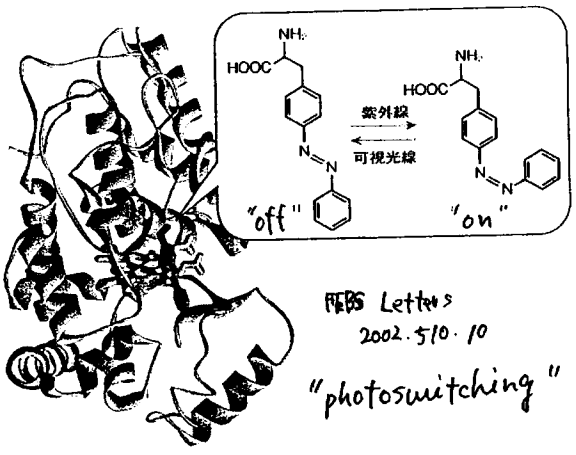
Genetic Code Expansion
-- Incorporation of Non-Natural Amino Acids into Proteins--

2005.5.25
lit. seminar
Rie MOTOKI (M2)

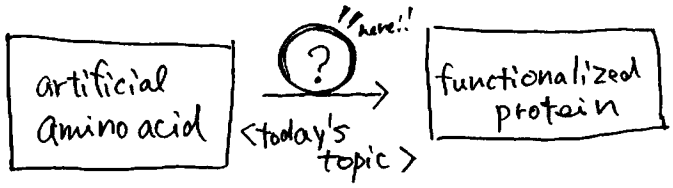
review : Schultz et al. *Angew. Chem. Int. Ed.* **2005**, *44*, 34-66
Sisido et al. *Curr. Opin. Chem. Biol.* **2002**, *6*, 809-815

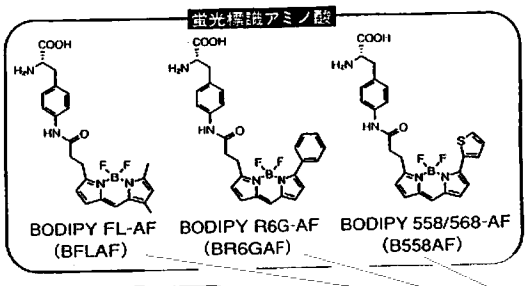
Introduction

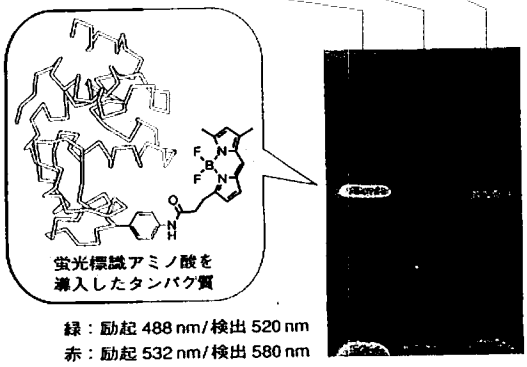


西洋ワサビ由来ペルオキシダーゼの活性中心ヘム近傍の68番目のフェニルアラニン部位にアゾベンゼンを側鎖にもつ非天然アミノ酸を導入した場合，酵素活性を紫外線・可視光線の照射により制御することができた．

非天然アミノ酸の導入による酵素活性の光制御







蛍光標識アミノ酸が導入されたタンパク質が，蛍光検出装置により直接確認できる．

蛍光標識アミノ酸を導入したストレプトアビジンのSDS-ポリアクリルアミドゲル電気泳動

Biological background

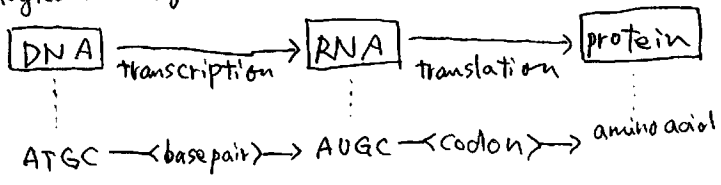


表 4・2 遺伝暗号（RNA からアミノ酸）

1番目 (5′末端)	2番目 U	2番目 C	2番目 A	2番目 G	3番目 (3′末端)
U ウラシル	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	終止(och)	終止	A
	Leu	Ser	終止(amb)	Trp	G
C シトシン	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu(Met)	Pro	Gln	Arg	G
A アデニン	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met(開始)	Thr	Lys	Arg	G
G グアニン	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val(Met)	Ala	Glu	Gly	G

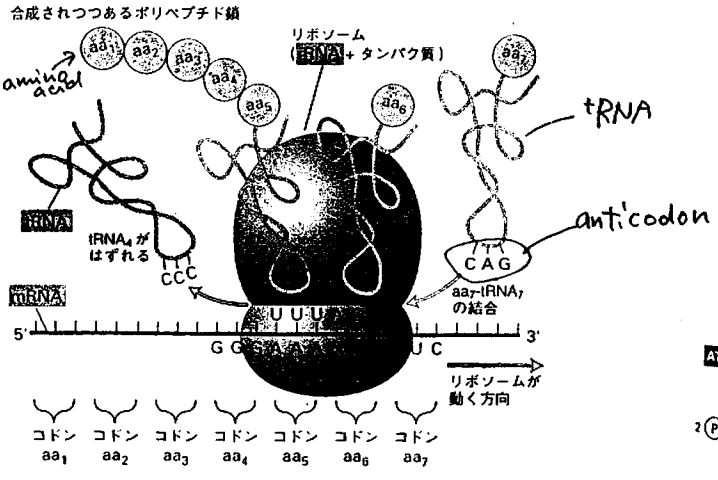
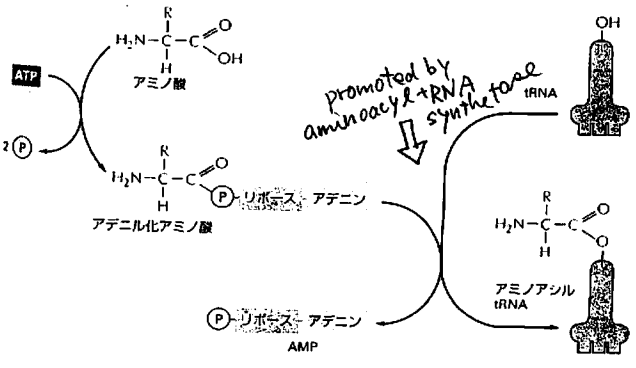


図 4・20 タンパク質合成における3種類の RNA の役割．mRNA は tRNA とリボソームの協調作業によってタンパク質に翻訳されていく．リボソームは多数のタンパク質と2種類の rRNA から構成されている．

