Metabolic Engineering of *Aspergillus niger* for production of aromatic compounds

Takasumi HATTORI

Research Collaboration and Promotion Center, Waseda University

Research aims

A filamentous fungus *Aspergillus niger* is capable of high-yield production of citric acid from molasses and other raw materials containing saccharides, and widely used for industrial production of citric acid, which reaches 1.6 million tons per year in the world¹). On the other hand, aromatic compounds are important precursors of many useful polymers, pharmaceuticals, and agrochemicals, etc. Presently, the aromatic compounds are mostly prepared from petroleum. However, in near future, production of aromatic compounds from renewable raw materials would be highly desirable from the view point of building a low-carbon society. In this study, we examine the possibility that aromatic compounds can be produced from glucose, a renewable carbon source, in *A. niger*, using fungal type III polyketide synthases.

Methods

Two fungal type III polyketide synthase (PKS) genes, AB206758 $(csyA)^{2}$ and XM_001390834 (An-csyA), were amplified by RT-PCR from *Aspergillus oryzae* RIB40 and *A. niger* NRRL328, respectively. These genes are cloned into the pET-21a(+) vector (Merck, Darmstadt, Germany), and *Escherichia coli* BL21 (DE3) was transformed with the recombinant plasmids. The recombinant PKS proteins were produced by culturing the transformed *E. coli* strains for 16 hours at 16°C. For transformation of *A. niger*, a *pyrG*⁻ mutant of citric acid-producing *A. niger* strain WU-2223L was co-transformed with each of the PSK genes cloned in the pNAN8142 vector³⁾ and a fragment of *pyrG* gene by protoplast-PEG method. The standard conditions for citric acid production were essentially the same as described previously⁴⁾.

The reaction mixture for the assay of PKS contains 125 mg/l of purified PKS obtained through Ni²⁺-affinity

chromatography, 0.3 mM starter substrate, and 0.7 mM malonyl-CoA in 50 mM K_2 HPO₄-KH₂PO₄ buffer (pH 7.0). The reaction was performed at 30°C for 16 hours.

The extracellular metabolites and reaction products were extracted from supernatant by ethylacetate-HCl, dried, resuspened in methanol, and analyzed by HPLC. The ODS-100V column (Tosoh, Tokyo, Japan) and PDA (SPD-M20A, Shimadzu, Kyoto, Japan) or ESI-IT-MS (LCQ-Deca, ThermoFishier, MA, USA) detectors were used for the HPLC analyses.

The intracellular metabolome analysis was performed at Human Metabolome Technology Inc., Tsuruoka, Japan.

Results

In this study, we focused on PKS as a key enzyme for production of aromatic compounds (Fig. 1), and cDNAs of A. oryzae PKS gene (csyA) and its homologue from A. niger (An-csyA) were cloned for overexpression of the genes. The deduced amino acid sequence of the An-csvA gene product contains the residues and active centers that are conserved among PKSs (Fig. 2). To examine the enzymatic activity of the An-cysA gene product, the recombinant An-CsyA protein was incubated with each of fifteen different fatty acyl-CoAs (a starter substrate) and malonyl-CoA (an elongation substrate). The LC-MS/MS analysis of extracted reaction mixture revealed structures of reaction products as shown in Table 1. The reaction products were pyrone-type polyketides, which are made up of a molecule of a starter substrate (C2-C12 fatty acyl-CoA) and 2-5 molecules of the elongation substrate (malonyl-CoA). Therefore, An-CsyA is a novel type III PKS capable of using a smaller starter substrate, a property that is suitable for the aromatic compounds synthesis⁵⁾.

The genes, *An-csyA* and *csyA*, were introduced into *A*. *niger* WU-2223L and placed under the control of a promoter for high level expression. The transcripts of *An-csyA*



Fig. 1 Synthesis of aromatic compounds from renewable glucose by A. niger.

