Title: Coding triplets in the transfer RNA arm and their role in present and past tRNA recognition

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Authors contributions:

IA came up with the research idea and did the biological analysis. TM, IF, and AM defined the conserving AA and performed the statistical analyses. IA defined the main method and TM defined the benchmarking test. IA wrote the manuscript with the help of TM.

Abstract

Background

The evolutionary pathway of tRNA established the linking of the genetic code and protein synthesis, thus, revealing the characteristics of the prebiotic tRNA is crucial for presenting a feasible scenario for the emergence of life.

Results

Analysis of bacterial tRNA sequences reveals that for nine amino acids, mostly those considered to be the most ancient ones, the acceptor-TΨC arm carries cognate coding triplets far beyond statistical expectation. The contemporary determinants for the recognition of tRNAs and minihelices by the cognate synthetases and by EF-Tu, are part of these conserved triplets. We also find a strong correlation between the extent of the coding triplet conservation and amino acids whose contemporary synthetases belong to class IIa.

Conclusions

Identification of conserved coding triplets within the tRNA acceptor-TΨC stem, along with the analysis of their distinctive layout, is suggestive of a continuous evolutionary path that could have led to the appearance of the early translation system.

Keywords: tRNA, tRNA aminoacylation, Origin of the genetic code, Translation, Origin of life, Synthetase

Background

tRNA is a critical component of the translation system due to its role in linking the genetic code with the synthesized protein. The modern tRNA is an L-shaped molecule. One end of the molecule is the universally conserved single stranded NCCA tail which carries the cognate amino

acid (aa), while at the other end, about 75Å away, the anticodon (AC) loop that fully characterizes the attached amino acid is located. The mature tRNA interacts with three key components of the translation system - the cognate aminoacyl-tRNA-synthetase (aaRS) that specifically aminoacylates it; the elongation factor (EF-Tu in bacteria) that accommodates the aa-tRNAs and carries them, in a ternary complex, to the ribosome; and the ribosome where the attached amino acid is incorporated into the growing polypeptide.

Recognition of aa-tRNA on the ribosome is accomplished via the interactions of the mRNA codon accommodated on the small subunit, with the AC loop. EF-Tu, which carries a variety of different tRNAs, recognizes each of them specifically [1]. The minimal fragment of tRNA that can interact efficiently with EF-Tu, consists of a 10 base-pair helix from the acceptor-TΨC stem, nucleotides 63-72, linked to the aa-3'end tail [1], from which nucleotides 63–67 form the contact interface [2].

Aminoacyl-tRNA-synthetase typically acquires the identity of the cognate amino acid from the anticodon stem-loop [3,4], from the discriminator base (N73) and from specific nucleotides in the acceptor stem of the tRNA, named identity determinants [5,6,7,8]. Minimal RNA substrates comprising 4-12 base-pairs, derived from the acceptor-TΨC stem of the tRNA, were shown to be specifically aminoacylated by their cognate synthetases [5,6,7,9,10,11], even though the main provider of identity information, i.e. in the AC loop, was absent. This non-AC aminoacylation process was proposed to be controlled by an “operational code” [6] that relates few nucleotides in the acceptor stem to specific amino acids. The major recognition determinants for this enzymatic process were found to lie in the first five base-pairs (nucleotides 68-72) of the acceptor stem [7,9,11]. They were proposed to be the remnants of an early mechanism of specifically aminoacylating proto-tRNAs, possibly associated with a second code [11,12], distinct from the nucleotide triplets of the standard genetic code [6,11].

The possible existence of non-enzymatic aminoacylation process, which could have preceded the enzymatic charging, is of significance because it could have provided aa-tRNAs for the synthesis of the initial coded peptides, prior to the appearance of the proto-synthetase. Such processes were demonstrated experimentally: Hairpins were aminoacylated with their cognate amino acids by reaction with aminoacyl-adenylates in the presence of a dipeptide, where the

specificity was likely to be provided by the codon at the 5'end [13]. In a different line of research, aminoacylation of tRNAs with their cognate amino acids, in the absence of the specific aminoacyl-tRNA synthetase and ATP, was reported to occur at high pressure of 6 kbar [14,15]. The aa-tRNAs obtained were shown to bind to ribosomes and participates in the synthesis of a polypeptide in vitro. Specificity was assumed to be encoded in a nucleotide sequence, probably of the amino acid acceptor stem.

Here we analyze sequence data from bacterial tRNA genes and report the occurrence, to large extent, of cognate coding triplets in the acceptor-T⍰C arm of the modern bacterial tRNAs belonging to nine amino acids (the "conserving" amino acids), and to a very high extent in six of these nine amino acids (the "robustly conserving" amino acids"): Ala, Asp, Gly, His, Pro and Ser. High correlation is found between the 9 conserving amino acids and the amino acids believed to be among the most ancient ones [16,17]. High correlation is also found between the robustly conserving amino acids and the amino acids that are charged by class IIa synthetases [23].

The highly conserved coding triplets of six amino acids, which accommodate the identity determinants for the recognition of minihelices and tRNAs by their cognate synthetases and by EF-Tu, are proposed to be the precursors of the modern recognition determinants. Their possible involvement in a non-enzymatic aminoacylation of proto-tRNAs, which could have preceded the enzymatic process, is suggestive of an evolutionary scheme that resolves the chicken and egg paradox concerned with the appearance of translation, where a synthetase is required for charging tRNAs, but charged tRNAs are required for the formation of synthetases [18]. The conserved coding triplets, assumed to have a prebiotic origin, constitute a subset of the contemporary codon set, suggesting the significance of this subset of the standard codon table in the emergence of life as we know it.

Results

In bacterial tRNAs, the sequence of the string preceding the 3'end (pre-3'end), ranging from nucleotide 63 to 72 is diverse [19] even among different strains of the same bacterium (Tab. 1). Statistical analysis of these strings was performed on sequences of the pre-3'end strings, acquired from the tRNAdb database[20,21], http://trna.bioinf.uni-leipzig.de which holds 128

bacterial tRNA sequences obtained from elongator-tRNAs involved in protein synthesis, and over 6200 bacterial tRNA genes, belonging to the 20 canonical amino acids. To avoid the inclusion of pseudo-tRNA genes, i.e. those not coding for elongator-tRNAs, only tRNA sequences having a full CCA 3'end and a consensus discriminator base [8] (N73) were incorporated into the statistics (Materials and Methods), retaining only 60% of the original sequences.