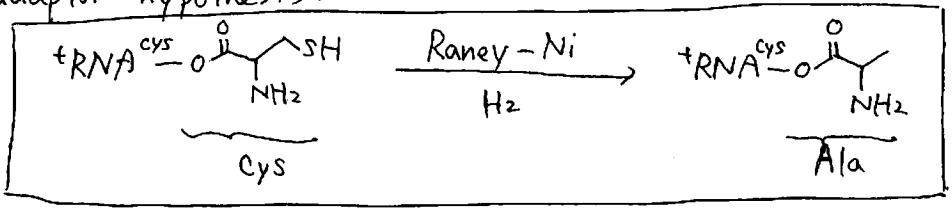


· specific aminoacyl tRNA synthetase exists for each amino acid
(= ARS)

$tRNA^{Cys} \xrightarrow{ARS^{Cys}} tRNA^{Cys}—Cys$

$tRNA^{Cys} \xrightarrow{ARS^{Ala}} tRNA^{Cys}—Ala$ // proof leading

• adaptor hypothesis •

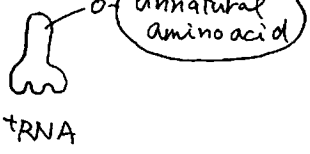


PNAS 1962. 48. 1086

· not determined by amino acid itself
· hydrogen bond between codon-anticodon is important

works as Ala donor in response to Cys codon

■ Strategy to synthesize unnatural amino acid contained protein.



[construction of misacylated tRNA might be the way for "artificial protein"!]

1) How to construct misacylated tRNA ?
- chemical acylation
- mutation of aminoacyl tRNA synthetase (enzyme) } talk later
- de novo aminoacylation by ribozyme

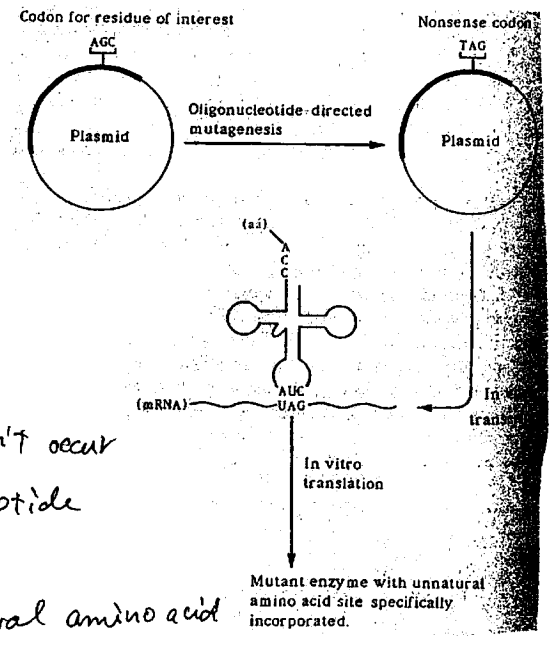
2) How to incorporate unnatural amino acid site specifically into protein ?

① using amber codon (rare codon for E. coli system)
└ UAG (stop codon)

A General Method for Site-Specific Incorporation of Unnatural Amino Acids into Proteins

CHRISTOPHER J. NOREN, SPENCER J. ANTHONY-CAHILL, MICHAEL C. GRIFFITH, PETER G. SCHULTZ*

Science. 1989. 244. 183



advantage
· competition with other amino acid doesn't occur
→ truncated peptide or mutated peptide (短い)

limitation
· incorporation of more than two unnatural amino acid into protein is difficult

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② Using four base codon

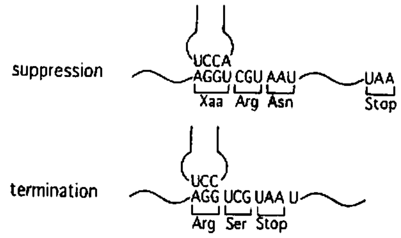
J. Am. Chem. Soc. 1996, 118, 9778–9779

Incorporation of Nonnatural Amino Acids into Streptavidin through *In Vitro* Frame-Shift Suppression

Takahiro Hohsaka, Yuki Ashizuka, Hiroshi Murakami, and Masahiko Sisido*

Department of Bioscience and Biotechnology
Okayama University
3-1-1 Tsushimanaka, Okayama 700, Japan

Received April 30, 1996



Y. 20%

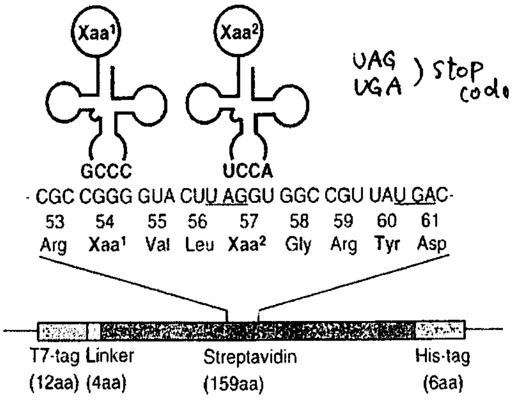
codon for Arg (E. Coli)
CGU, CGC, CGA,
AGA, AGG CGG
↳ rarely used in E. Coli system
four base codon AGGN might be possible

(Sisido, et al Nucleic acids research 2001. 29. 3646
"five-base codon"
efficiency: lower than four-base codon)

Incorporation of Two Different Nonnatural Amino Acids Independently into a Single Protein through Extension of the Genetic Code

Takahiro Hohsaka, Yuki Ashizuka, Hiroshi Sasaki, Hiroshi Murakami, and Masahiko Sisido*

J. Am. Chem. Soc. 1999, *121*, 12194–12195



UAG
UGA) stop codon

Y. 9% (comparing with WT)
↓
using CGGG & GGGU (Y. ~80%)
(high efficiency) Shishido. et. al Biochemistry. 2001. 40. 11060

• merit to incorporate two different unnatural amino acid
(· site to site electron transfer
· energy transfer (FRET etc.))

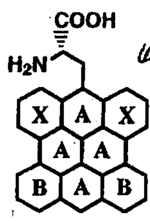
Y. ~20%

oc. 1999, 121, 34–40

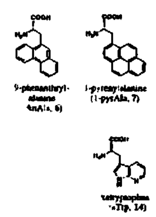
Amino Acids with Large
Vitro Protein Synthesizing

