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ORIGINAL RESEARCH PAPER

Construction of an L-phenylalanine-producing tyrosine-prototrophic *Escherichia coli* strain using *tyrA* ssrA-like tagged alleles

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Abstract To construct a Phe-producing Tyr^+ *Escherichia coli* strain, TyrA (chorismate mutase/prephenate dehydrogenase) activity was varied by engineering a proteolytically unstable protein. The *tyrA* in the *E. coli* BW25113 was altered to include ssrA-like tags. The tagged *tyrA* genes, which ensured different growth rates in M9 medium, were introduced into a Phe-producing strain to replace Δ*tyrA*. Strains with unstable TyrA-(A)ANDENYALAA proteins had a lower biomass yield and a higher Phe accumulation than strains generating the more stable TyrA-(A)ANDENYALDD. The Tyr/Phe ratio produced by the TyrA-tag strains was 10-fold less than that produced by the $TyrA^{wt}$ strain.

Keywords Phenylalanine production · Proteolysis · TyrA-tag · Tyrosine by-production

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Introduction

L-Phenylalanine (Phe) is an important commercial amino acid. Recombinant Phe-producing *Escherichia coli* strains are usually constructed from wild-type strains through several defined steps based on the pathways of aromatic amino acid biosynthesis. One of these steps is disruption of the *tyrA* gene coding for chorismate mutase (E.C. 5.4.99.5)/prephenate dehydrogenase (E.C.1.3.1.12), resulting in a Tyr^- mutant. Disruption of *tyrA* prevents the branching of chorismate (the common precursor of the aromatic biosynthetic pathway, Fig. 1) and decreases the accumulation of L-tyrosine (Tyr) as a by-product. The presence of Tyr creates problems in downstream processing, and the need for its removal increases the cost of the production of Phe.

To construct a Phe-producing prototrophic strain, we modulated the protein levels of the enzyme TyrA using ssrA-mediated tagging. The ssrA tag is an 11-aa peptide with the sequence AANDENYALAA, and this tag is added to the C terminus of proteins that stall during translation (Keiler et al. 1996). These tagged proteins are recognized and subsequently degraded by specific *C*-terminal proteases present in both the cytoplasm and periplasm. ClpXP, ClpAP and, to a lesser extent, HflB (FtsH) are proteases that degrade ssrA-tagged proteins in the cytoplasmic compartment (Herman et al. 1998). The ssrA tag contains independent but overlapping determinants for its interactions with ClpX, ClpA, and SspB, a specificity-enhancing factor for ClpX (Fig. 2).

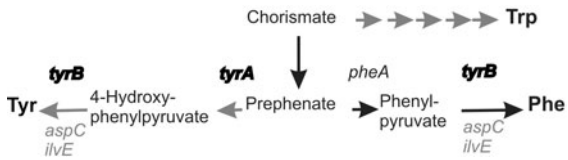

Fig. 1 Phe biosynthesis in *E. coli* cells. The side pathways for Tyr and Trp biosynthesis are indicated by the *gray arrows*. The genes coding for complementary functions are shown in *gray*. The genes repressed by TyrR in the presence of Tyr and ATP are shown in bold