When read from the 5' to the 3', this string was found to hold excessive codon and/or anticodon triplets of its cognate amino acid, termed here C/ACs, in the tRNAs of nine amino acids (Tab. 1, 2, Supplemental Tab. S1a and Tab. S1b, and Supplemental DataSet S1). One class of bacteria, the Mollicutes, deviated from the statistical behavior exhibited by the other bacteria (Tab. 1, Supplemental Tab. S2, Supplemental DataSet S1), and its exclusion resulted in a sample of 3324 tRNA gene-sequences from over 100 bacterial species. Statistical analysis that took into account the 350 tRNA-genes of Mollicutes did not differ significantly from the results obtained with the data sample used in the current study.

Table 1. Pre-3'end sequences

Amino acid (codons/anti-codons)	Pre-3'end sequence (nucleotides 63-72)**	Organism	Code in tRNAdb*
Ala (Conserved codons-GCC,GCU)	GCUAGGCUCC	*Bacillus subtilis*	tdbR00000007
	GCUUAGCUCC	*Escherichia coli*	tdbR00000008
	GCAUAGCUCC	*Escherichia coli*	tdbR00000010
	GUUAGGGUCC	*Mycoplasma capricolum*	tdbR00000005
Pro (Conserved codon-CCG)	UGUCUUCCCG	*Bacillus subtilis*	tdbR00000316
	UCUAUCACCG	*Escherichia coli*	tdbR00000623
	UCUCGUGCCG	*Salmonella typhimurium*	tdbR00000318
	UGUCUUCCCG	*Mycoplasma mycoides*	tdbR00000413
Gly (Conserved Anticodons-UCC, CCC)	CAUCACCCGC	*Bacillus subtilis*	tdbR00000116
	CUUCGCCCGC	*Escherichia coli*	tdbR00000117
	CGUUUCCCGC	*Escherichia coli*	tdbR00000118
	CGCUGCCCGC	*Salmonella typhimurium*	tdbR00000121
	UAUCUUCCGC	*Staphylococcus_epidermidis*	tdbR00000114
	CGUCACCCGC	*Streptomyces_coelicolor_A3*	tdbR00000113
	CUUCACCUGC	*Mycoplasma capricolum*	tdbR00000111

* All the unique sequences existing for the elongator-tRNAs of Ala, Pro and Gly, as found in the tRNAdb database [20,21].

**The conserved codons are framed and the anticodons underlined. In $tRNA^{Ala}$ the location of the conserved coding triplets varies, in $tRNA^{Pro}$ (codon) and in $tRNA^{Gly}$ (anticodon) it keeps a fixed position. The Mycoplasma (genus of the Mollicutes class) sequences, at the bottom of each box, often do not comply with the other bacteria.

The occurrence of the cognate codons and anticodons in the pre-3'end strings of the 20 canonical amino acids was determined. Two distinct groups were observed with a gap containing no amino acids at all between 40% and 85%, as follows: the pre-3'end strings of the constituents of the first group, i.e. of Ala, Arg, Asp, Gly, His, Leu, Pro, Ser and Val, were found to contain cognate C/ACs in over 85% of their strings. These amino acids were denoted “conserving amino acids” (Materials and Methods). In the remaining 11 "non-conserving amino acids", less than 40% of their pre-3'end strings carried a cognate C/AC. The highly occurring C/ACs for the amino acids of the first group typically constitute only a subset of the standard codons and anticodons table of each amino acid and these C/ACs form its ”conserved C/ACs set” (Materials and methods).

For each amino acid, the observed occurrence of a signal was compared to the expected occurrence of that signal: for the conserving amino acids, the occurrences of the conserved set of C/AC were checked, and for the non-conserving amino acids the occurrences of all C/AC were checked (since there is no conserving set for those amino acids).

The expected occurrences were obtained from randomized 10-mer oligonucleotides simulation. The construction of the randomized sequences and the estimation of the expected occurrence of the full set and of a specific set of C/ACs are described in the Materials and Methods section. The randomized strings were constructed with three nucleotide distributions: 1. Distribution identical to the average found in all the bacterial pre-3'end strings analyzed (“sample” distribution), i.e.: A=10%, C=46%, G=24%, T=20%; 2. Equal distribution of the four nucleotides; 3. The individual nucleotide distribution found in the pre-3'end string of each amino acid (Supplemental Tab. S1 ; to be split in a later version into S1a , S1b).

Analysis of the observed vs. expected C/AC occurrences supported the dichotomy between the two distinct groups. The conserved cognate C/AC of the nine conserving amino acids (the first

group) are present (Materials and Methods) in more than 80% of the pre-3'end strings. This observed occurrence is found to be far beyond the statistical expectation (P<<0.01), as obtained with all three distributions (Tab. 2, Supplemental Tab. S1a). In contrast, the pre-3'end strings of the non-conserving amino acids (the second group) exhibit the (full list of) cognate C/ACs (Materials and Methods) in less than 40% of their sequences, which, for Gln, Glu, Lys, Met,Thr and Tyr is less than statistically expected and for the remaining five amino acids it practically accords with the statistical expectations (Supplemental Tab. S1b). Interestingly, the cognate C/ACs conserved in the pre-3'end strings is often not correlated with the anticodon triplet in the AC loop (Supplemental DataSet S1).

