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Recent Research on the Development of Microbial Strains for Amino-Acid Production

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Introduction

Amino acids are now widely used for their flavour-enhancing, nutritional and physiological properties, involving the food, animal feed and pharmaceutical industries. The annual production of amino acids in the world has an estimated value of about US$1·5 × 10^9 or over 500 000 t in 1980 (Kitamura, 1982). Monosodium glutamate, a well-known flavour enhancer, is still the predominant product, with a world-wide annual production of about 340 000 t. About 120 000 t of DL-methionine and 34 000 t of L-lysine are produced for animal-feed additives. The demand for other amino acids is small, mostly in the pharmaceutical field. However, the demand will grow with progress in production technology to reduce costs, and on further expansion of their uses (*Table 1*); for example, tryptophan and threonine may be used as animal-feed additives.

Table 1. World supply (t) of amino acids (1980). From Kitamura (1982)

Fermentation		Enzymatic synthesis	
L-Glu	340000	L-Asp	450
L-Lys	34000	L-Met	150
L-Arg	500	L-Ala	130
L-Gly	500	Chemical synthesis	
L-His	200	DL-Met	120000
L-Thr	160	Gly	6000
L-Ile	150	DL-Ala	2000
L-Val	150	L-DOPA	200
L-Pro	100	L-Trp	200
L-Cit	50	L-Phe	150
L-Orn	50	Extraction	
L-Ser	50	L-Cys, L-$(Cys)_2$	700
		L-Leu	150
		L-Asn	50
		L-Hyp	50
		L-Tyr	50

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Of the various methods for production of amino acids, e.g. protein hydrolysis, chemical synthesis and microbiological methods, the latter now dominate industrial production, especially for optically active amino acids. The selection of new microbial strains has been very important for the establishment and improvement of amino-acid production. Considerable efforts have also been made to improve process control and operation.

Within normal wild-type microbial cells, synthesis of amino acids is restricted to the amounts needed for cell growth. Wasteful overproduction is prevented by feedback inhibition and repression. Accordingly, the overproduction of amino acids essential for industrial purposes requires the release of feedback inhibition and/or repression by environmental control of the fermentation processes, or genetic modification of micro-organisms. Until recently, genetic alteration of amino-acid producers was mostly by mutagenesis and selection of mutants to give various auxotrophic and regulatory strains with improved characteristics. Now, the newly developed technology of genetic manipulation *in vivo* (such as transduction, cell fusion) and *in vitro* (such as DNA recombination) is also applied to the construction of new types of amino-acid producer. Mutagenesis has provided new genetic characters such as drug resistance, the techniques of transduction and cell fusion have been used for the recombination of desired genetic characters, while the technique of DNA recombination has been used for the amplification of gene copy numbers.

This report describes the practical application of these new techniques to amino-acid overproduction.

Genetic manipulation *in vivo*

Genetic manipulation *in vivo*, such as transduction and cell fusion, has been applied to the genetic analysis of many bacteria and has contributed to the elucidation of the molecular basis of regulatory mechanisms of microbial metabolism. By these techniques, mutations carried by a donor strain can be combined with the other mutations carried by the recipient strain. Techniques of transduction and cell fusion therefore have been applied effectively also to the improvement of the genetic properties of microbial strains.

APPLICATION OF CELL-FUSION TECHNIQUE

Akamatsu and Sekiguchi (1982) and Kaneko and Sakaguchi (1979) were the first in Japan to establish the basis of cell fusion in *Bacillus* and *Brevibacterium* respectively, but they did not apply the technique to strains used to produce amino acids. Tosaka *et al.* (1982) successfully applied the technique to microbial breeding programmes for lysine and threonine using *Brevibacterium lactofermentum*.

Lysine-producing strain

Lysine productivity was improved stepwise by successive mutation in *Brevibacterium lactofermentum*. Some of the lysine producers obtained by such

repeated mutations had lowered rates of glucose consumption and growth. The technique of cell fusion was applied to get better strains with improved fermentation rates (Tosaka *et al.*, 1982).