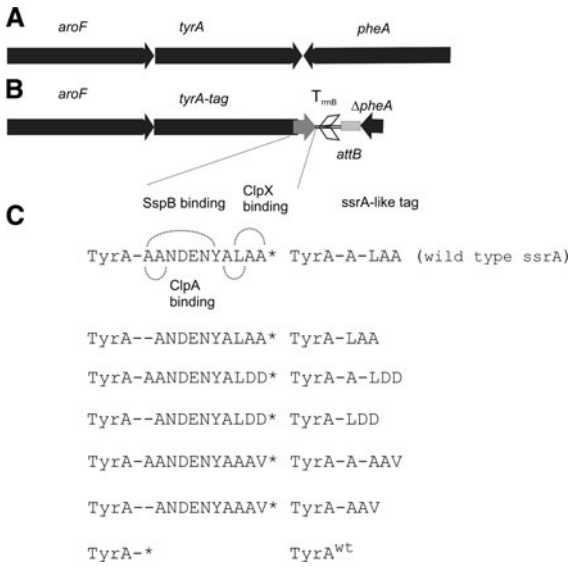

Fig. 2 Modification of the *tyrA-pheA* chromosomal locus of the *E. coli* chromosome. The structures of the native (**a**) and modified (**b**) loci and the structures of the ssrA-like tags (**c**) are shown. Genes, the ssrA-like tag, and the terminator of *rrnB* (T_{rrnB}) are shown as *black arrows*, a *gray arrow* and a *white angle bracket*, respectively. The attachment site of phage λ, *attB* (*gray square*), remained after removal of the *cat* gene. The recognition sites for SspB, ClpA and ClpX are shown according to Flynn et al. (2001)

To modify the activity of TyrA, we integrated the customary ssrA tag and its variants into the chromosome of *E. coli* BW25113 towards the 3′-end of *tyrA*. A few *tyrA*-tag alleles ensuring different growth rates of BW25113 cells in minimal medium without Tyr were transferred into an *E. coli* Phe-producing $TyrR^+$ strain. The TyrR repressor controls the aromatic biosynthetic pathway (Pittard et al. 2005), and a Δ*tyrR* strain usually produces more Phe than its $TyrR^+$ variant. At the same time, Tyr is the main cofactor of TyrR, and TyrR-mediated repression of Phe biosynthesis could be an internal indicator of the by-production of Tyr during batch cultivation. In the $TyrR^+$ genetic background, we selected a *tyrA*-tag allele that resulted in the production of Phe up to the level of the Phe-producing strain (Δ*tyrR* Δ*tyrA*).

Materials and methods

Bacterial strains

All ssrA-like tags were introduced into the chromosome of *E. coli* BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR*514 $\Delta araBA\text{-}D_{AH33}$ $\Delta rhaBAD_{LD78}$) using the λRed recombination system, followed by λ Xis/Int-mediated Cm^r marker excision, as described previously (Doroshenko et al. 2007). The entire fragment containing (ssrA-tag)-T_{rrnB}-$\lambda attR$-*cat*-$\lambda attL$ was integrated toward the 3′-end of *tyrA* as shown in Fig. 2. The terminator of *rrnB* was concurrently introduced immediately after the tag to cut off any convergent to *tyrA* transcription.

E. coli DV269 (Δ*tyrA* *htrE*:(P_L-*yddG*) [MUD-*aroG*4-$pheA^B$-*aroL*]) denoted below as DV269(Δ*tyrA*) and its derivative DV269(Δ*tyrA* Δ*tyrR*), both of which are Phe producers, harbor mutated *aroG* and *pheA* genes encoding insensitive to feedback inhibition by Phe 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and chorismate mutase/prephenate dehydratase, respectively (Imaizumi et al. 2007). The *tyrA*-tag and $tyrA^{wt}$ genes were transferred to strain DV269 (Δ*tyrA*) using P1*vir* transduction.

Growth conditions and analytical procedures

To compare BW25113-like strains for growth rates, colonies of freshly grown cells from LB agar plates were resuspended in M9 medium, and aliquots (to generate an initial $OD_{600} \sim 0.2$) were inoculated into 30 ml M9 medium supplemented with 50 mg Phe/l in 0.75 l flasks. The cells were cultivated at 37°C with vigorous shaking (240 rpm).

Batch cultivation was carried out in a Biostat Q using 1 l glass vessel with a working volume of 0.3 l (BBI, Germany). The medium contained 50 g glucose/l, 0.6 g $(NH_4)_2SO_4$/l, 0.6 g KH_2PO_4/l, 10 mg $FeSO_4$/l, 10 mg $MnSO_4$/l and 2 g yeast extract/l. In the culture of ΔtyrA strains, the medium was supplemented with 125 mg Tyr/l. The cells were cultivated aerobically at

37°C, and the pH of the medium was maintained at 6.7 using aqueous NH_3. Seed cultures (0.03 l) were cultivated in 0.75 l flasks in LB medium with aeration (240 rpm).

