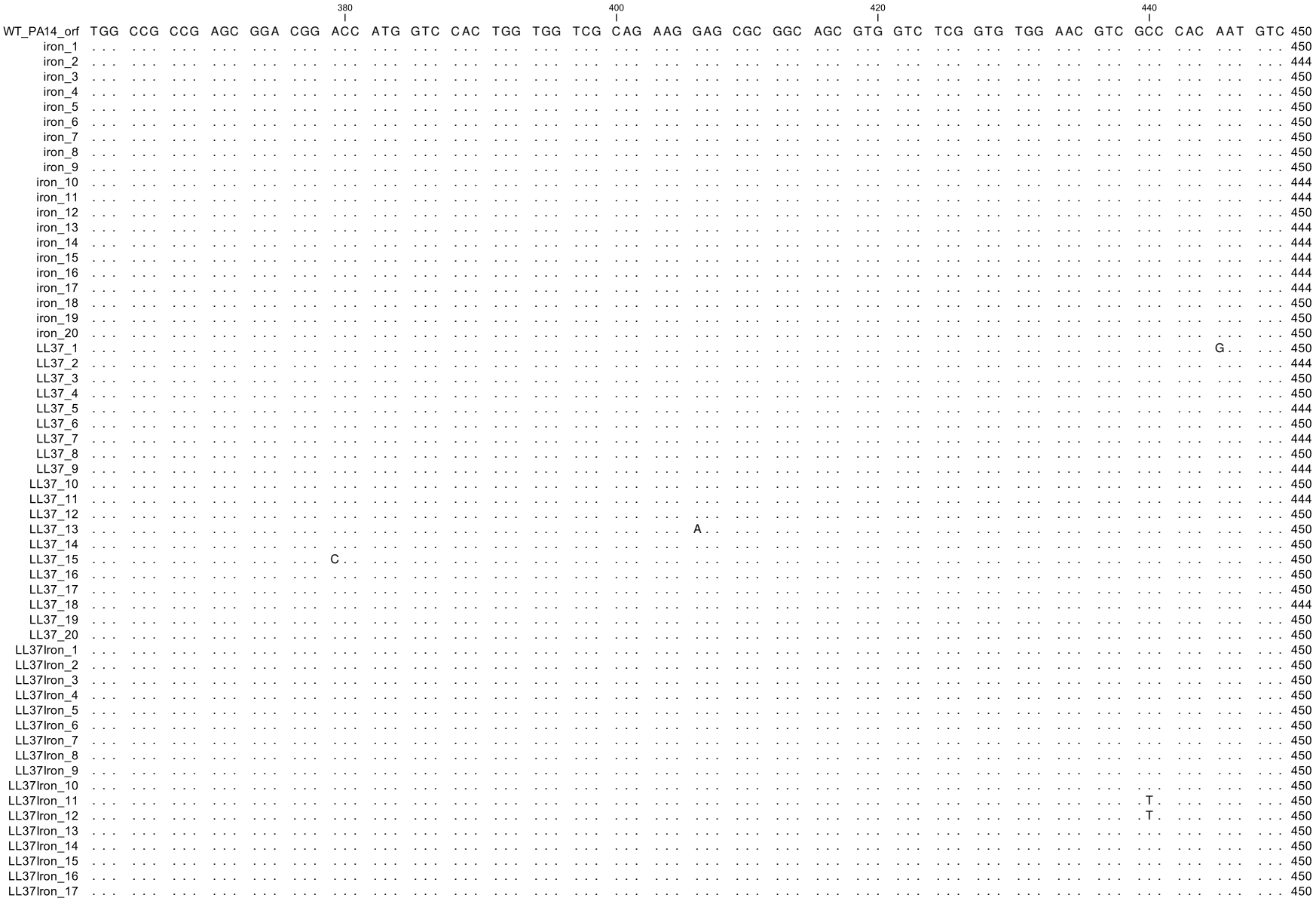
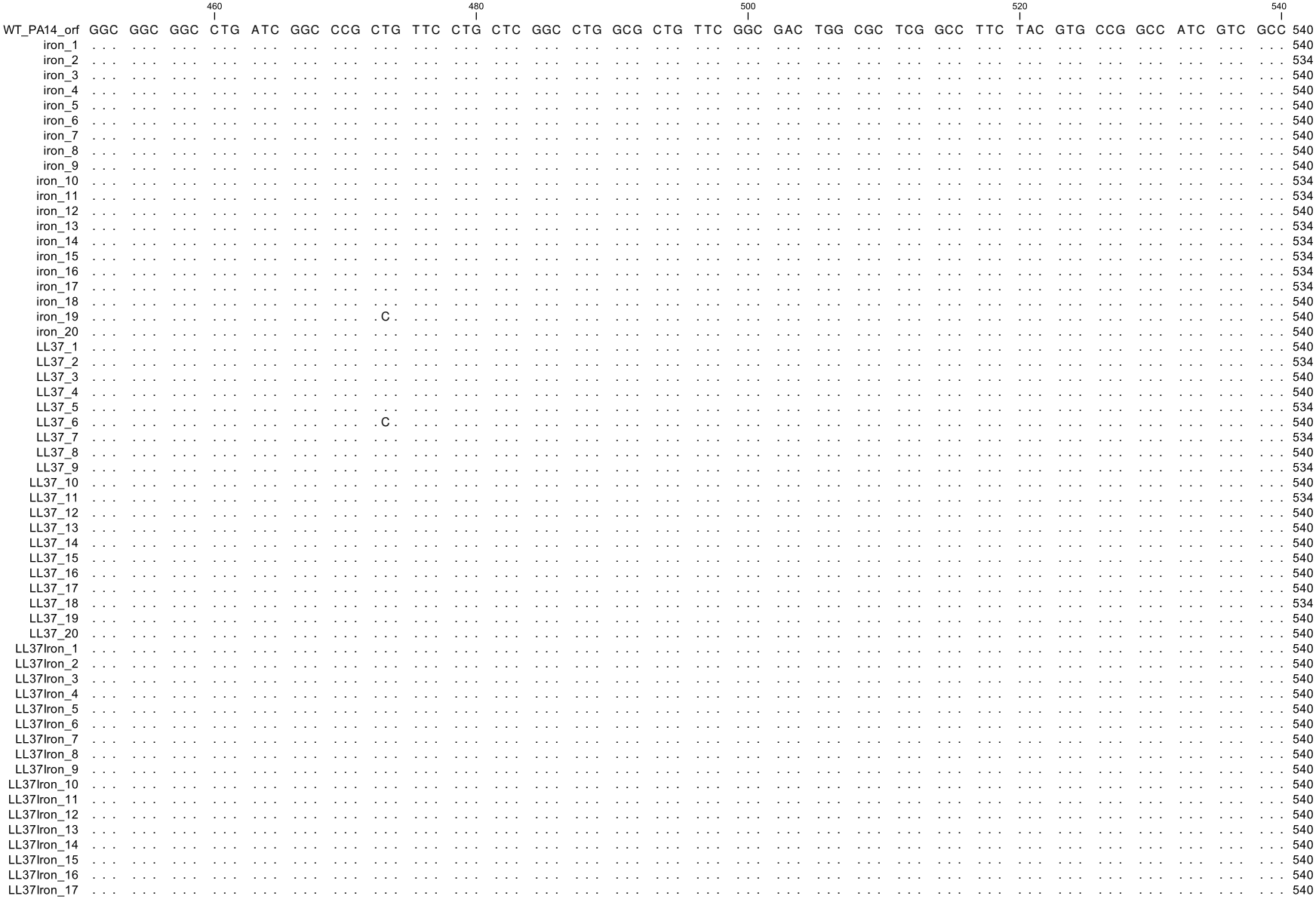

380
400
420
440
WT_PA14_orf TGG CCG CCG AGC GGA CGG ACC ATG GTC CAC TGG TGG TCG CAG AAG GAG CGC GGC AGC GTG GTC TCG GTG TGG AAC GTC GCC CAC AAT GTC 450
iron_1 450
iron_2 444
iron_3 450
iron_4 450
iron_5 450
iron_6 450
iron_7 450
iron_8 450
iron_9 450
iron_10 444
iron_11 444
iron_12 450
iron_13 444
iron_14 444
iron_15 444
iron_16 444
iron_17 444
iron_18 450
iron_19 450
iron_20 450
LL37_1 . . . . . G 450
LL37_2 444
LL37_3 450
LL37_4 450
LL37_5 444
LL37_6 450
LL37_7 444
LL37_8 450
LL37_9 444
LL37_10 450
LL37_11 444
LL37_12 450
LL37_13 . . . . . A 450
LL37_14 450
LL37_15 . . . . . C 450
LL37_16 450
LL37_17 450
LL37_18 444
LL37_19 450
LL37_20 450
LL37Iron_1 450
LL37Iron_2 450
LL37Iron_3 450
LL37Iron_4 450
LL37Iron_5 450
LL37Iron_6 450
LL37Iron_7 450
LL37Iron_8 450
LL37Iron_9 450
LL37Iron_10 450
LL37Iron_11 . . . . . T 450
LL37Iron_12 . . . . . T 450
LL37Iron_13 450
LL37Iron_14 450
LL37Iron_15 450
LL37Iron_16 450
LL37Iron_17 450

LL37Iron_18	... ...	449
LL37Iron_19	... ...	450
LL37Iron_20	... ...	450


460
480
500
520
540
WT_PA14_orf GGC GGC GGC CTG ATC GGC CCG CTG TTC CTG CTC GGC CTG GCG CTG TTC GGC GAC TGG CGC TCG GCC TTC TAC GTG CCG GCC ATC GTC GCC 540
iron_1 540
iron_2 534
iron_3 540
iron_4 540
iron_5 540
iron_6 540
iron_7 540
iron_8 540
iron_9 540
iron_10 534
iron_11 534
iron_12 540
iron_13 534