Table 2: Statistics of the C/ACs occurrence in the pre-3'end of bacterial tRNA genes

Amino acid (no. sequences)	Conserved C/ACs set*		Observed Occurrence **	Expected occurrence *** (±0.8%)	*P*
Conserving amino acids	codons	anticodons			
Ala (209)	**GCU**, GCC		83 %	50 %	E-22
Asp (110)		**GUC**	96 %	17 %	E-106
Gly (243)		**CCC,UCC**	94 %	57 %	E-31
His (83)	**CAC, CAU**		90 %	22 %	E-51
Pro (180)	**CCG**		98 %	37 %	E-64
Ser (290)	**UCA,UCC,UCU**		99 %	46 %	E-72
Arg (305)	CGN, AGG		92 %	68 %	E-20
Leu (366)	CUN		81 %	57 %	E-20
Val (201)	GUN	NAC	88 %	58 %	E-17

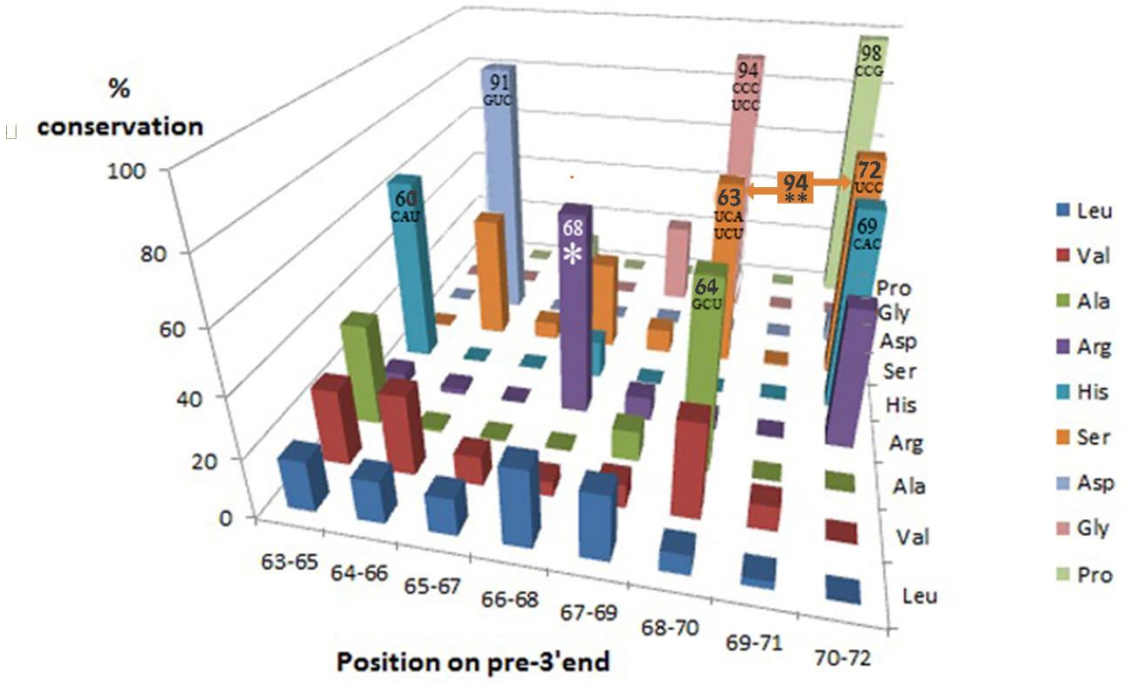
***** Cognate triplets whose conservation is considered as robust (Materials and Methods) are depicted by bold letters, along with their corresponding amino acids.

** % of pre-3'end strings holding at least one C/AC from their conserved set at any location along the string.

*** Derived from random oligonucleotides simulation with "sample" distribution (A,C,G,U)=(10%,46%,24%,20%). The statistics of the two other distributions and for sample distribution calculated for the remaining 11 amino acids is given in Supplemental Tab. S1 (to be split in a later version into Tab S1a, and Tab. S1b.

N- any nucleotide. *P*- the probability that the observed occurrence of a particular C/AC set is random. P ≤ 0.01 is considered statistically significant.

Figure 1: Occurrence of the conserved codons and/or anticodons in the pre-3'end string of the conserving amino acids.



The column of each "C/AC conservation site" is marked by the identity of its conserved triplets and by the percentages of sequences carrying these triplets in the site.

*- Arg codons CGA, CGC CGG, CGT and AGG.

**- The overall occurrence of the conserved C/ACs of Ser in its two partially overlapping conservation sites (positions 68-70 and 70-72).

To further benchmark these statistical results, the occurrence of the triplet CCC, which is expected to be the most prevalent in 14 out of 20 amino acids due to the excess of C in their sequences, was examined. Strings from the conserving amino acids display CCC considerably less than inferred from their nucleotide distribution and surprisingly, Ala, His and Asp, hardly show any CCC triplets in their strings (Supplemental Fig. S1). Conversely, amino acids whose

tRNAs display C/ACs significantly less than statistically expected (Gln, Tyr, Lys) exhibit the most significant access of CCC.

The C/ACs in the pre-3'end string of the conserving amino acids may appear as a single site which is fully conserved although the rest of the sequence varies (Pro, Tab. 1, Fig. 1) or as an accumulation of occurrences from as many as seven different sites (Leu, Val, Fig. 1). The conserved C/ACs of Ala, Asp, Gly, His, Pro and Ser are concentrated in one or two sites, each holding one or two coding triplets. In contrast, the overall high occurrence of the conserved C/ACs of Leu, Val and Arg is the sum of small occurrences scattered throughout their pre-3'end strings (Leu, Val) or of five different codons largely concentrated in a single position (Arg).

Components of a conserved set are considered as "robustly conserved" when they appear, alone or with an additional coding triplet, in a specific location in more than 60% of the pre-3'end strings belonging to a certain amino acid. This location is termed a "C/AC conservation site". Overall the occurrence histogram (Fig. 1) contains eight such sites: one for Pro (positions 70-72, 98% occurrence of the codon CCG), Gly (positions 68-70, 94% occurrence of the anticodons CCC or UCC), Asp (positions 64-66, 91% occurrence of the anticodon GUC), Ala (positions 68-70, 64% occurrence of the codon GCU. In the remaining sequences only C69 and U70 are conserved), and two for Ser (in two partially overlapping positions; 68-70, 63% occurrence of the codons UCU or UCA and positions 70-72, 72% occurrence of UCC, which combine to 94% occurrence of a conserved codon at positions 68-72) and His (positions 63-65, 60% occurrence of the codon CAU. In the remaining sequences only C63 and U65 are conserved; Positions 70-72, 69% occurrence of the codon CAC. In the remaining sequences only C70 and C72 are conserved).