Phe and Tyr concentrations were measured using HPLC. Prephenate dehydrogenase (PDH) activity was measured in the crude cell extracts as described previously (Davidson and Hudson 1987).

Results and discussion

Construction of the BW25113(Δ*pheA*) strains containing different *tyrA*-tag genes

To obtain mutant TyrA proteins of varying stability, we constructed variants of the *tyrA* gene carrying ssrA-like tags with minor alterations in the ssrA consensus sequence as has been previously described (Andersen et al. 1998). The adjacent *pheA* gene encoding a sensitive to feedback inhibition by Phe enzyme was deleted (Fig. 2). This wild-type gene was not needed in the Phe-producing strain carrying the integrated copy of $pheA^B$ in its chromosome.

We proposed that TyrA proteins carrying ssrA, i.e.—AANDENYALAA or the modified tag-AANDENYALDD at their *C*-termini (TyrA-A-LAA or TyrA-A-LDD) would be protease-sensitive and protease-resistant, respectively, and that TyrA ending with AANDENYAAAV (TyrA-A-AAV) would be a protein with intermediate sensitivity to proteolysis. We also constructed mutants encoding TyrA with truncated variants of these tags: TyrA-NDENYALAA (TyrA-LAA), TyrA-ANDENYAAAV (TyrA-AAV) and TyrA-ANDENYALDD (TyrA-LDD). To control the level of $TyrA^{wt}$ activity, we obtained strain BW25113(Δ*pheA*) containing wild-type *tyrA* using the same scheme (Fig. 2). The modified chromosomal regions of all the constructed strains were verified by sequencing.

Growth of BW25113(Δ*pheA tyrA*-tag) strains in M9 medium

As seen from Fig. 3, the BW25113(Δ*pheA tyrA*-tag) strains were divided into three groups according to their growth in M9 medium supplemented by Phe but not Tyr. Three strains of the first group, expressing

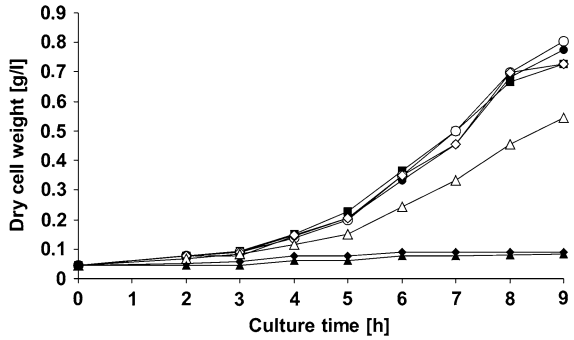

Fig. 3 Typical time profiles of cell growth of BW25113(Δ*pheA*) strains with $TyrA^{wt}$ (*closed boxes*), TyrA-LDD or TyrA-A-LDD (*open* or *closed circles*), TyrA-AAV or TyrA-A-AAV (*open* or *closed diamonds*), TyrA-LAA or TyrA-A-LAA (*open* or *closed triangles*) in M9 medium. The means deviations of each curve from three experiments were ~0.01 g/l

TyrA-LDD, TyrA-A-LDD and TyrA-AAV, grew at the same rate as BW25113(Δ*pheA* $tyrA^{wt}$). The 'second group' strain generated TyrA-LAA. The most poorly growing strains synthesized TyrA-A-AAV or TyrA-A-LAA (group III), and the addition of Tyr (50 mg/l) restored their growth (data not shown). Therefore, the poor growth of the latter strains was caused by decreased TyrA activity.