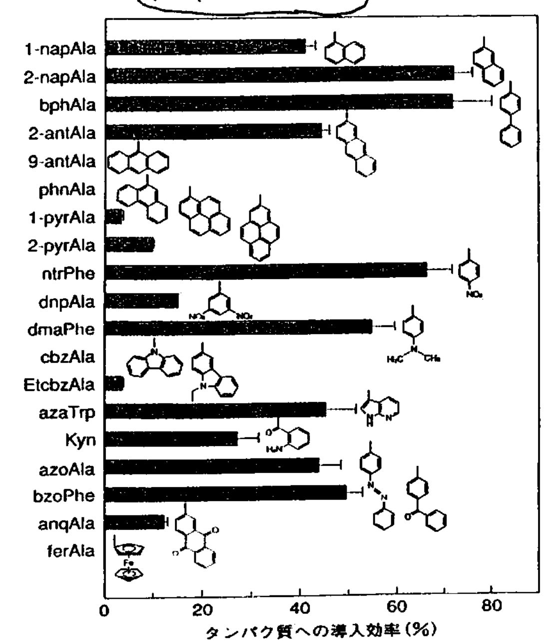
…Murakami, and

…Engineering



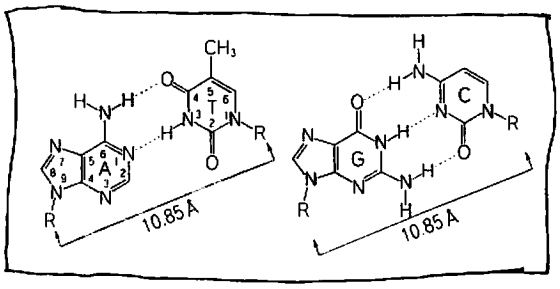
A: O.K
B: acceptable
X: less favored



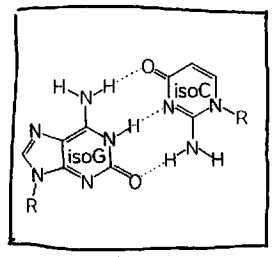


protective group is unnecessary
in NH3 aq.

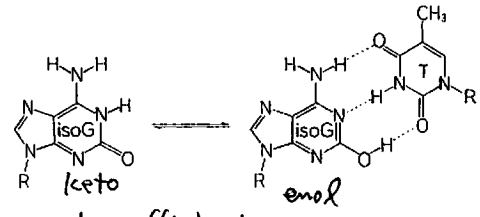
③ expansion of base Benner et al Nature 1992. 356. 537



A-T . G-C pair



new base pair



not so efficient
(enol form of isoG can make pair with T)

base 4 → 6 kinda
$4^3 = 64$ $6^3 = 216$
→ extra base pair
would accomodate natural amino acid incorporation into peptide

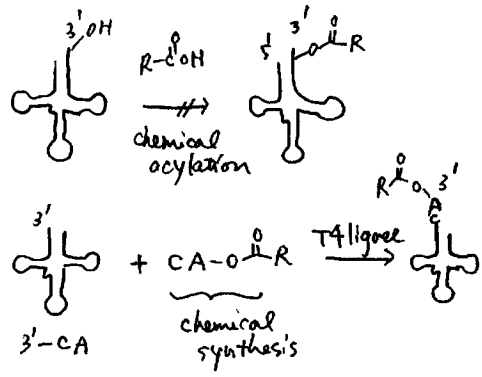
---

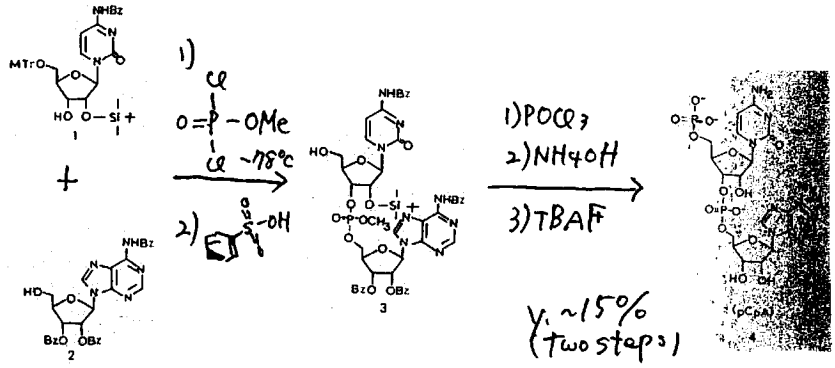
1) Synthesis of aminoacylated ^{t}RNA

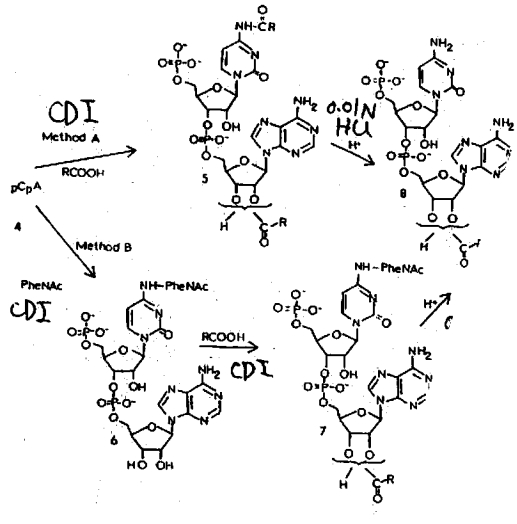
① Chemical acylation *Biochemistry* 1984, *23*, 1468–1473

T4 RNA Ligase Mediated Preparation of Novel "Chemically Misacylated" tRNA$^{Phe}_S$†

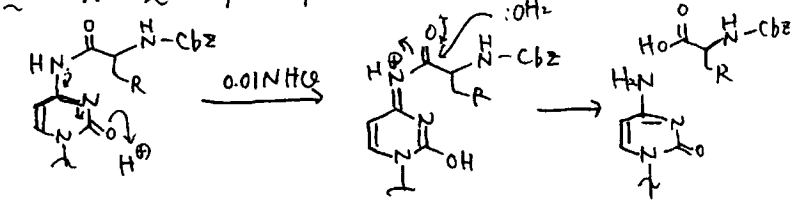
Thomas G. Heckler, Li-Ho Chang, Yoshiyuki Zama, Takehiko Naka, Mukund S. Chorghade, and Sidney M. Hecht*



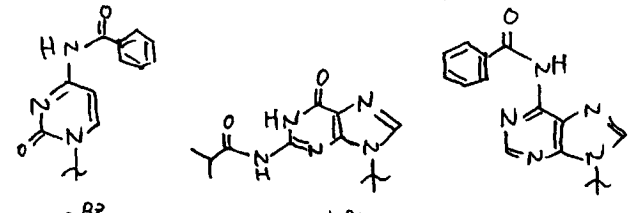




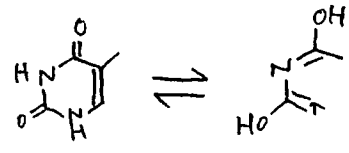
… 5 → 8 or 7 → 8 hydrolysis of amide



… in the case of DNA automated synthesis



necessary protective group is need to increase solubility



T

protective group

A.C.G → amide-type cleaved wi

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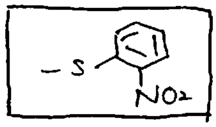
J. Am. Chem. Soc. 1991, 113, 2722-2729