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To benchmark the choice of robustly conserving amino acids [see Materials and Methods], the triplet-occurrences per each 3 mer location was counted in the pre-3'end strings of each amino acid (data regarding highly appearing triplets, both coding and non-coding, appears in DataSet S4), without relating to the nucleotide distribution. Assuming that an occurrence of a cognate coding triplet in the majority of the strings implies high conservation we define in this alternative method such C/AC as firmly conserved (to distinguish from robustly conserved C/AC). We then call "firmly conserving" any amino acid having firmly conserved C/AC. We obtain that the same six robustly conserving amino acids (Tab. 2) are also firmly conserving, while all other 14 AAs are neither firmly conserving nor robustly conserving.

While the conserving amino acids in this alternative method are defined exactly as in the main method, the conserved C/AC per each conserving amino acid is defined, for the benchmarking test, simply as any C/AC appearing in a specific location more in more than 20% of the strings. We note that the conserving set is then unique, that Leu has no C/AC in its (empty) conserving set, and that the threshold has been chosen such that the conserved set for any of the non-conserving amino acids is also empty. High conservation is then defined to be at the same location in the majority of the strings. Any C/AC having such a high conservation is named firmly conserved, and any amino acids having a firmly conserved C/AC is named firmly conserving amino acid.

Table 3 compares the resulting conserved sets and robustly versus firmly conserved sets, for the two methods. Let us observe, for example, Gly, Val and Ser in Table 3. For Gly, the third most appearing triplet is a coding one (CCC), found in 78% of the strings, at site 68-70, and UCC is not firmly conserved according to the alternative method. For Val, the most appearing coding triplet is GUC yet 6 non-coding triplets appear more times than GUC, and three coding triplets are conserved according to the main method while not conserved according to the alternative method. The most striking difference is for Ser, having UCA as robustly conserved according to the main method, and not conserved at all using the alternative method - as can be seen in additional file 2, DataSet S4, UCA (namely TCA in the dataset) appears only in 17% of the sequences, namely slightly below the threshold of 20%.

TABLE 3: The list obtained using the alternative assignment method

	Main method	Alternative method			
	Conserved set	Conserved set*	Percentage of appearance**	The relevant site	"Relevant" non-coding triplets***
Ala	**GCU**, GCC	**GCU** [AGC,GGC]	64%	68-70	2
Asp	**GUC**	**GUC** [GAC]	91%	64-66	0
Gly	**CCC,UCC**	**CCC** [UCC,ACC,GCC]	78%	68-70	2
His	**CAC,CAU**	**CAC** [**CAU**]	69%	70-72	0
Pro	**CCG**	**CCG** [CCC]	98%	70-72	0
Ser	**UCA,UCC,UCU**	**UCC** [UCU]	74%	70-72	0
Arg	CGN,AGG	CGG [GCG,CGC,UCG,CCG]	49%	66-68	1
Val	GUN	GUC	23%	63-65	6
Leu	CUN	CUU (ought to be deleted from list)	14%	66-68	11

This table compares the Results of the main method and the alternative method. In bold we note the robustly/firmly conserved coding triplets, and the robustly/firmly conserving aa's.

**For the Conserved C/AC set, out of parenthesis appears the most conserved C/AC. As in Table 2, robustly/firmly conserved aa's and coding triplets are in bold-face. Inside parenthesis there is no repetition in case a coding triplet is conserved more than once (appearing in different sites).*

***Percentage of appearance stands for the percentage of 10-mer strings containing the most appearing coding triplet (per its site), while that relevant site is presented in the next column.*

****The last column presents the number of non-coding triplets that are more common than the most common coding triplet.*

It is important to note that in both methods, setting the threshold anywhere between 50% and 60% has no influence, yet a slight lowering of that threshold to be below 50% adds Arg to the list of robustly/firmly conserved aa's.

Five out of the six robustly/firmly conserving amino acids, i.e., Ala, Gly, His, Pro and Ser, have synthetases that belong to class IIa. We tested statistically the correlation between the list of amino acids that robustly/firmly conserve the C/ACs (Ala, Asp, Gly, His, Pro and Ser) and those whose synthetases belong to class IIa (Ala, Gly, His, Pro, Ser and Thr (ref)). The probability to obtain m=5 (or more, namely m=6) common amino acids between two lists of n1=n2=6 amino acids chosen randomly out of pile of n=20 amino acids, is 0.002 (Materials and Methods), indicating that the null hypothesis of no correlation between the C/AC conservation and belonging to class IIa is wrong.

We note that if Arg is added so the list of firmly conserving amino acids , as would be the case if threshold is slightly lowered (say to 49%), then both Asp and Arg are not in the list of class IIa synthetase; this change then leads to a probability P=~0.00722, still quite significant as it is below the conventional threshold (for P-value for example) of 0.01.

To provide a proof of correlations between conservation and robust/firm conservation, and the antiquity of aa's is more subtle, as there is no agreed list of antiquity. Yet, Gly, Ala, Asp, Val, Pro and Ser are commonly considered among the most antique aa's [16,17].

The genomic tRNA sequences of archaea and eukarya were not subjected to the aforementioned analysis due to the difficulty to distinguish pseudo-tRNA gene-sequences, which are prevalent in the database (Sprinzl, M., private communication), from the authentic ones. The 3'end tail – NCCA – used for screening the bacterial data, which excluded 40% of the bacterial sequences, appears only in few archaeal tRNA-genes and is absent in eukaryotes. The more reliable elongator-tRNA sequences are too scarce to ensure any statistical validity. Part of the 63 unique archaeal and 117 unique eukaryotic sequences, belonging to the tRNAs of the 20

amino acids, do display access of cognate C/ACs in their pre-3'end strings (additional file 2 DataSets S2, S3), mostly for the same amino acids classified as conserving in bacteria. However, the position and identity of these cognate triplets differ from bacteria, leaving open the question of whether the cognate C/ACs are conserved in the other realms of life besides bacteria.

Discussion

In the discussion we focus on the results of the main method.