G.h	2PS	1	MGSYSSDDVEVIREAGRAQGLATILAIGTATEPNCVAQADYADYYFRVTKSEHMVDLKEKFKRICEKTAIKKEYLALTEDYIQENETMCEFMAES	95
M.s	CHS	1	MVSVSEIRKAQRAEGPATILAI <mark>G</mark> TANPANCVEQSTYPDFYFKITNSEHKTELKEKFQRMCDKSMIKR <mark>y</mark> MYLTEEILKENPSVCEYMAPS	90
N.C	ORAS	1	MAASTVAGELGLSITGLGVQYPPYSLGPDAIDILSKRYHPESPAMKKVLAINRYTGIDQRSSIGNPDHPLVNKPNPPT	78
R.p	ALS	1	MADVLQEIRNSQKASGPATVLAIGTAHPPTCYPQADYPDFYFRVCKSEHMTKLKKKMQFICDRSGIRQRFMFHTEENLGKNPGMCTFDGPS	91
A.a	OKS	1	MSSLSNASHLMEDVQGIRKAQRADGTATVMAIGTAHPPHIFPQDTYADFYFRATNSEHKVELKKKFDRICKKTMIGKRYFNYDEEFLKKYPNITSFDEPS	100
A.n	AnCsyA	1	MGNTFSASP-SDGQPPKQQVPLKGDEAVITGMGTEWESRLVVPEELSDYAKKIYPDNPPWLKAMLKINTQTGTETRPVVDLWDDERWLGTSPET	93
G.h	2PS	96	INARQDLVVTGVPMIGKEAAVKAIDEWGLPKSKITHLIFCITAGVDMEGADYQLVKLLGISPSVKRYMLYQQGGAAGGTVLRLAKDLAENNKGSRV	191
M.s	CHS	91	LDARQDMVVVEVPRIGKEAAVKAIKEWGQPKSKITHLIVCTISGVDMEGADYQLIKLLGURPYVKRYMMYQQGGGFAGGTVLRLAKDLAENNKGARV	186
N.C	ORAS	79	VKELHEVFMSDGVPLAVEASRKAMABARLVPAQTTHMVSTTCTDSANEGYDHYVAKELGUSDRLEKVLLHGIGSSGGLAALRTAANLCLGHTARGKPARI	178
R.p	ALS	92	LNARQDMLIMEVPKLGAEAAEKAIKEWGQDKSRITHLIFCTTTSNDMEGADYQFATIFGINPGVSRTMVYQQGGFAGGTVLRLVKDIAENNKGARV	187
A.a	OKS	101	LNDRQDICVPGVPALGADAAVKAIADWGRPKSEITHLVFCTSCGVDMESADFQCAKLLGLRTNVNKYCVYMQGCYAGGTVMRYAKDLAENNRGARV	196
A.n	AnCsyA	94	TEDVDTEFRKFSVELSKNAALKALRESNIEPSSITHIVSVTVTNGGAEGFDQLVARELGLSPTAERILLSGIGGGGCAALRVASTIASAATYRKQEARI	193
			197 215 256 265	
G.h	2PS	192	IIVCSEITAILFHGPNENHLDSLVAQALEGDGAAALIVGSGPHLAVERE-IFEIVSTDQTILEDTEKAMKHHLREGELTFQLHRDVELMVAKNIENA	287
M.s	CHS	187	DVVCSDVTAVTFRGPSDTHLDSLVGQALEGDGAAALIVGSDPVPEIEKP-IFEMVWTAQTIABDSEGAIDGHLREAGLTFHLLKDVPGIVSKNIDKA	282
N.C	ORAS	179	IVLALEVSTIMVRSELESIDALQETRIGIALESDCESAVILSNGIGEAPGKEAIYDLLGWENRVIEDSEHDLGEDVDPMGWKMVLSPRVEVLAKASLQPT	278
R.p	ALS	188	DVVCSEIVAFAFRGPHEDHIDSLIGQLLEGDGAAALVVGTDIDESVERE-IFQIMSATQATIENSLHTMALHLTEAGLTFHLSKEVEKVVSDNMEEL	283
A.a	OKS	197	DVVCABLTIIGLRGPNESHLDNAIGNSLEGDGAAALIVGSDPIIGVEKP-MFEIVCAKQTVIBNSEDVIHLHMREAGLMFYMSKDSPETISNNVEAC	292
A.n	AnCsyA	194	UVVACELCSIHLRAELHASSQSGMTGVAPALFSDCAAALVVCNPLGMSDKTEKQFAIVDQRTGVTEGTLDEMSMTVSTHEFIASISKNIEKLAVASIQAP	293
			303 <u>336</u> <u>338</u>	
G.h	2PS	288	AEKALSPLGITDWNSVFWMVHPGGRAILDQVERKLNLKEDKLRASRHVLSEYGNLISACVLFIIDEVRKRSMAEGKSTTGEGLDCGVLFGFGPG	381
M.s	CHS	283	LVEAFQPLGISDYNSIFWIAHPGGPAILDQVEQKLA <mark>H</mark> KPEKMRATREVLSEY <mark>GN</mark> MS <mark>S</mark> ACVLFILDEMRKKSTQDGLKTTGEGLEWGVLFGFGPG	376
N.C	ORAS	279	YADLLSSLQDQLPSSYQKPADFDMAMHPGGATILSGAESAMGUTPEHMRASYDRYINHGNSSSATIFSVLNRL <mark>R</mark> EKDMDALAPG-GKVKEYVVGCAFGPG	377
R.p	ALS	284	MLEAFKPLGITDWNSIFWQVHEGGRAILDKIEEKLELTKDKMRDSRYILSEYGNLTSACVLFVMDEMRKRSFREGKQTTGDGYEWGVAIGLGPG	377
A.a	OKS	293	LVDVFKSVGMTPPEDWNSLFWIPHPGGRAILDQVEAKLKURPEKFRATRTVLWDCONMVSACVLYILDEMRRKSADEGLETYGEGLEWGULLGFGPG	389
A.n	AnCsyA	294	FQSLIQSNGMSSASPTDFHMAL <mark>HEGG</mark> KAVIQGAQDALNHPDDALAASKEIYRTK <mark>GN</mark> TS <mark>S</mark> VAVLAVLEKV <mark>R</mark> GLKLQSPNVWACSFGEG	380
G.h	2PS	382	MTVDTVVHRSVRVTAAVANGN	402
M.s	CHS	377	LEIDTVVURSVAI	389
N.C	ORAS	378	INVEMCMEKRMNAPARTTTGLDTPPETDDSEGPGPGSSAGSDDGESIEGGEKEEKFINEALDNVELD	445
R.p	ALS	378		391
A.a	OKS	390		403
A.n	AnCsyA	381	LTTOMTLURRMV	392