A General and Efficient Route for Chemical Aminoacylation of Transfer RNAs

Stephanie A. Robertson, Jonathan A. Ellman, and Peter G. Schultz*

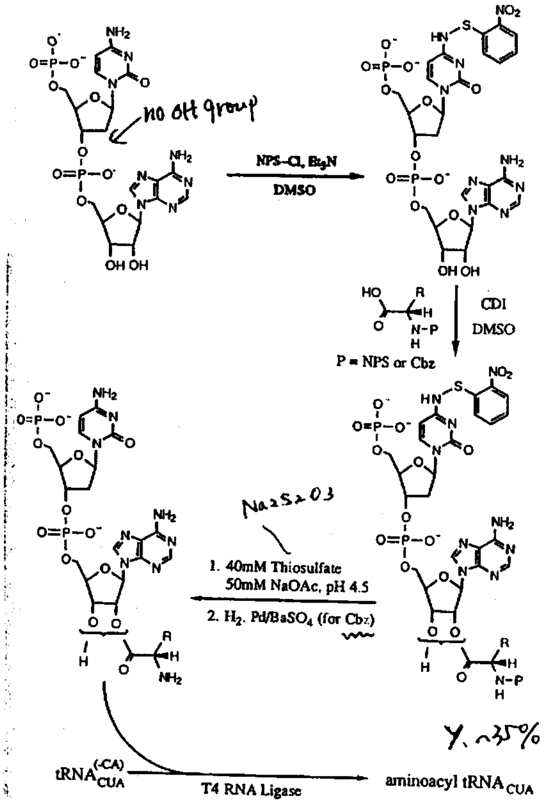
Contribution from the Department of Chemistry, University of California, Berkeley, California 94720. Received August 30, 1990

NPS: 2-Nitrobenzenesulfenamide



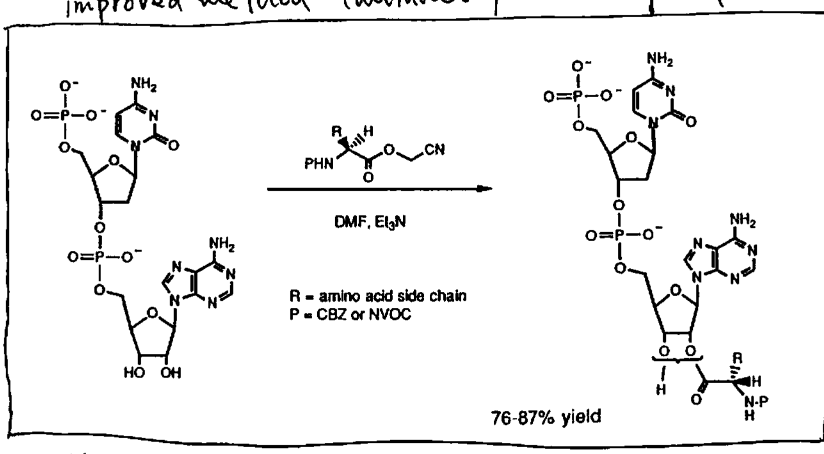
formation
o-$NO_2C_6H_4SCl$, NaOH, dioxane

cleavage: HCl / Et_2O or EtOH, 0°C, 1hr
PhSH, HS⌒COOH rt, 1hr
NaI, CH_3OH 0°C, 20min
Raney-Ni / DMF



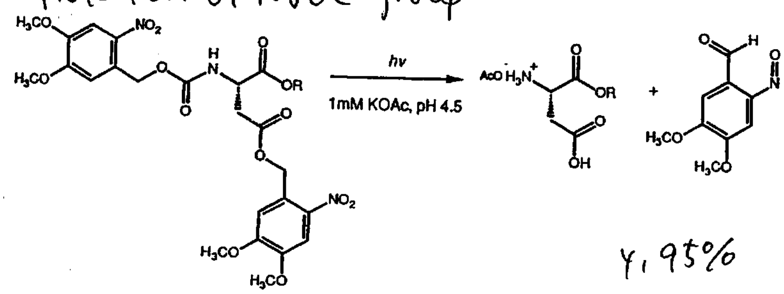
Y. ~35%

improved method (without protective group)

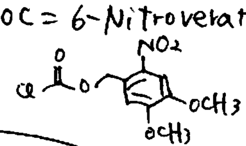


◎ cyanomethyl group ⌒CN
mildly activated esters
(too strong activation → dec.)

deprotection of NVOC group

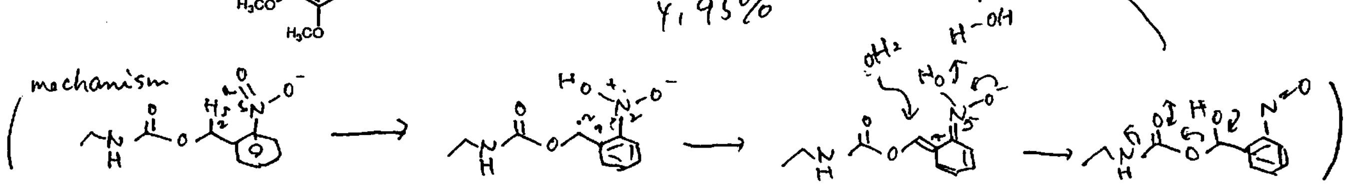


NVOC = 6-Nitroveratryloxycarbonyl



Y. 95%

mechanism



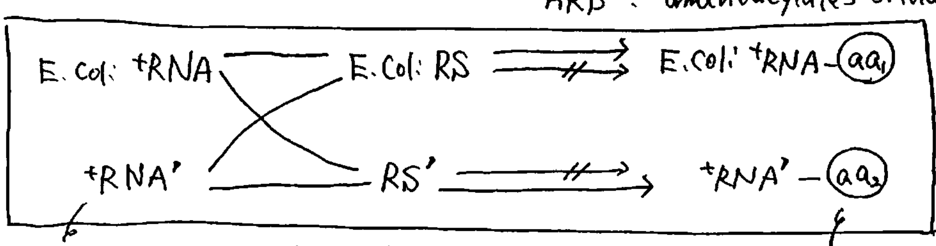
② mutation of aminoacyl tRNA synthetase (enzyme)

Expanding the Genetic Code of *Escherichia coli*

Lei Wang,[1] Ansgar Brock,[2] Brad Herberich,[1] Peter G. Schultz[1,2*]

Science. 2001. 292. 498

"orthogonal" tRNA / Synthetase pair is necessary
tRNA: must not be recognized by E.Coli ARS but function efficiently in translation
ARS: aminoacylates orthogonal tRNA, but doesn't recognize endogenous E.Coli tRNA



例えば: 終止codonに対応した外来のtRNA

導入したい非天然 or 天然アミノ酸

Orthogonal tRNA / ARS pair might be obtained by using Archaebacteria derived tRNA / ARS

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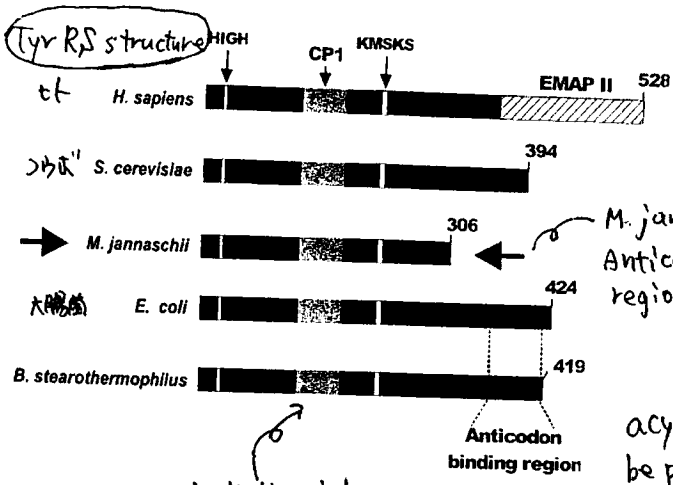
Major Anticodon-binding Region Missing from an Archaebacterial tRNA Synthetase*