1. Coding triplets in the acceptor-TΨC stem

In total, over 90% of the bacterial pre-3'end strings from the conserving amino acids contain a conserved codon and/or anticodon triplet (Tab. 2), although the data probably includes unidentified pseudo-tRNA genes randomly carrying the consensus discriminator base, and possibly sequences from additional species besides the Mollicutes, whose tRNAs do not comply with the bacterial pattern of C/ACs conservation. Moreover, tRNAs from the three most populated bacterial genera in the data, i.e. *Bacillus*, *E. coli*, and *Salmonella* (comprising 20% of the data; 659 sequences altogether), having varying phylogeny, exhibit complete conservation (99% occurrence) of the cognate C/ACs in the pre-3'end strings of the conserving amino acids (additional file 1 Tab. S2). Coding triplets, which are complementary to the conserved C/ACs presented here, were previously identified at positions 3-5 of prokaryotic tRNAs from four of the conserving amino acids, i.e. ala, Asp, Gly and Val [22]. Collectively, these observations point to a highly non-random occurrence of conserved C/ACs, distributed along the pre-3'end strings of the conserving amino acid.

Nucleotide C is the most abundant (53%) of the nucleotides constituting the 10 robustly-conserved C/ACs (Tab. 2). The conservation is therefore facilitated by the elevated occurrence of nucleotide C (46%) and reduced occurrence of A (10%) in the bacterial pre-3'end sequences. Yet, the C/ACs occurrence in the pre-3'end strings of the nine conserving amino acids is of consequence with regard to both the expectation values derived from the randomized oligonucleotides constructed with the C-rich and A-poor "sample" distribution (Tab. 2) and with

the individual nucleotide distributions (additional file 1 Tab. S1). Thus, the conservation of the cognate codons and/or anticodons in the pre-3'end string is a genuine phenomenon and not an artifact of the nucleotide distribution. Correspondingly, the triplet CCC, which is expected to be highly frequent due to the significant access of C nucleotides in the pre-3'end of 14 amino acids, is nearly absent in the sequences of the robustly conserving amino acids (additional file 1 Fig. S1), being probably correlated with the marked occurrence of the conserved C/ACs.

For the six robustly conserving amino acids, i.e. for Ala, Asp, Gly, His, Pro and Ser, the conserved C/ACs are mainly located in the C/AC conservation sites (Fig. 1), by definition. All of their tRNAs are charged by class IIa synthetases [23], except from $tRNA^{Asp}$ which is charged by class IIb synthetase. Similarly, all of them except $tRNA^{Asp}$, exhibit conservation sites in the range 68-72, which forms a contact interface with the synthetase. In the data of Gly, Pro and Ser the conservation sites show essentially full occupancy (94-98% occurrence), but for Ala and His, although a large majority of their pre-3'end sequences contain a coding triplet (83% and 90%, respectively), only about 2/3 of the sequences have a full codon located at their conservation sites (Fig. 1). In the remaining third of the data, only two out of the three coding letters are fully conserved at the conservation sites, possibly reflecting the involvement of distinct nucleotides in the contemporary aminoacylation process, either directly as identity determinants, or indirectly by providing a certain sequence contexts essential for the tRNA recognition [6].

At the other half of the pre-3'end string, nucleotides 63–67 from the TΨC-acceptor stem establish the contact interface with EF-Tu [2]. The C/AC conservation sites in that range, i.e. the Asp codon GUC at positions 64-66, which is conserved in 91% of the sequences and the His codon CAU at positions 63-65, which is conserved in 60% of the sequences (Fig. 1), are suggested to contribute to the binding affinity for EF-Tu. In particular, EF-Tu was shown to use the $tRNA^{Asp}$ base-pairs G51:C63, C50:**G64** and G49:**U65** for tightly binding it [24], where **G64** and **U65** are included in the conserved Asp codon GUC. For His, only C63 and U65 are completely conserved, which suggests, analogously to synthetase recognition scheme, that the present role in the recognition of tRNA by EF-Tu is likely to determine the degree of nucleotide conservation.

The C/ACs conserved in tRNAs of the non-robustly conserving amino acids Arg, Leu and Val, which are charged by class I synthetases, seem to lack a clear evolutionary role, as far as our

results shows: The conserved C/ACs of Leu and Val are dispersed throughout the pre-3'end string (Fig. 1) rather than holding specific sites, as would be expected from functional C/ACs. 68% of the sequences of the pre-3'end from $tRNA^{Arg}$ contain a conserved codon at positions 66-68, but this site is unlikely to serve as an identity determinant, because the elevated presence of conserved C/ACs is the sum of occurrences of four different codons, and because its location partially overlaps the ranges of tRNA interaction with EF-Tu and with the synthetase. Correspondingly, the level of C/ACs conservation in the 3'end strings of these three amino acids is somewhat lower compared to the six robustly-conserving amino acids (Tab. 2), and the number of conserved C/ACs per amino acid is larger (four, five and eight for Leu, Arg and Val, respectively, compared to one-three for the robustly conserving amino acids). These statistical observations may point to a gradual process by which, in the absence of contemporary function, the conserved C/ACs were shifted throughout the acceptor-TΨC stem, while random mutations supplemented the list of conserved C/ACs and lowered the overall level of conservation.

Interestingly, eight out of the nine conserving amino acids, i.e. - all except His, are considered to be among the ten earliest amino acids to emerge in the prebiotic world, according to consensus chronology of amino acids built on 60 criteria [16] and to the Miller-Urey experiment [17]. In contrast, amino acids whose pre-3'end string is devoid from the cognate triplets or exhibit statistically-expected occurrence, are considered to be rather late arrivals.

2. Implications of the common properties of Ala, His, Gly, Ser and Pro

Five robustly-conserving amino acids, i.e. Ala, Gly, His, Pro and Ser, possess common properties:

1. Their synthetases are classified as belonging to class IIa on the basis of their active site architecture [23].

2. Their synthetases were shown to be the most efficient in aminoacylating minihelices [25], except from Pro where relevant data is currently unavailable.