Measurement of PDH activity revealed instability of the mutant TyrA-tag in crude cell extracts

To confirm the variability in the TyrA, we measured the PDH activity in crude cell extracts of some *tyrA*-tag strains. Before sonication, the cells were resuspended in buffer in the presence or absence of glycerol, a compound that is known to increase the conformational stability of enzymes (Bradbury and Jakoby 1972).

As seen from Table 1, PDH activity for $TyrA^{wt}$ and TyrA-LDD was practically the same with and without glycerol. PDH activities decreased in the order of restoration of ssrA. This decrease was mainly detected in crude extracts without glycerol. Moreover, for the glycerol-free extracts containing TyrA-LAA, the low PDH activity could not be accurately measured. It appears probable that the TyrA-tags could have problems with maintenance of the folded conformation in the cell glycerol-free crude extracts, and these proteins were apparently degraded by proteases recognizing ssrA.

Table 1 Prephenate dehydrogenase (PDH) activity of the TyrA^{wt} and TyrA-tag mutants

BW25113(Δ*pheA*)	PDH activity (nM/min mg)[a]		
	1% glycerol	Without glycerol	
	Without Tyr	Without Tyr	50 μM Tyr
TyrA^{wt}	31	29	9
TyrA-LDD	32	28	6
TyrA-A-LDD	28	9	NT
TyrA-LAA	20	<1	NT

[a] The specific enzyme activities were measured in crude extracts of the cells harvested at 4–5 h (Fig. 3). The data presented are the means from triplicate samples. The standard deviations did not exceed 1 nM/min mg. *NT* not tested

The effect of the TyrA-tags on Phe accumulation and Tyr by-production

Genes encoding TyrA^{wt}, TyrA-LAA, TyrA-A-LAA, TyrA-LDD and TyrA-A-LDD were introduced into the Phe-producing strain DV269(Δ*tyrA*). Figure 4 shows the time profiles of cell growth and Phe production in typical batch cultivations of DV269(*tyrA*-tag), DV269(TyrA^{wt}), DV269(Δ*tyrA*) and DV269(Δ*tyrA* Δ*tyrR*) strains. In comparison with BW25113(Δ*pheA tyrA*-tag) strains grown in M9 medium (Fig. 3) DV269(*tyrA*-tag) strains grown in complex medium were divided into two groups according to their growth rates: DV269(TyrA-A-LAA) and other three strains (Fig. 4a).

In the cultures of DV269(TyrA-LDD), DV269-(TyrA-A-LDD) the final DCW was higher (3.2 g/l) and in the cultures of DV269(TyrA-LAA) or DV269(TyrA-A-LAA) the final DCW was lower (2.1 or 1.9 g/l) than the final DCW of DV269(TyrA^{wt}) (2.9 g/l, Fig. 4a). Nevertheless, all strains containing *tyrA*-tag genes produced less Tyr (not more than 0.1% of Phe production) than DV269(TyrA^{wt}) (Table 2).

Among the Tyr-prototrophic strains, only DV269(TyrA-LAA) approached the level of Phe production demonstrated by the Tyr-auxotrophic DV269(Δ*tyrA* Δ*tyrR*) strain (Fig. 4b). These results showed that DV269(TyrA-LAA) cells produced sufficient Tyr for growth but did not accumulate Tyr in amounts sufficient for the TyrR-mediated repression of Phe biosynthesis.