(Received for publication, August 4, 1999, and in revised form, September 2, 1999)

Brian A. Steer‡ and Paul Schimmel§
From The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037

Tyr RS structure



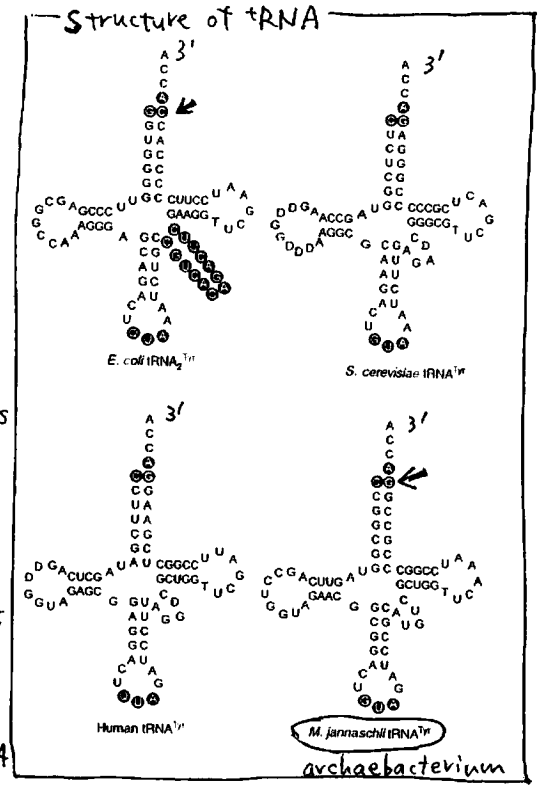
M. jannaschii lacks Anticodon binding region

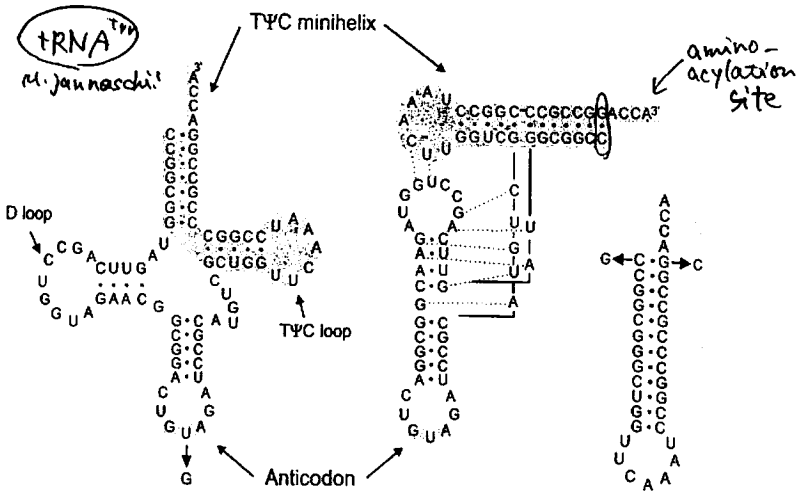
↓

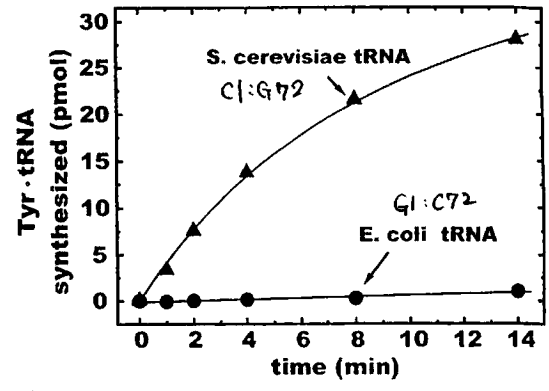
acylation might be possible if we change anticodon of tRNA

to distinguish GC or CG in acceptor stem

Structure of tRNA







↑ Aminoacylation using M. jannaschii Tyr RS

minihelixTyr → minihelix can become the substrate of aminoacylation

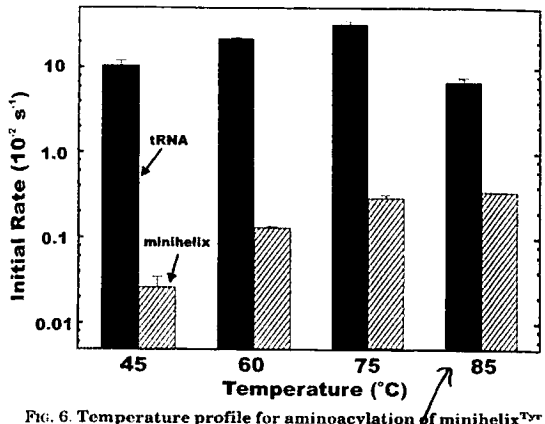


FIG. 6. Temperature profile for aminoacylation of minihelix^{Tyr} and tRNA^{Tyr}

"anticodon might not be particularly important for M. jannaschii Tyr RS"

↕

negative control

① in the case of E. Coli
U36G mutation of central anticodon based on E. coli tRNA^{Tyr}
→ 200-fold less reactive
(M. jannaschii → ~6-fold)

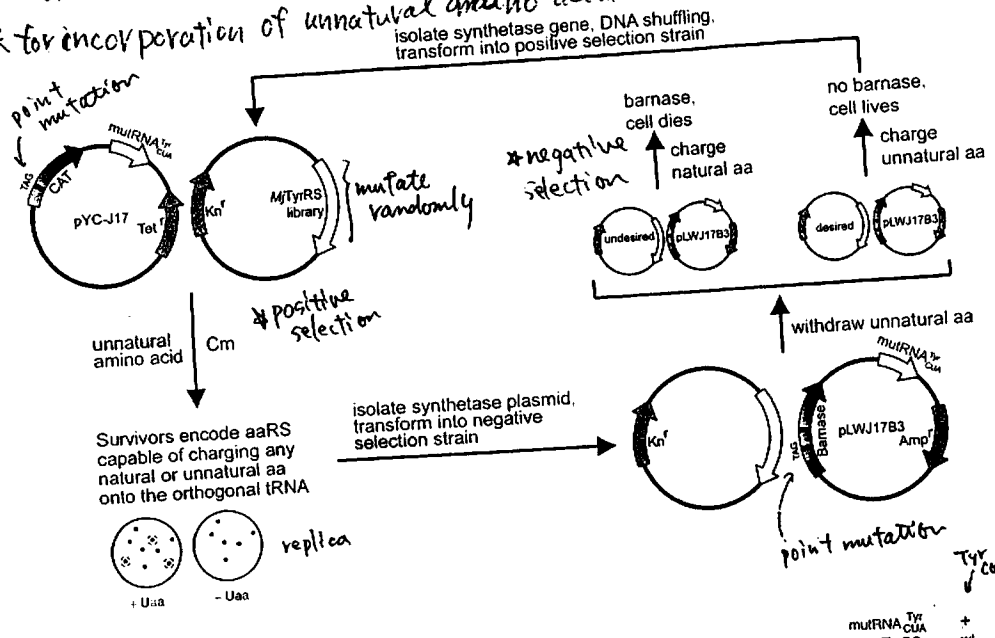
② 16-nucleotide single stranded RNA at the 3′ end of minihelix
→ can't work as substrate