3. All these amino acids, except for His, are believed to be among the most ancient ones [16,17].

4. Their tRNAs exhibit C/AC conservation sites in the range 68-72, which forms a contact interface with the synthetase.

5. Nearly all the identity determinants for the synthetase recognition, present in the 3' side of minihelices derived from the acceptor stem of these five tRNAs, are contained in the conserved triplets (Tab. 4).

Table 4. Synthetase recognition determinants vs. conserved C/ACs

Amino acid		**Ala**	**Gly**	**Ser**		**His**	**Pro**
C/AC conservation sites in the 68-72 range	Positions	68-70	68-70	68-70	70-72	70-72	70-72
	Conserved C/ACs*	GC**U**	CC**C**/UC**C**	**UCU/UCA**	**UCC**	**CA**C	**C**C**G****
Recognition determinants for minimal substrate aminoacylation*		G3**·U70** [5]	G3:**C70,** C2:G71† [30]	(C/**U)68**, (**C**/U)**69**, (**A/U**)**70** [9]	(A/**U)70 C71, C72** [9]	**C70, A71** [30]	No data

**In bold - nucleotides from the conserved coding-triplets serving as identity determinants.*

*** These identity determinants refer to the recognition of full tRNAs [27].*

† The identity determinant for aminoacylating minihelices of Gly, C2:G71, are not part of the conserved coding triplet.

These apparently unrelated properties may all be correlated. Properties 1 and 2, i.e. having class IIa synthetases which perform non-AC aminoacylation efficiently, can be linked by suggesting that the efficiency stems from the distinct recognition mode carried out by bacterial class IIa synthetases, which interact with the tRNA acceptor stem in a base-specific fashion via their characteristic longer motif 2 loop [26]. Biochemical studies [27] revealed interactions of this loop with G72 of bacterial $tRNA^{Pro}$, while the X-ray structure of a complex of $tRNA^{Ser}$ with SerRS, showed the loop interactions with U68 and C69 [28], establishing the loop ability to scan the entire 68-72 region. Linking the non-AC aminoacylation efficiency with this distinctive recognition mode is consistent with the lack of C/AC conservation in the pre-3'end strings of the sixth amino acid charged by a class IIa synthetase, Thr (additional file 1 Tab. S1). In bacterial ThrRS, motif 2 loop contacts only the CCA end while the stem identity determinants are

recognized by the N terminal domain of the enzyme [29], a functional exception that is likely to account for the lack of C/AC conservation. Interestingly, Thr is listed among the ten amino acids considered to be the most ancient ones [16,17].

The contemporary non-AC aminoacylation, which is efficiently carried out by class IIa synthetases, was suggested to be a relic from the primordial aminoacylation process [6,12,31] and consistently, Ala, Gly, Ser, and Pro are considered to be among the earliest amino acids [16,17]. This correlation may be explained by proposing, in accord with previous suggestions, that the earliest synthetase that catalyzed the primordial enzymatic charging was the ancestor of class II synthetases [10], or particularly of class IIa synthetases [32].

2.1 Possible implications on the origin of the genetic code

Generating a primordial synthetase, which could have utilized the identity determinants for aminoacylating tRNAs via the ancient non-AC aminoacylation process, would have required a primitive translation system relying on aminoacylated proto-tRNAs, thus presenting a chicken and egg paradox [18]. However, attributing the conserved coding-triplets to a non-enzymatic aminoacylation process that preceded the appearance of a synthetase seems to resolve the dilemma, as the non-enzymatically charged aa-tRNAs could have been engaged in the prebiotic synthesis of the proto-synthetase. A non-enzymatic charging process could have possibly relied on direct interaction between an amino acid and its cognate coding triplet, i.e. on the stereochemical interaction [33] which suggests specific affinity between amino acids and their cognate coding triplets. Alternatively, the acceptor stems can perhaps acquire subtly-distinct conformations, which are particularly suited for accommodating the cognate amino acid [14,15,34]. Such a specific conformation was possibly induced by the presence of a coding triplet in the sequence. According to both mechanisms, an amino acid would reside on the acceptor stem and its proximity to the 3'end would facilitate the tRNA charging.

The existence of stereochemical affinity between amino acids and their coding triplets is considered non-conclusive at best [6,34], yet, the possible relevance of this idea seems to be supported by an experimental demonstration of non-enzymatic aminoacylation. The specific charging of C4N hairpins, which was catalyzed by di-peptides that were unlikely to provide the specificity, is attributed to the interaction of the amino acid with the cognate codon at the 5'end

[13], pointing to the possible existence of sequence-specific affinity. The specificity reported in the self-aminoacylation of tRNAs, which occurred under high pressure in the absence of a synthetase or ATP, is proposed to be encoded in a nucleotide sequence, probably of the amino acid stem [14,15]. This process was demonstrated for $tRNA^{Phe}$ from *E. coli* and yeast. In accord with the two mechanisms proposed for the specific accommodation of amino acids on the acceptor stem, the two unique sequences of elongator-tRNAs and genomic tRNAs from yeast, found in the tRNAdb (additional file 2 DataSet S3), include the Phe codon UUC at positions 68-70. However, the only sequence of $tRNA^{Phe}$ from *E. coli* (additional file 2 DataSet S1), does not display a cognate C/AC of Phe in its pre-3'end, possibly pointing to an alternative self-aminoacylation mechanism. The misfit between the processes implied by the yeast and *E. coli* aminoacylation, calls for further experimental studies of this non-enzymatic mechanism.