Two kinds of strain with different properties were used as parent strains: parent A was a glutamic-acid producer resistant to decoyinine (Dec) and ketomalonate (KM), which was able to consume glucose rapidly (decoyinine is an analogue of adenosine). Parent B was a lysine producer with a low glucose consumption rate, resistant to *S*-(β-aminoethyl)-L-cysteine (AEC), γ-chlorocaprolactam (CCL), γ-methyllysine (ML), sensitive to β-fluoropyruvate, and requiring L-alanine (Tosaka *et al.*, 1982). *Figure 1* is a flow diagram of intraspecific fusion between parent A and parent B, and *Figure 2*

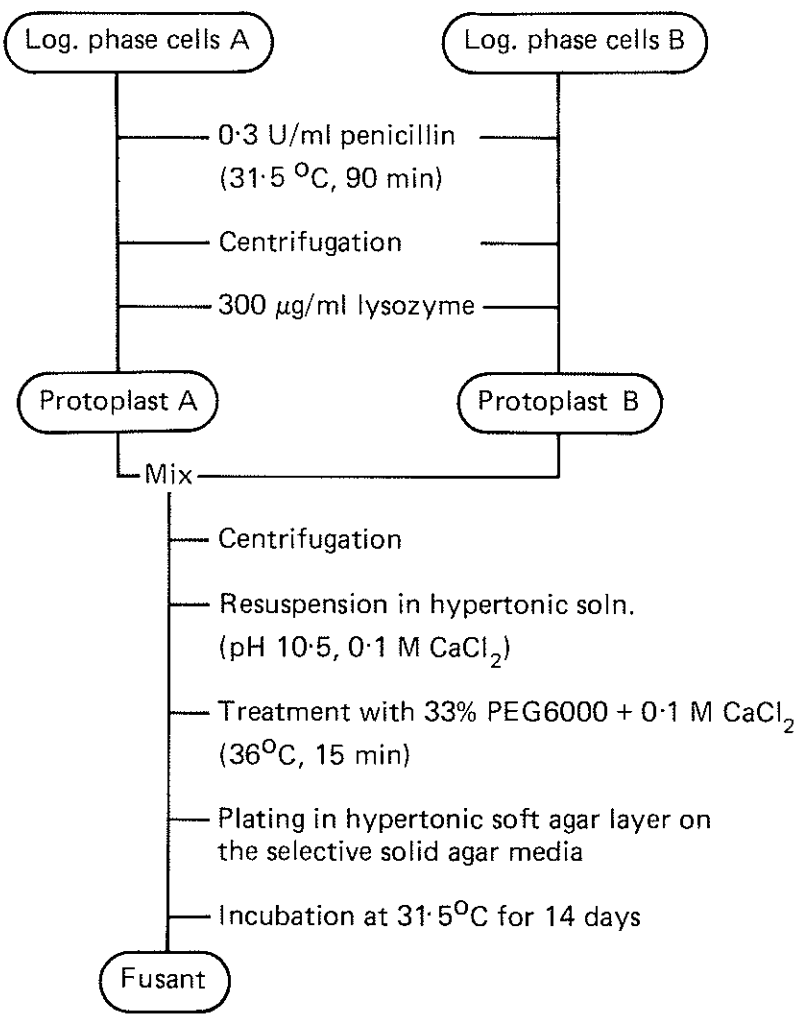


Figure 1. Diagram of cell fusion procedure in *Brevibacterium*. Tosaka *et al.* (1982).