tertiary structure of the tRNA might be disrupted

6/10

By using orthogonal M.jannaschii tRNA/Synthetase pair, E.Coli can encode amino acid in response to amber codon in vivo !! (Schultz' paper : page 5)

* for incorporation of unnatural amino acid

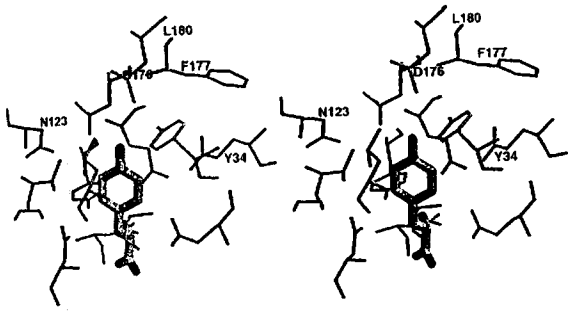


* positive selection
cf. β-lactamase + Amp selection

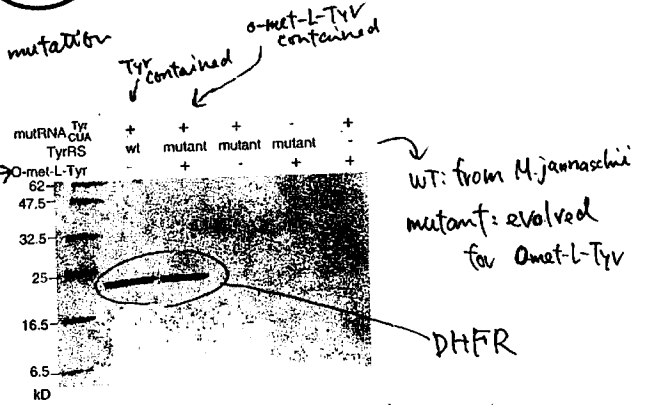
survive: → AA (unnatural or natural)

* negative selection
barnase : poison

survive: MjTyrRS → AA (unnatura



Crystal structure of homologous TyrRS from Bacillus stearothermophilus



Synthesis of E.Coli DHFR (dihydrofolate reductase) under different conditions

conclusion

M.jannaschii $tRNA^{Tyr}_{CUA}$ // TyrRS } orthogonal pair in E.Coli system (stop codon)

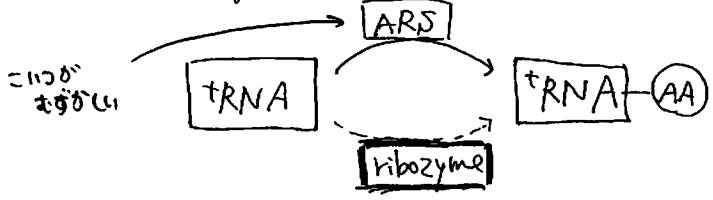
Tyr in response to stop codon (amber)

in vitro evolution { · mutation · selection

M.jannaschii $tRNA^{Tyr}_{CUA}$ mut TyrRS → (confirmed by Mass)

O-met-L-Tyr in response to stop codon

③ Using "Ribozyme" as novel aminoacylating catalyst



catalytic role for aminoacylation

「RNA-World」

RNA played a key role in ancient world catalytic system

ARS-like ribozyme should exist!!

(protein) (RNA)

7/10

The EMBO Journal Vol. 20 No. 7 pp. 1797–1806, 2001

An *in vitro* evolved precursor tRNA with aminoacylation activity

Hirohide Saito[1,2], Dimitrios Kourouklis[1] and Hiroaki Suga[1,3]

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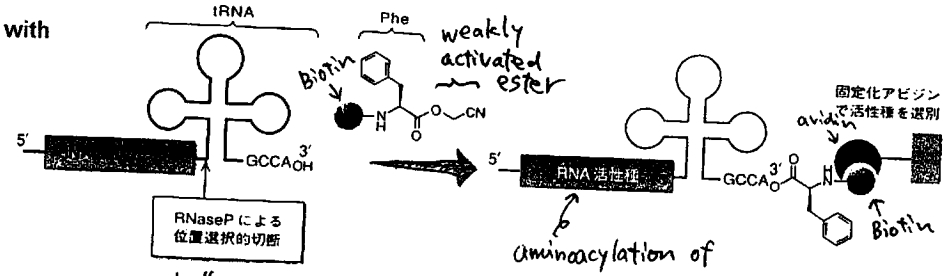