iron_14 534
iron_15 534
iron_16 534
iron_17 534
iron_18 540
iron_19 . . . . . C 540
iron_20 540
LL37_1 540
LL37_2 534
LL37_3 540
LL37_4 540
LL37_5 534
LL37_6 . . . . . C 540
LL37_7 534
LL37_8 540
LL37_9 534
LL37_10 540
LL37_11 534
LL37_12 540
LL37_13 540
LL37_14 540
LL37_15 540
LL37_16 540
LL37_17 540
LL37_18 534
LL37_19 540
LL37_20 540
LL37Iron_1 540
LL37Iron_2 540
LL37Iron_3 540
LL37Iron_4 540
LL37Iron_5 540
LL37Iron_6 540
LL37Iron_7 540
LL37Iron_8 540
LL37Iron_9 540
LL37Iron_10 540
LL37Iron_11 540
LL37Iron_12 540
LL37Iron_13 540
LL37Iron_14 540
LL37Iron_15 540
LL37Iron_16 540
LL37Iron_17 540

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LL37Iron_18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 539
LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 540
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 540
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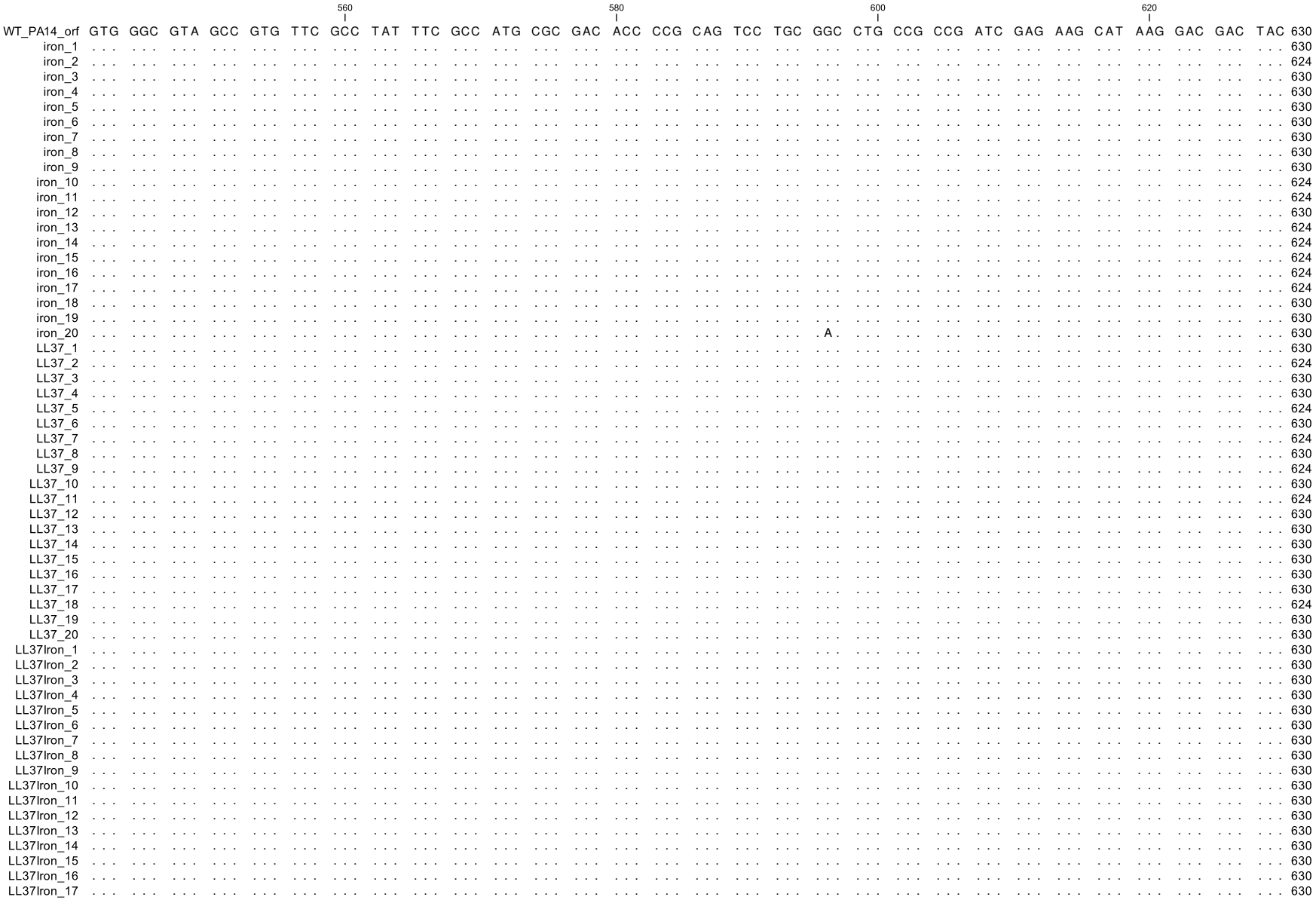
560
580
600
620
WT_PA14_orf GTG GGC GTA GCC GTG TTC GCC TAT TTC GCC ATG CGC GAC ACC CCG CAG TCC TGC GGC CTG CCG CCG ATC GAG AAG CAT AAG GAC GAC TAC 630
iron_1 630
iron_2 624
iron_3 630
iron_4 630
iron_5 630
iron_6 630
iron_7 630
iron_8 630
iron_9 630
iron_10 624
iron_11 624
iron_12 630
iron_13 624
iron_14 624
iron_15 624
iron_16 624
iron_17 624
iron_18 630
iron_19 630
iron_20 A 630
LL37_1 630
LL37_2 624
LL37_3 630
LL37_4 630
LL37_5 624
LL37_6 630
LL37_7 624