Fig. 2 Comparison of the deduced amino acid sequences of *A. niger* An-CysA and other type III PKSs. G.h 2PS, *G. hybrida* 2PS; M.s CHS, *M. sativa* CHS; N.c ORAS, *N. crassa* ORAS; R.p ALS, *R. palmatum* ALS; A.a OKS, *A. arborescens* OKS; A.n AncsyA, *A. niger* An-CysA. The critical active-site residue 197 is marked in bold number, the active center (Cys164, Phe215, His303, Asn336) are marked in italicized numbers, and the residues lining the active site (Gly256, Phe265, Ser338) are marked in underlined numbers.

Table	1.	Polyketides	synthesized	by	reactions	with A	An-CsyA
		~		~			~

Starter substrate	Side chain	Triketide pyrone ¹⁾	Tetraketide pyrone ²⁾	Pentaketide pyrone ³⁾	Hexaketide pyrone
Acetyl-CoA	R=CH ₃	+	+	_	-
Acetoacetyl-CoA	R=CH ₃	+	+	_	_
Malonyl-CoA	R=CH ₃	+	+	_	_
Methylmalonyl-CoA	R=CH(CH ₃)COOH	+	_	_	_
Isobutyryl-CoA	$R = CH(CH_3)_2$	+	+	_	_
Benzoyl-CoA	$R = C_6 H_5$	+	+	_	_
Butyryl-CoA	$R = (CH_2)_2 CH_3$	+	_	_	_
Hexanoyl-CoA	$R = (CH_2)_4 CH_3$	+	+	-	-
Octanoyl-CoA	$R = (CH_2)_6 CH_3$	+	+	_	_
Decanoyl-CoA	$R = (CH_2)_8 CH_3$	+	+	+	$+^{4)}$
Lauroyl-CoA	$R = (CH_2)_{10}CH_3$	+	+	+	$+^{4),5)}$
Myristoyl-CoA	$R = (CH_2)_{12}CH_3$	_	_	_	-



or *cysA* were detected in transformants by RT-PCR analysis. The transformants were then cultivated under the conditions for citric acid production for 7 days, and the extracellular metabolites were extracted and analyzed. However, we detected no remarkable accumulation of polyketides in the cells (data not shown).

The metabolome analysis of *A. niger* WU-2223L cells after 7 days cultivation under the conditions for citric acid production revealed the intracellular accumulation of acetyl-CoA (0.3 nmol/g-wet cell). But malonyl-CoA, which can serves as an elongation substrate in An-CysA-mediated reaction, was not detected in the cells.

Conclusion

In this study, we examined the properties of a novel type III PKS from *A. niger* (An-CysA). The An-CysA exhibited activities towards many different fatty acyl-CoAs and produced pyrone-type polyketides made up of a molecule of C2–C12 fatty acyl-CoA and 2–5 molecules of malonyl-CoA. The simple overexpression of these PKS genes in *A. niger* did not resulted in the production of polyketides under the conditions described previously for citric acid production.

The pyrone-type polyketides are precursors of resolcinoltype polyketides, which are aromatic compounds. At present, we are attempting to modify the metabolic pathways in *A. niger* to increase intracellular levels of malonyl-CoA for production of polyketides.

References

- Kirimura, K., Honda, Y., and Hattori, T. (2011) Citric Acid, p. 135– 142. *In* Moo-Young, M., Butler, M., Webb, C., Moreira, A., Grodzinski, B., Cui, Z. F., and Agathos, S. (eds.), Comprehensive Biotechnology, 2 nd ed. Elsevier
- Seshime, Y., Juvvadi, P. R., Kitamoto, K., Ebizuka, Y., Nonaka, T., and Fujii, I. (2010) *Aspergillus oryzae* type III polyketide synthase

CsyA is involved in the biosynthesis of 3,5-dihydroxybenzoic acid. *Bioorg. Med. Chem. Lett.* **20**: 4785–4788.

- 3) Minetoki, T., Kumagai, C., Gomi, K., Kitamoto, K., and Takahashi, K. (1998) Improvement of promoter activity by the introduction of multiple copies of the conserved region III sequence, involved in the efficient expression of *Aspergillus oryzae* amylase-encoding genes. *Appl. Microbiol. Biotechnol.* **50**: 459–467.
- Hattori, T., Kino, K., and Kirimura, K. (2009) Regulation of alternative oxidase at the transcription stage in *Aspergillus niger* under the conditions of citric acid production. *Curr. Microbiol.* 58: 321–325.
- Hansen, C. A., and Frost. J. W. (2002) Deoxygenation of polyhydroxybenzenes: an alternative strategy for the benzene-free synthesis of aromatic chemicals. J. Am. Chem. Soc. 124: 5926–5927.