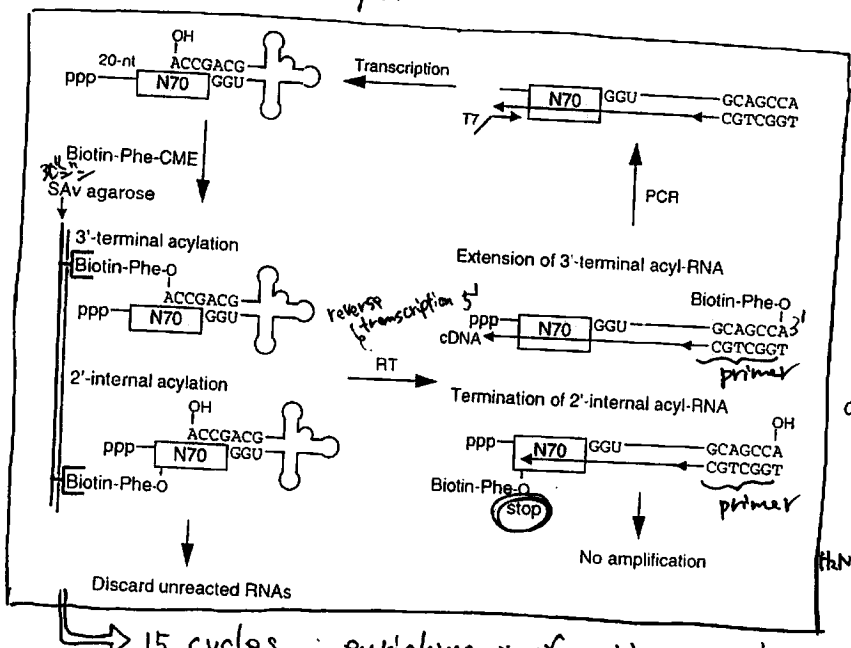
advantage of ribozyme

↳ "trans" acting catalyst in the future

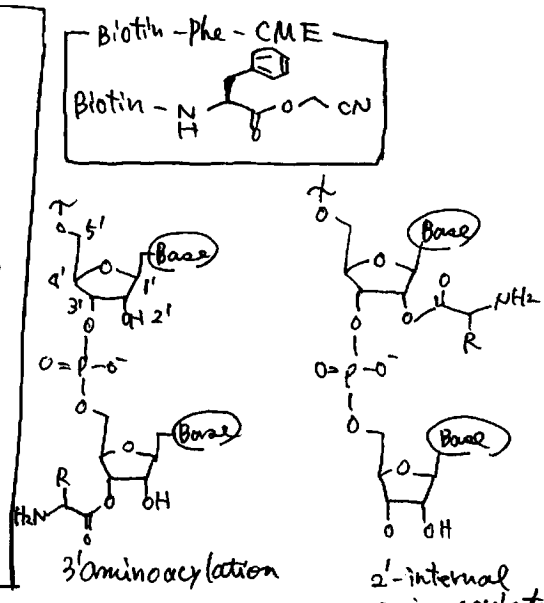
aminoacylation of tRNA 3' end

DNA ↓↑ RNA → ribozyme ..... easy to replication by RT. PCR.

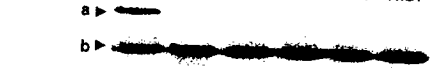
RNA → protein → general enzyme (protein) ... protein cannot amplify itself!!



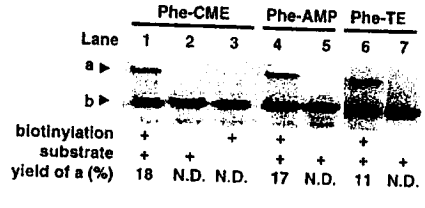
15 cycles : enrichment of active species



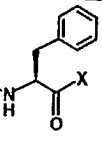
amino acid	Phe	Leu	Met	Gln	Gly	Val
rel. activity	1	0.02	< 0.01	N.D.	0.01	N.D.

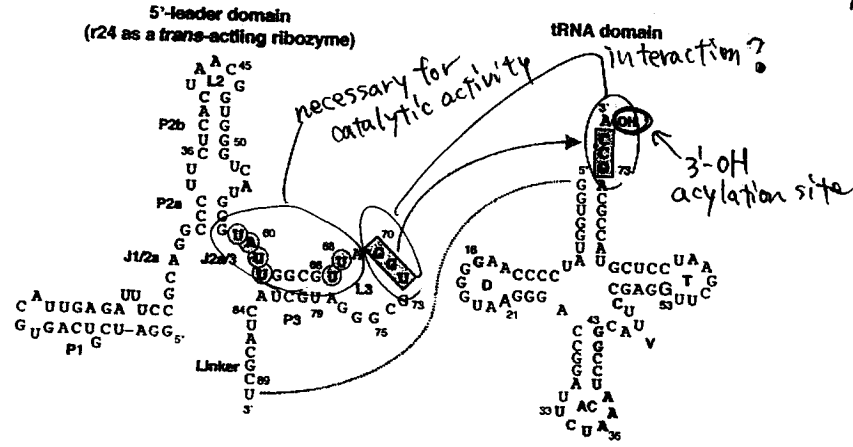


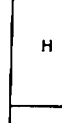
Biotin - aa - CME as donor the selective



Most critical recognition element of the substrate is Phe side chain, not leaving group or biotinyl group.

Biotin or H → 



R	X	Abbreviation
Biotin		Biotin-Phe-CME
H		Phe-CME
H		Phe-AMP
H		Phe-TE

"How ribozyme works ?" Nucleic acids Research 2002. 30. 5151 Suga. et al.

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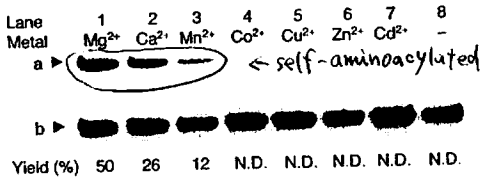
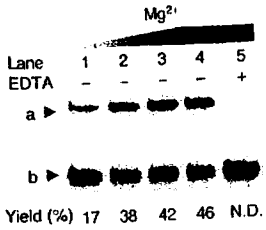
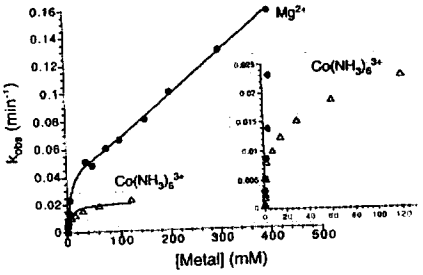
• metal dependency ?



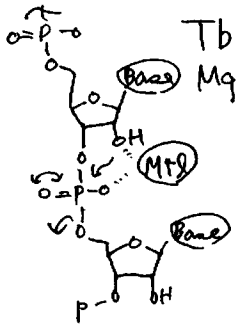
Mg^{2+}, Ca^{2+} dependant





hypothesis : two metal binding site exists
- high affinity site
- low affinity site

• where metals coordinate ?



Tb^{3+} ionic radius 0.92 Å
Mg^{2+} 0.72 Å
} same preference for coordination to oxygen ligands.

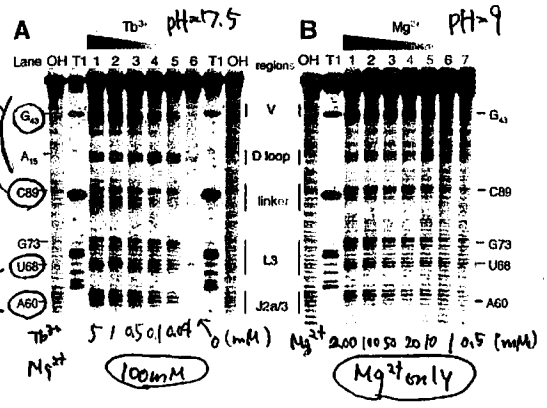
Tb^{3+} → cleaves RNA at neutral pH
Mg^{2+} → cannot cleave "
but cleaves at ~ pH 9

→ but... high concentration of Tb^{3+} cleaves single-stranded or structurally relaxed region