LL37_8 630
LL37_9 624
LL37_10 630
LL37_11 624
LL37_12 630
LL37_13 630
LL37_14 630
LL37_15 630
LL37_16 630
LL37_17 630
LL37_18 624
LL37_19 630
LL37_20 630
LL37Iron_1 630
LL37Iron_2 630
LL37Iron_3 630
LL37Iron_4 630
LL37Iron_5 630
LL37Iron_6 630
LL37Iron_7 630
LL37Iron_8 630
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LL37Iron_11 630
LL37Iron_12 630
LL37Iron_13 630
LL37Iron_14 630
LL37Iron_15 630
LL37Iron_16 630
LL37Iron_17 630

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LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 630
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 630
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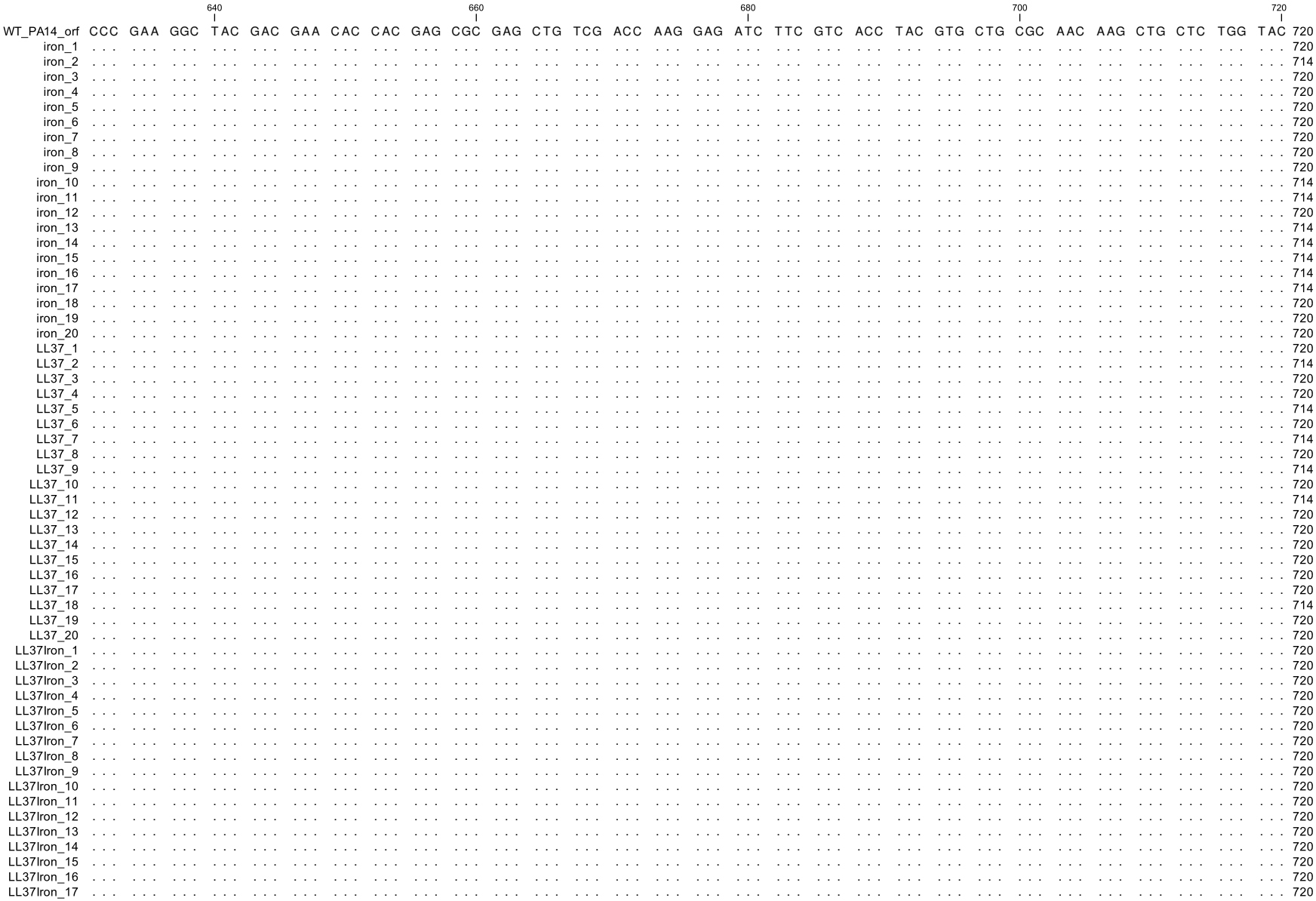
640
660
680
700
720
WT_PA14_orf CCC GAA GGC TAC GAC GAA CAC CAC GAG CGC GAG CTG TCG ACC AAG GAG ATC TTC GTC ACC TAC GTG CTG CGC AAC AAG CTG CTC TGG TAC 720
iron_1 720
iron_2 714
iron_3 720
iron_4 720
iron_5 720
iron_6 720
iron_7 720
iron_8 720
iron_9 720
iron_10 714
iron_11 714
iron_12 720
iron_13 714
iron_14 714
iron_15 714
iron_16 714
iron_17 714
iron_18 720
iron_19 720
iron_20 720
LL37_1 720
LL37_2 714
LL37_3 720
LL37_4 720
LL37_5 714
LL37_6 720
LL37_7 714
LL37_8 720
LL37_9 714
LL37_10 720
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LL37_12 720
LL37_13 720
LL37_14 720
LL37_15 720
LL37_16 720
LL37_17 720
LL37_18 714
LL37_19 720
LL37_20 720
LL37Iron_1 720
LL37Iron_2 720