The dependence of the non-enzymatic aminoacylation, as well as of the non-AC enzymatic aminoacylation, on a particular combination of nucleotides located in the acceptor-stem, offers two possible scenarios concerned with the origin of the genetic code. Triplets of the standard genetic code could have originated, at least in part, from the acceptor-stem determinants for enzymatic aminoacylation by a proto-synthetase [6,11]; Or alternatively, coding triplets suggested here to be engaged in an earlier process of non-enzymatic aminoacylation, formed the basis for the current genetic code. In the latter case, the ensuing appearance of enzymatic aminoacylation would have retained, as identity determinants, only those nucleotides required for the recognition by the proto-synthetase. The second scenario seems more reasonable because, evolutionarily speaking, non-enzymatic processes are expected to precede the enzymatic ones. Moreover, the second scenario is supported by the partial conservation of the coding triplets observed in the acceptor stems of Ala and His, where two third of the sequences carry a full cognate codon, but one third conserves only two of the three coding letters. The possibility that random mutations could have supplemented the correct third letter and completed the codon triplet in the majority of Ala and His sequences appears less feasible than the alternative process, by which one letter from the initial triplet was abandoned due to the relaxation of functional requirements in the transition from non-enzymatic to enzymatic aminoacylation. A scenario, in which the identity determinants for non-AC aminoacylation were singled out from their former conserved C/ACs, links the conserved triplets and the identity determinants contained in them, i.e. properties 4 and 5, supporting the notion that the origin of

the conserved coding triplets and of the non-AC determinants, is likely to be prebiotic [22]. However, the “operational code” residing in the tRNA acceptor stem, which was associated with a second code distinct from the nucleotide triplets of the standard genetic code [6,11,12], consists primarily of elements from the current coding-triplets set, suggesting the singularity of a subset of the standard genetic code in the evolution of life as we know it.

3. Discussing the results of the alternative method

The alternative method, also named the benchmarking test, gave similar results when it comes to the list of robustly-firmly conserving aa's, hence benchmarking the main results of this paper. On the other hand, the list of conserved and robustly conserved coding triplets (per each conserving amino acid) is less similar; hence these notions might deserve more research in the future.

4. Proposed evolutionary path

Incorporating the insight gained from stem coding triplets into the vast experimental and theoretical data accumulated in the field of Origin of Life, may suggest a continuous evolutionary scenario: Long oligonucleotides, reaching over 100 mer, which were demonstrated, both experimentally and theoretically, to polymerize in solution under a thermal gradient [35], would have autonomously folded in the primordial environment into stable RNA molecules, mostly into hairpins consisted of a single or two helices [36]. Two matching hairpins were proposed to combine to form an L-shaped proto-tRNA carrying a coding triplet both in its AC loop and in the acceptor stem [11,37,38]. Self-aminoacylation, occurring either under high-pressure or via another yet unknown process, is suggested to have allowed certain hairpins, as well as L-shaped RNAs to be autonomously charged with their cognate amino acid.

Self-aminoacylation occurring under high pressure is a tempting possibility, because it matches up with the widespread belief that life started at hydrothermal vents [39], where elevated pressure is prevalent and where liquid under thermal gradient, that can induce the formation of long RNA chains [35], is found. It follows that attempts to reproduce prebiotic processes with contemporary chemistry may benefit from working under high pressure. In particular, it may prove useful to conduct the experimental search for the stereo-chemical effect under high

pressure conditions. RNA helices derived from the acceptor stems of Ala, Gly, His, Pro or Ser, which carry full codons and were suggested to accommodate amino acids prebiotically, may offer suitable binding sites for testing the specific affinity of amino acids for their coding triplets.

These self-assembled products of the prebiotic chemistry, i.e. charged hairpins and charged L-shaped RNAs could have been floating around in the prebiotic environment. Charged hairpins were assigned a possible role in prebiotic replication [40], while charged L-shaped RNAs could have started acting as proto-tRNAs with the appearance of a coding proto-ribosome. This ribozyme, that was capable of translating a code into a peptide, could have possibly evolved via the addition of an RNA element that accommodated the translated RNA, i.e. the initial version of the ribosomal small subunit, into an earlier proto-ribosome that catalyzed the association of random amino acids [41,42]. Translation of random RNA chains would produce random polypeptides, which could, in certain cases, possess weak catalytic capabilities, maybe yielding in such manner a simple proto-synthetase. When the earliest specific synthetase emerged, the conserved coding triplets at positions 68-72, which, until that stage are suggested to control the non-enzymatic aminoacylation, could have constituted the template for establishing the mutual recognition scheme between the proto-tRNAs and the primordial synthetases. The vestige of the primordial enzyme was linked with the catalytic core of the modern synthetase, while the AC binding domain would have ensued [6,10,31]. Charging of full tRNAs by this composite synthetase relies, in certain cases, on acceptor-stem determinants distinct from those of the non-AC aminoacylation [10], expanding the conserved part of the acceptor stem, as is the case with the conserved base-pairs C2:G71, G1:C72 of $tRNA^{Gly}$ [43]. The interaction of tRNA with EF-Tu would join when protein factors were integrated into the function of an elaborated ribosome, giving rise to higher efficiency and accuracy. The three key components of the protein-synthesis machinery, i.e. the tRNA, synthetase and EF-Tu, along with the ribosome, would have coevolved into the intricate contemporary translation system, still keeping traces of their prebiotic roots in the form of the conserved coding-triplets. The evolutionary path proposed for the coding triplets conserved in the tRNA acceptor stem, commencing from specificity control in a prebiotic non-enzymatic aminoacylation to the involvement in the specificity of the contemporary synthetase, outlines a continuous route from the prebiotic era to modern biology.

Conclusions

Our results detect highly-conserved cognate coding triplets in the acceptor-TΨC stem of the bacterial tRNAs of six amino acids, mostly those considered to be the earliest. These triplets allow to suggest a temporally order for principal events in the prebiotic emergence of translation. They are suggested to have been involved in an initial non-enzymatic aminoacylation process, a process whose existence can resolve the chicken and egg paradox concerned with the appearance of translation. The triplets are proposed to be the precursors of modern recognition determinants located the acceptor-TΨC arm, and the fact that they constitute a subset of the contemporary codon set, suggests the high significance of (this part of) the current set of codons in the emergence of life. The triplets also point at the ancestor of the class IIa synthetases as the earliest aminoacylating enzyme. Overall, the conserved coding triplets are suggestive of a coevolution scheme of the three major components of the protein-synthesis machinery, i.e. the tRNA, synthetase and EF-Tu, laying the ground for the emergence of coded translation.