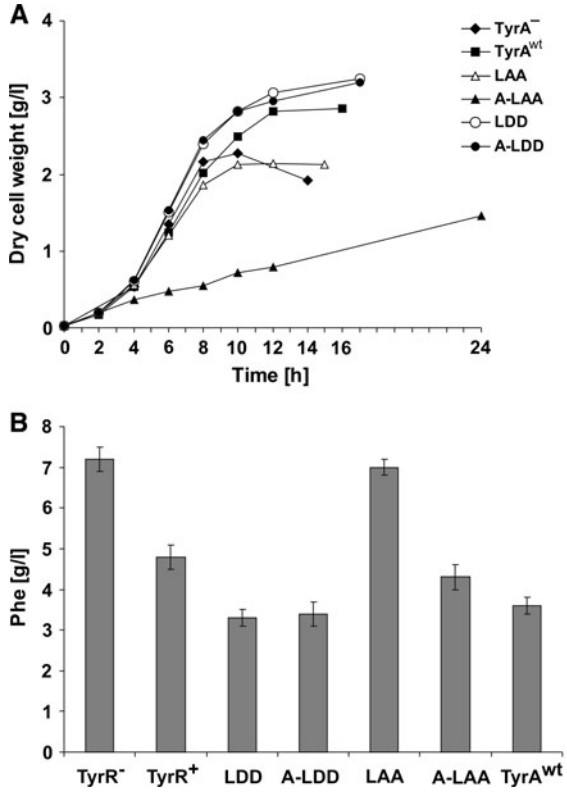

Fig. 4 The time profiles of cell growth (**a**) and Phe production (**b**) of *E. coli* strains cultivated in batch. Strain abbreviations: DV269(Δ*tyrA*)–TyrA^{-}, DV269(TyrA^{wt})–TyrA^{wt}, DV269(TyrA-LAA)–LAA, DV269(TyrA-A-LAA)–A-LAA, DV269(TyrA-LDD)–LDD, DV269(TyrA-A-LDD)–A-LDD and DV269(Δ*tyrA* Δ*tyrR*)–TyrR^{+}. Strains were cultivated up to complete glucose consumption in the medium, with the exception of DV269(TyrA-A-LAA), where the remaining glucose was ~8–10 g/l. **a** Standard deviations of each curve (three experiments) did not exceed 0.1 g/l. **b** The *vertical bars* show the standard deviation obtained from three independent experiments

Table 2 Tyrosine (Tyr) production in cultures of DV269(*tyrA*-tag) *E. coli* strains

TyrA-tag	TyrA^{wt}	TyrA-LAA	TyrA-A-LAA	TyrA-LDD	TyrA-A-LDD
Tyr, mg/l[a]	50 ± 10	4 ± 2	<2	4 ± 2	4 ± 2
Tyr/Phe, %	1.3	0.06	<0.04	0.1	0.1

[a] The mean results of three batch cultivations where Phe production is shown in Fig. 4

In conclusion, a new approach in reduction of unwanted side reaction in an amino acid-producing strain was demonstrated. To eliminate a drain of carbon through Tyr production in Phe-producing

strain, the overall cellular activity of the TyrA enzyme was changed by increasing its susceptibility to proteolysis. The approach described here should be generally useful in the rational design of bacterial strains producing cell metabolites.

References

Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64:2240–2246

Bradbury SL, Jakoby WB (1972) Glycerol as an enzyme-stabilizing agent: effects on aldehyde dehydrogenase. Proc Natl Acad Sci USA 69:2373–2376

Davidson BE, Hudson GS (1987) Chorismate mutase-prephenate dehydrogenase from *Escherichia coli*. Methods Enzymol 142:440–450

Doroshenko V, Airich L, Vitushkina M, Kolokolova A, Livshits V, Mashko S (2007) YddG from *Escherichia coli* promotes export of aromatic amino acids. FEMS Microbiol Lett 275:312–318

Flynn JM, Levchenko I, Seidel M, Wickner SH, Sauer RT, Baker TA (2001) Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. Proc Natl Acad Sci USA 98:10584–10589

Herman C, Thevenet D, Bouloc P, Walker GC, D'Art R (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). Genes Dev 12:1348–1355

Imaizumi A, Airikh LG, Doroshenko VG, Tsyrenzhapova IS (2007) The method for producing of L-amino acid. WO 2007/088977 A1, PCT/JP2007/051815

Keiler KC, Waller PR, Sauer RT (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science 271:990–993

Pittard J, Camakaris H, Ji Yang (2005) The TyrR regulon. Mol Microbiol 55:16–26