LL37Iron_3 720
LL37Iron_4 720
LL37Iron_5 720
LL37Iron_6 720
LL37Iron_7 720
LL37Iron_8 720
LL37Iron_9 720
LL37Iron_10 720
LL37Iron_11 720
LL37Iron_12 720
LL37Iron_13 720
LL37Iron_14 720
LL37Iron_15 720
LL37Iron_16 720
LL37Iron_17 720

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LL37Iron_18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 719
LL37Iron_19 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 720
LL37Iron_20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 720
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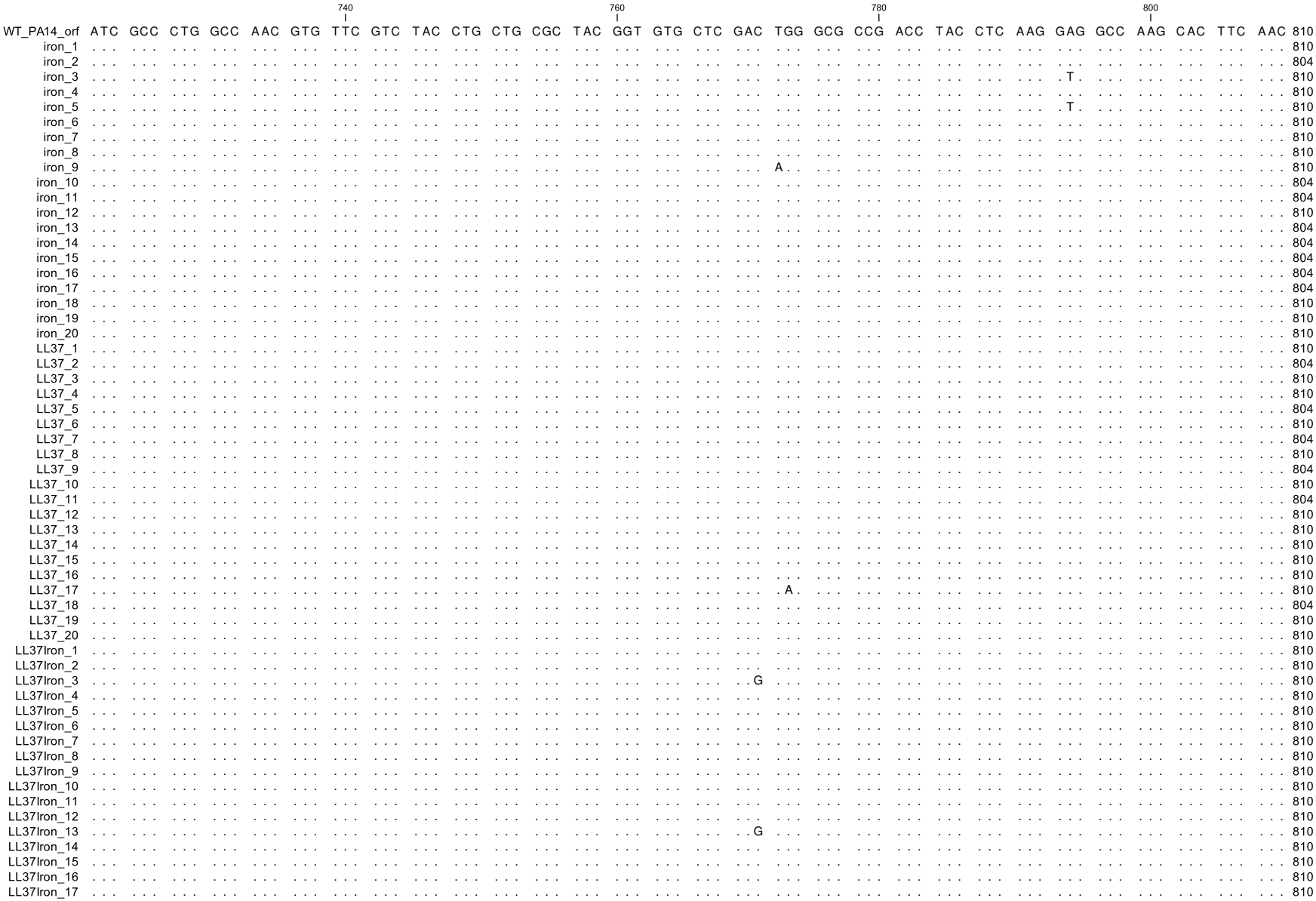
740
760
780
800
WT_PA14_orf ATC GCC CTG GCC AAC GTG TTC GTC TAC CTG CTG CGC TAC GGT GTG CTC GAC TGG GCG CCG ACC TAC CTC AAG GAG GCC AAG CAC TTC AAC 810
iron_1 810
iron_2 804
iron_3 . . . . . T 810
iron_4 810
iron_5 . . . . . T 810
iron_6 810
iron_7 810
iron_8 810
iron_9 . . . . . A 810
iron_10 804
iron_11 804
iron_12 810
iron_13 804
iron_14 804
iron_15 804
iron_16 804
iron_17 804
iron_18 810
iron_19 810
iron_20 810
LL37_1 810
LL37_2 804
LL37_3 810
LL37_4 810
LL37_5 804
LL37_6 810
LL37_7 804
LL37_8 810
LL37_9 804
LL37_10 810
LL37_11 804
LL37_12 810
LL37_13 810
LL37_14 810
LL37_15 810
LL37_16 810
LL37_17 . . . . . A 810
LL37_18 804
LL37_19 810
LL37_20 810
LL37Iron_1 810
LL37Iron_2 810
LL37Iron_3 . . . . . G 810
LL37Iron_4 810
LL37Iron_5 810
LL37Iron_6 810
LL37Iron_7 810
LL37Iron_8 810
LL37Iron_9 810