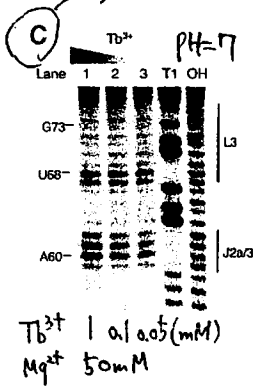
tRNA site

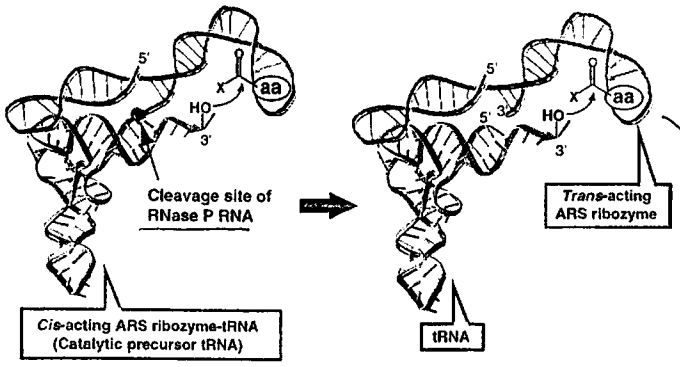
not so important ? from mutation study

Mg^{2+} binding site ?



similar to A

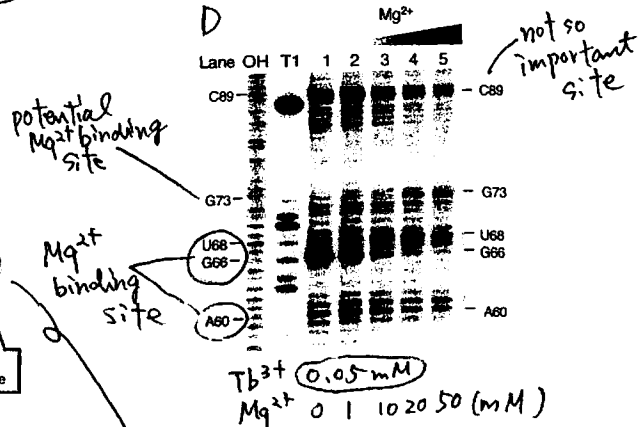


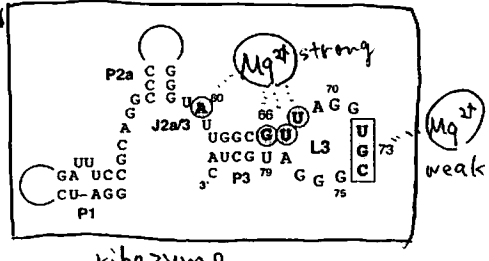


ribozyme works as trans fashion

potential Mg^{2+} binding site

Mg^{2+} binding site





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A Versatile tRNA Aminoacylation Catalyst Based on RNA

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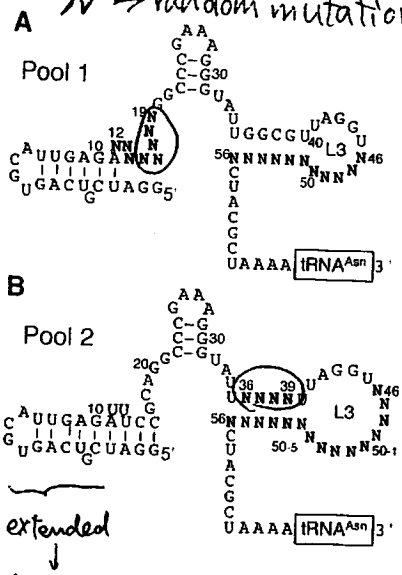
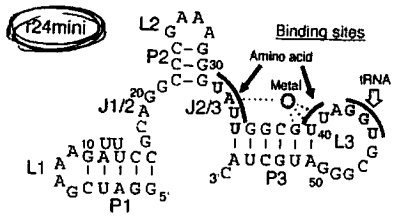
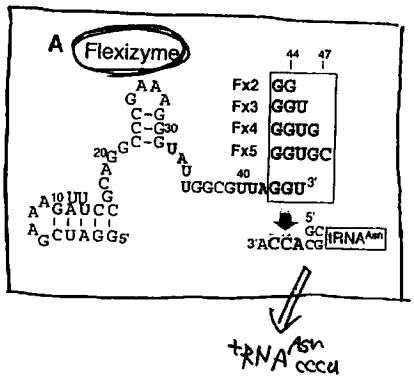
N → random mutation (result)

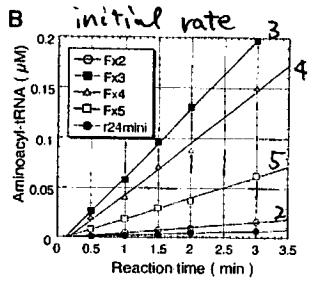


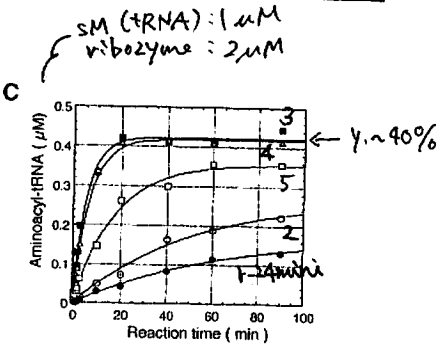
pool 1 :
- 12-14 not conserved
- 15-19 WT sequence
- 46-56 not conserved (no similar sequence)

pool 2 :
- 36-39 completely conserved
- 46-56 no consensus sequence

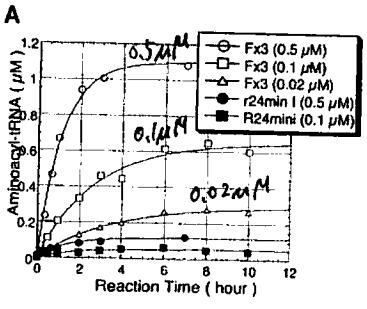


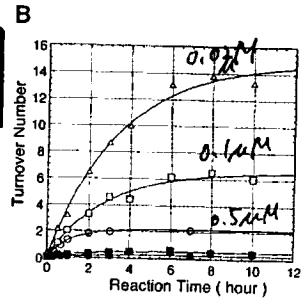




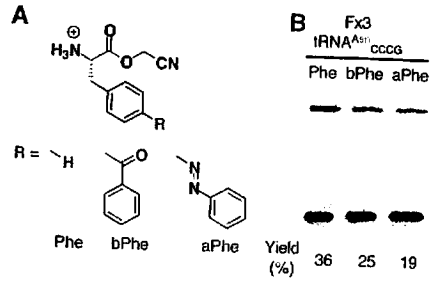


Fx3 is the best catalyst!
(rate and yield)





catalytic amount of ribozyme → 0.1 μM
high conc. → TON ↓ due to product inhibition?



versatile substrate generality

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Aminoacyl-tRNA Synthesis by a Resin-Immobilized Ribozyme

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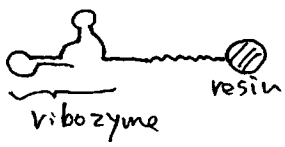

"recyclable"
"easy to use"

<Summary>

works as Unnatural Amino acid donor

- acylation
  - chemical acylation
  - orthogonal ARS
  - ribozyme
- in competition with natural AA
  - amber (stop) codon
  - four (five) base codon
  - newly synthesized base

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