Materials and Methods

Dataset construction:

Sequences of the 3' side of the acceptor-TΨC stem from the bacterial tRNAs of each of the 20 canonical amino acids, were obtained from the tRNAdb database [20,21], http://trna.bioinf.uni-leipzig.de. [Year for when the data has been collected will be added here]. The tRNAdb database holds over 6200 bacterial tRNA genes, belonging to the 20 canonical amino acids. To avoid the inclusion of pseudo-tRNA genes, i.e. those not coding for elongator-tRNAs, only tRNA sequences having a full CCA 3'end and a consensus discriminator base (N73) [8] were incorporated into the statistics, with one exception; The data for Glu contained similar amount of sequences with the canonical G73 and with the non-canonical A73, both groups displaying the same pattern of C/AC occurrence. In order to obtain as much data as possible, tRNAs with non-canonical A73 were incorporated.

The observed occurrence of the cognate codon and anticodon triplets was determined in 10 mer strings composed of nucleotides 63-72 (termed pre-3'end), by scanning the sequences. Nucleotides 61 and 62 were not included in the study, being conserved and semi-conserved, respectively. The scanning indicated that one class of bacteria, i.e. species from six genera of the Mollicutes class, which constituted about 9% of the initial data set, i.e. 350 gene-sequences, does not share the statistical behavior of the other bacteria (Tab. 1, Supplemental Tab. S2, Supplemental DataSet S1) and it was excluded. No other species in the data contained enough entries to be reliably recognized as diverging from the conservation pattern. Under these restrictions, only 60% of the original sequences were retained, comprising an initial sample of 3324 tRNA gene-sequences from over 100 bacterial species.

Conserving amino-acids and conserved sets assignment:

Amino acids for which the pre-3'end strings were found to contain cognate C/ACs in over 85% of those strings are denoted "conserving amino acids", while amino acids for which the pre-3'end strings were found to contain cognate C/ACs in less than 40% of those strings are denoted "non-conserving amino acids".

For the conserving amino acids, the minimal set of cognate triplets accounting for 95% of the sequences carrying codons and/or anticodons at any position along the 10-mer pre-3'end string (hence appearing in more than 80% of the strings), was assigned as the conserved set.

Robustly conserving amino-acids and robustly conserved sets assignment:

The robustly conserved C/ACs are the C/ACs that are located at specific sites in the pre-3'end string, occurring, alone or with a single additional coding triplet, in more than 60% of the sequences. Robustly conserving amino acids (Pro, Asp, Ala, His, Glu, Ser) are those whose pre-3'end string contains at least one robustly conserved C/AC.

Benchmark test:

The test counts the occurrences of all (coding and non-coding) triplets according to their appearance per 3-mer site within the 10-mer pre-3'end string. The conserving amino acids are defined exactly as in the main method (namely, above 85% therefore above the empty gap that exists in the range 40-85%).

Any coding triplet appearing alone in a specific location, in the majority of the strings, is defined as "firmly conserved". Accordingly, amino acids having a firmly conserved C/AC are defined as firmly conserving amino acid. Any coding triplet appearing alone in a specific location, in more than 20% of the strings (but not in the majority of them), is defined as "conserved"; the threshold of 20% has been chosen such that the conserved set for any of the non-conserving amino acids is empty. We note that there is no possible choice of threshold that will make the conserved set for any of the non-conserving amino acids empty, while the conserving amino acids will all have non-empty sets.

Calculation of expected occurrences:

The expected occurrence of the coding triplets in the pre-3'end string was determined by analyzing their occurrence in simulated 10-mer randomized oligonucleotides. A pool of 10^5 randomized strings was generated using MATLAB and Statistics Toolbox (Release 2012b, The MathWorks, Inc., Natick, Massachusetts)**,** by producing a string of 10^6 nucleotides with a predetermined nucleotide-distribution, randomizing it and cutting it into 10^5 10-mer oligonucleotides. For the conserving amino acids, the percentage of the 10-mer randomized strings carrying at least one cognate triplet from the conserved triplet-set is defined as the expected occurrence. For the remaining amino acids (for which a conserved set of C/AC does not exist), the percentage of the 10-mer randomized strings carrying at least one cognate codon or anticodon triplet was set to be the expected occurrence. The error value for the expected percentage, ±0.8%, includes a statistical error of 0.6% and a rounding error of 0.5%. The

probability of random occurrence, *P*, was calculated for each amino acid, from the results of a χ^2 test, using a statistical cut off of $P \leq 0.01$.

Calculation of correlation:

The probability of obtaining exactly m common objects in two groups of n1,n2 objects chosen randomly out of a batch of n objects, is given by:

(n1 choose m) x ([n-n1] choose [n2-m]) / (n choose n2)

Where (n choose m) = n! / (m! x [n-m]!)

The probability of having m or more common object in two such groups is calculated via a summation from m till min(n1,n2); in our case, of overlap of 5 or more in two sets of 6 each, we get:

[(6 choose 6) x (14 choose 0) + (6 choose 5) x (14 choose 1)]/ (20 choose 6) ~ 0.002

Abbreviations:

aa- amino acid

aaRS - aminoacyl-tRNA synthetase

AC- anticodon

C/AC – codon and/or anticodon triplet

Pre-3'end – 10 mer string preceding the tRNA 3'end

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Appendix: Detailed calculation of correlations:

A more detailed calculations for proving the correlations between List-IIa and List-RC(K) might be helpful to the readers: For the case of k=6, there are (20 choose 6) possibilities to choose 6 aa's out of 20 for List-RC(6). Only one of these choices will contain exactly all the 6 from List-IIa, and another 6*14=84 of these choices will contain 5 out of the 6 from List-IIa (as there are 6 options for "which type IIa sythetase aminoacylated aa's to omit" and 14 options for its replacement in the 6 aa's we choose). Similarly, for the case of k=7, we calculated P=(14+(6*14*13/2))/(20 choose 7)=560/77520=~0.00722, as there are (20 choose 7) possibilities to choose 7 aa's out of 20 for List-RC(7). Exactly 14 of these choices will contain all the 6 from LIST-IIa plus one in the list complementary to List-IIa (a list of 14 aa's), and another 6*(14 choose 2) of these choices will contain 5 out of the 6 from LIST-IIa (6 options for omitting 1 aa), while having (14 choose 2) options for adding two aa's not in List-IIa.

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