LL37Iron_10 810
LL37Iron_11 810
LL37Iron_12 810
LL37Iron_13 . . . . . G 810
LL37Iron_14 810
LL37Iron_15 810
LL37Iron_16 810
LL37Iron_17 810

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LL37Iron_18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 809
LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 810
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 810
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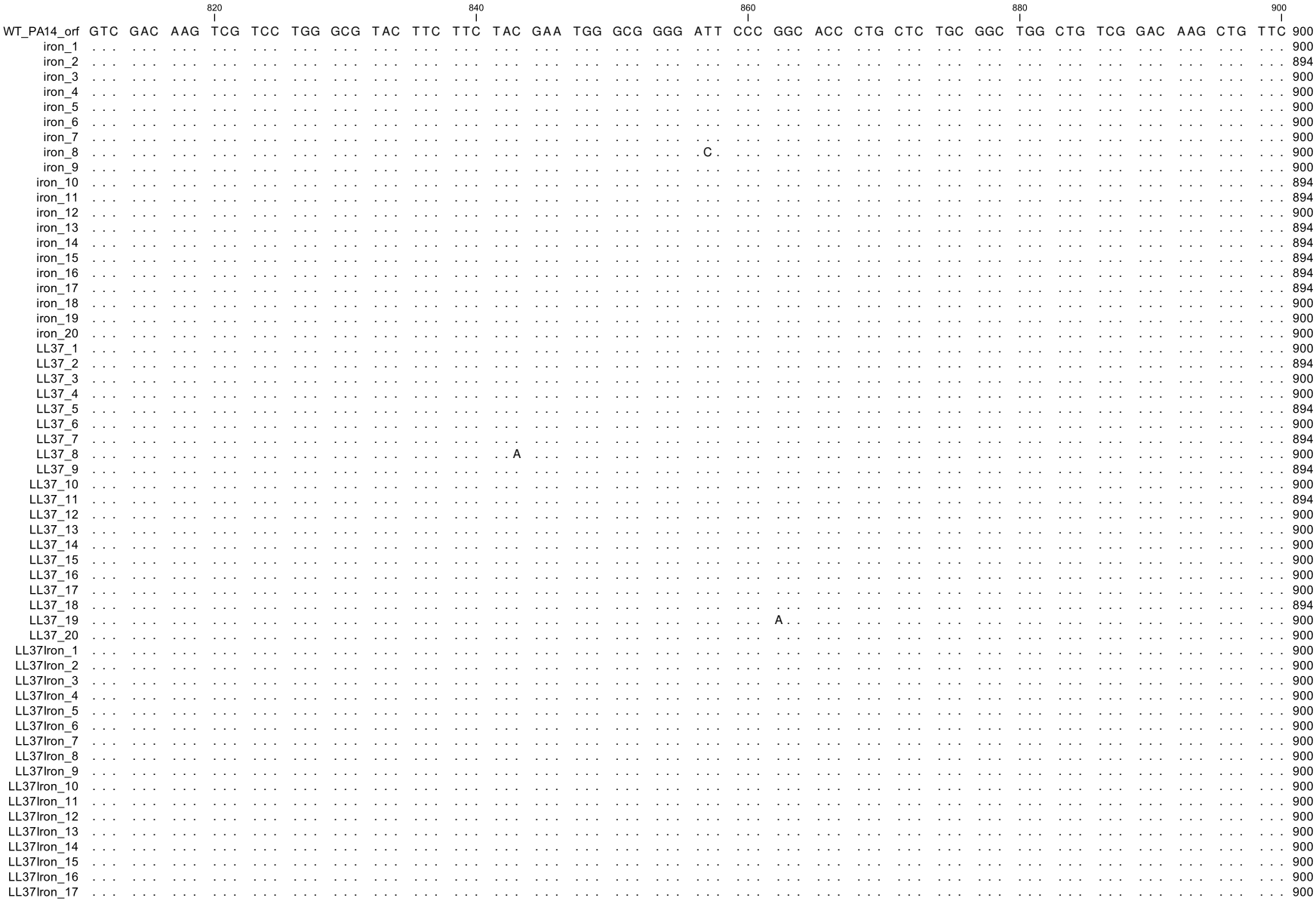
820
840
860
880
900
WT_PA14_orf GTC GAC AAG TCG TCC TGG GCG TAC TTC TTC TAC GAA TGG GCG GGG ATT CCC GGC ACC CTG CTC TGC GGC TGG CTG TCG GAC AAG CTG TTC 900
iron_1 900
iron_2 894
iron_3 900
iron_4 900
iron_5 900
iron_6 900
iron_7 900
iron_8 . . . . . C 900
iron_9 900
iron_10 894
iron_11 894
iron_12 900
iron_13 894
iron_14 894
iron_15 894
iron_16 894
iron_17 894
iron_18 900
iron_19 900
iron_20 900
LL37_1 900
LL37_2 894
LL37_3 900
LL37_4 900
LL37_5 894
LL37_6 900
LL37_7 894
LL37_8 . . . . . A 900
LL37_9 894
LL37_10 900
LL37_11 894
LL37_12 900
LL37_13 900
LL37_14 900
LL37_15 900
LL37_16 900
LL37_17 900
LL37_18 894
LL37_19 . . . . . A 900
LL37_20 900
LL37Iron_1 900
LL37Iron_2 900
LL37Iron_3 900
LL37Iron_4 900
LL37Iron_5 900
LL37Iron_6 900
LL37Iron_7 900
LL37Iron_8 900
LL37Iron_9 900
LL37Iron_10 900
LL37Iron_11 900
LL37Iron_12 900
LL37Iron_13 900
LL37Iron_14 900
LL37Iron_15 900
LL37Iron_16 900
LL37Iron_17 900

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LL37Iron_18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 899
LL37Iron_19 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 900
LL37Iron_20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 900
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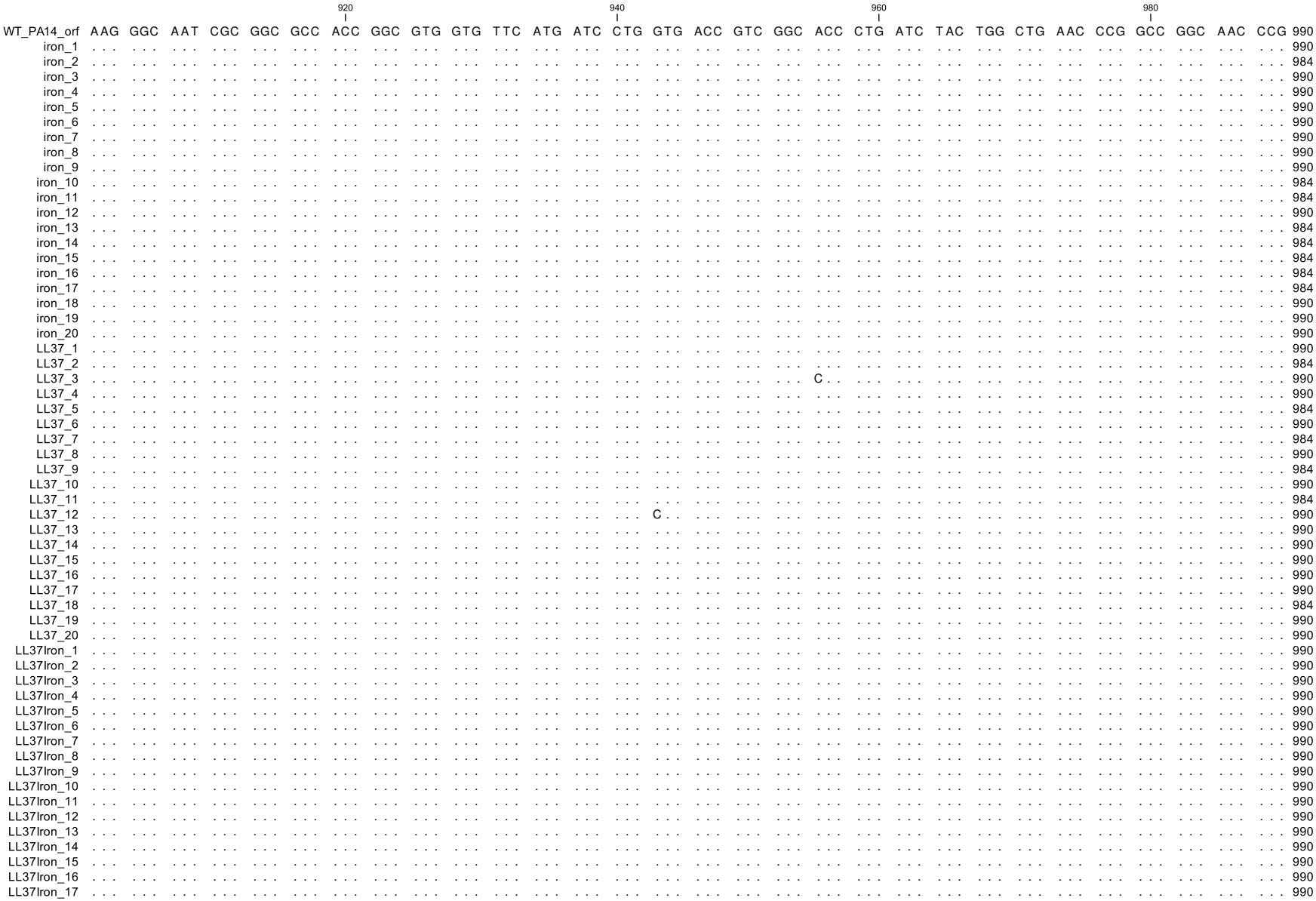
920
940
960
980
WT_PA14_orf AAG GGC AAT CGC GGC GCC ACC GGC GTG GTG TTC ATG ATC CTG GTG ACC GTC GGC ACC CTG ATC TAC TGG CTG AAC CCG GCC GGC AAC CCG 990
iron_1 990
iron_2 984
iron_3 990
iron_4 990
iron_5 990
iron_6 990
iron_7 990
iron_8 990
iron_9 990
iron_10 984
iron_11 984
iron_12 990
iron_13 984
iron_14 984
iron_15 984
iron_16 984
iron_17 984
iron_18 990
iron_19 990
iron_20 990
LL37_1 990
LL37_2 984
LL37_3 . . . . . C 990
LL37_4 990
LL37_5 984
LL37_6 990
LL37_7 984
LL37_8 990
LL37_9 984
LL37_10 990
LL37_11 984
LL37_12 . . . . . C 990
LL37_13 990
LL37_14 990
LL37_15 990
LL37_16 990
LL37_17 990
LL37_18 984
LL37_19 990
LL37_20 990
LL37Iron_1 990
LL37Iron_2 990
LL37Iron_3 990
LL37Iron_4 990
LL37Iron_5 990
LL37Iron_6 990
LL37Iron_7 990
LL37Iron_8 990
LL37Iron_9 990
LL37Iron_10 990
LL37Iron_11 990
LL37Iron_12 990
LL37Iron_13 990
LL37Iron_14 990
LL37Iron_15 990
LL37Iron_16 990
LL37Iron_17 990

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LL37Iron_18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 989
LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 990
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 990
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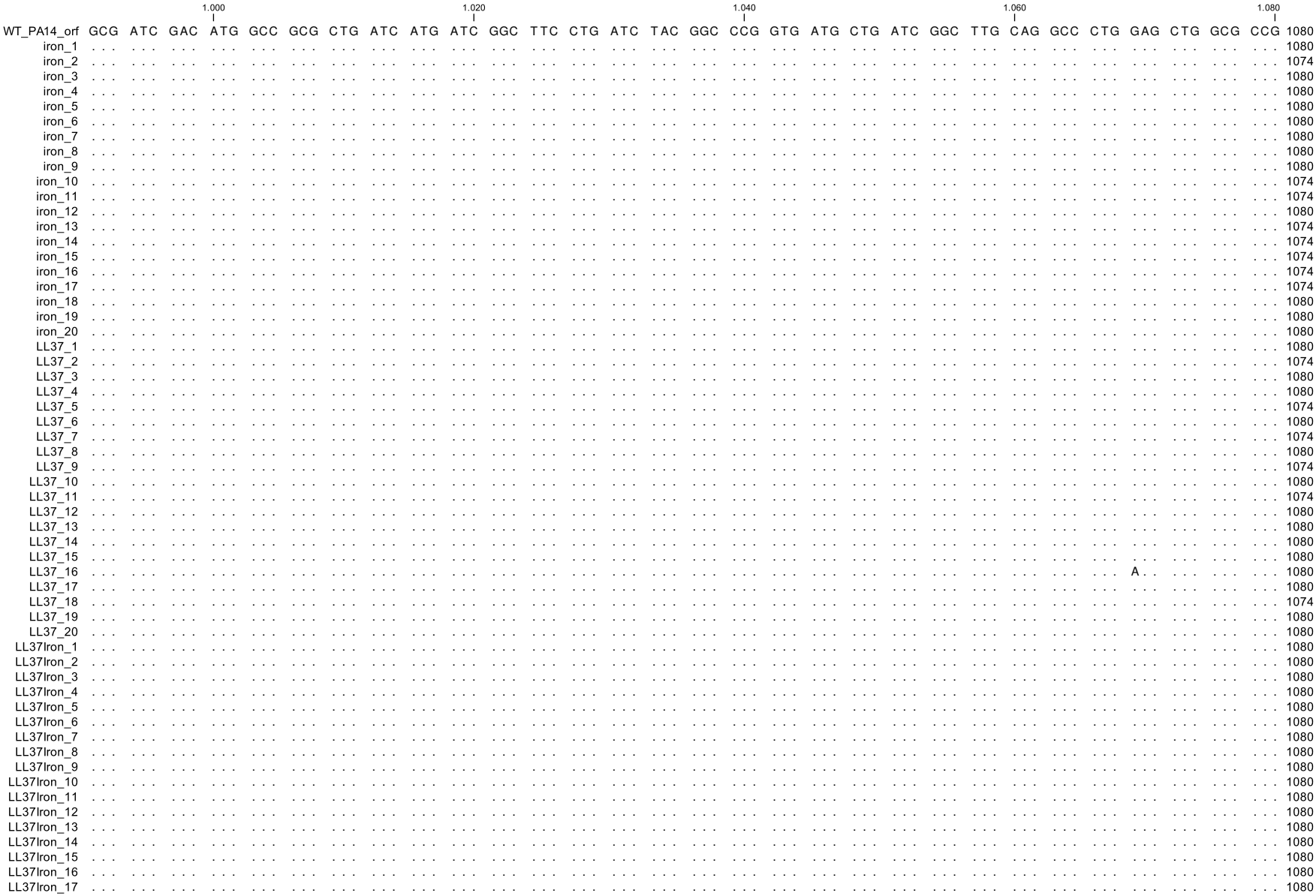
1.000
1.020
1.040
1.060
1.080
WT_PA14_orf GCG ATC GAC ATG GCC GCG CTG ATC ATG ATC GGC TTC CTG ATC TAC GGC CCG GTG ATG CTG ATC GGC TTG CAG GCC CTG GAG CTG GCG CCG 1080
iron_1 1080
iron_2 1074
iron_3 1080
iron_4 1080
iron_5 1080
iron_6 1080
iron_7 1080
iron_8 1080
iron_9 1080
iron_10 1074
iron_11 1074
iron_12 1080
iron_13 1074
iron_14 1074
iron_15 1074
iron_16 1074
iron_17 1074
iron_18 1080
iron_19 1080
iron_20 1080
LL37_1 1080
LL37_2 1074
LL37_3 1080
LL37_4 1080
LL37_5 1074
LL37_6 1080
LL37_7 1074
LL37_8 1080
LL37_9 1074
LL37_10 1080
LL37_11 1074
LL37_12 1080
LL37_13 1080
LL37_14 1080
LL37_15 1080
LL37_16 . . . . . A 1080
LL37_17 1080
LL37_18 1074
LL37_19 1080
LL37_20 1080
LL37Iron_1 1080
LL37Iron_2 1080
LL37Iron_3 1080
LL37Iron_4 1080
LL37Iron_5 1080
LL37Iron_6 1080
LL37Iron_7 1080
LL37Iron_8 1080
LL37Iron_9 1080
LL37Iron_10 1080
LL37Iron_11 1080
LL37Iron_12 1080
LL37Iron_13 1080
LL37Iron_14 1080
LL37Iron_15 1080
LL37Iron_16 1080
LL37Iron_17 1080

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LL37Iron_18 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 1079
LL37Iron_19 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 1080
LL37Iron_20 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 1080
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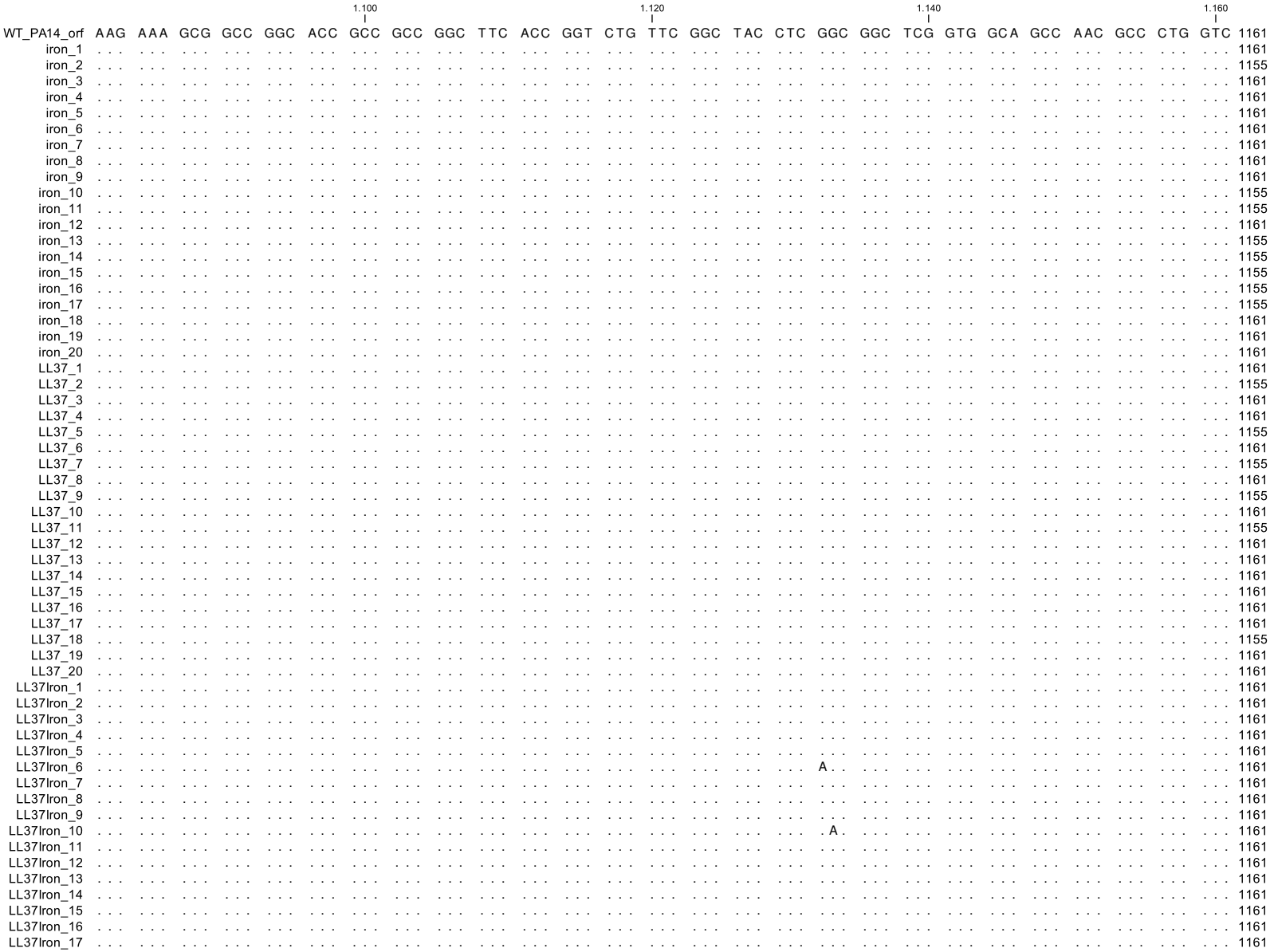
1.100
1.120
1.140
1.160
WT_PA14_orf AAG AAA GCG GCC GGC ACC GCC GCC GGC TTC ACC GGT CTG TTC GGC TAC CTC GGC GGC TCG GTG GCA GCC AAC GCC CTG GTC 1161
iron_1 1161
iron_2 1155
iron_3 1161
iron_4 1161
iron_5 1161
iron_6 1161
iron_7 1161
iron_8 1161
iron_9 1161
iron_10 1155
iron_11 1155
iron_12 1161
iron_13 1155
iron_14 1155
iron_15 1155
iron_16 1155
iron_17 1155
iron_18 1161
iron_19 1161
iron_20 1161
LL37_1 1161
LL37_2 1155
LL37_3 1161
LL37_4 1161
LL37_5 1155
LL37_6 1161
LL37_7 1155
LL37_8 1161
LL37_9 1155
LL37_10 1161
LL37_11 1155
LL37_12 1161
LL37_13 1161
LL37_14 1161
LL37_15 1161
LL37_16 1161
LL37_17 1161
LL37_18 1155
LL37_19 1161
LL37_20 1161
LL37Iron_1 1161
LL37Iron_2 1161
LL37Iron_3 1161
LL37Iron_4 1161
LL37Iron_5 1161
LL37Iron_6 . . . . . A 1161
LL37Iron_7 1161
LL37Iron_8 1161
LL37Iron_9 1161
LL37Iron_10 . . . . . A 1161
LL37Iron_11 1161
LL37Iron_12 1161
LL37Iron_13 1161
LL37Iron_14 1161
LL37Iron_15 1161
LL37Iron_16 1161
LL37Iron_17 1161

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LL37Iron_18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 1160
LL37Iron_19  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... 1161
LL37Iron_20  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 1161
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