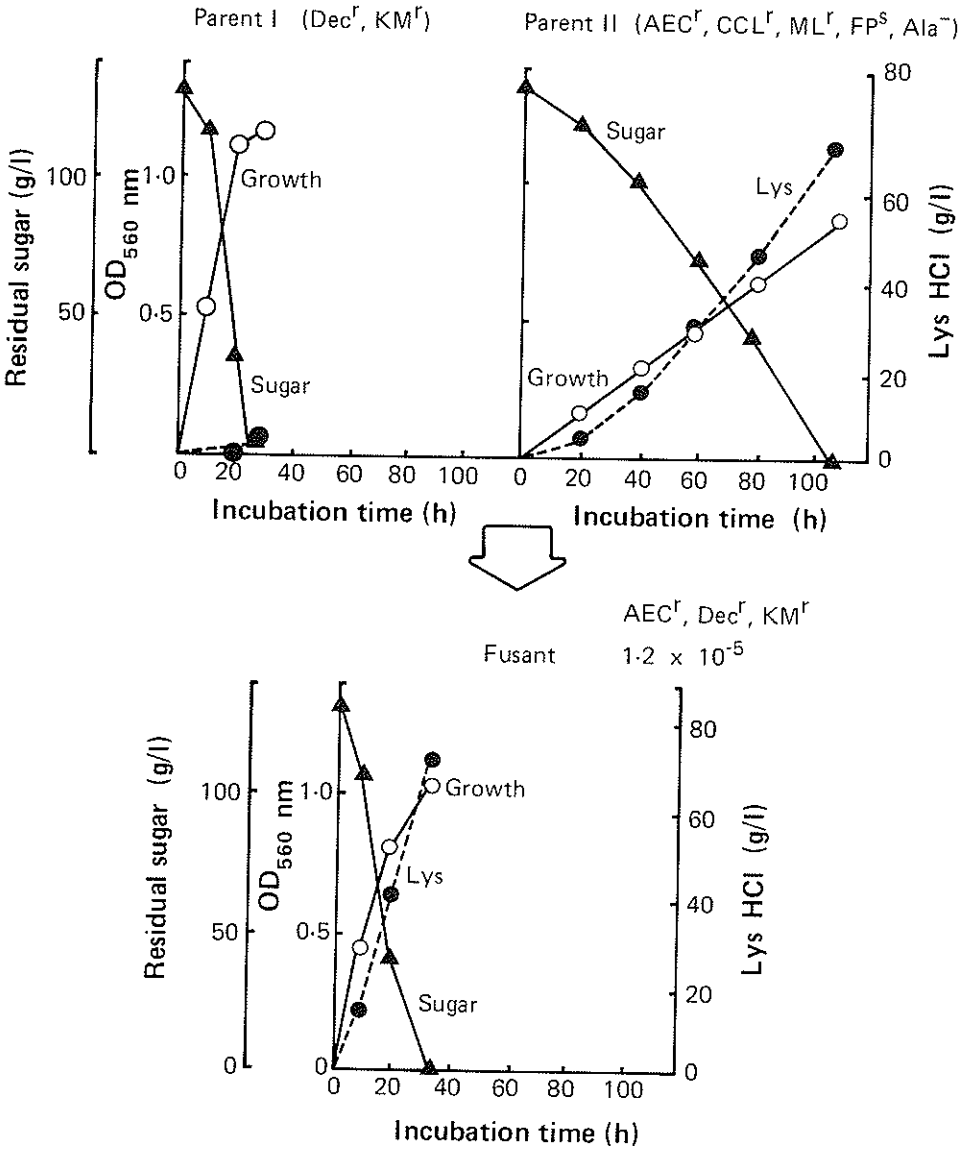


Figure 2. Time course of lysine fermentation by fusant and parent strains in a jar fermenter. Dec: decoyinine; KM: ketomalonate; AEC: *S*-(β-aminoethyl)-L-cysteine; CCL: γ-chlorocaprolactam; ML: γ-methyllysine; FP: β-fluoropyruvate. (Tosaka *et al.*, 1982).

shows a typical example of fermentation of fusant and parent strains. AEC, DC and KM resistances were used as selection markers. Among the fusants, one strain appeared at a frequency of $1{\cdot}2 \times 10^{-5}$ and consumed 130 g/ℓ of initial glucose in 30 h, thus showing a reduction of about 70 h compared with the parent lysine producer. The rate of glucose consumption of the fusant was increased threefold from 0·13 to 0·40 g glucose/h/g dry cell weight.

Threonine-producing strain

Tosaka *et al.* (1982) also used the cell-fusion technique to obtain another type of threonine producer from a threonine- and lysine-producing strain of *Brevibacterium lactofermentum*. Strain AJ11786 (threonine and lysine producer) and strain AJ11787 (lysine auxotroph) were fused at a frequency of 2/10 000 lysine-dependent organisms. The parent strain AJ11786 produced 13 g/ℓ of threonine and 6 g/ℓ of lysine simultaneously in culture broth, whereas one of the lysine-auxotroph fusants produced 18 g/ℓ of threonine only. The genetic property of lysine auxotrophy of AJ11787 may thus be transferred to strain AJ11786 (*Figure 3*).

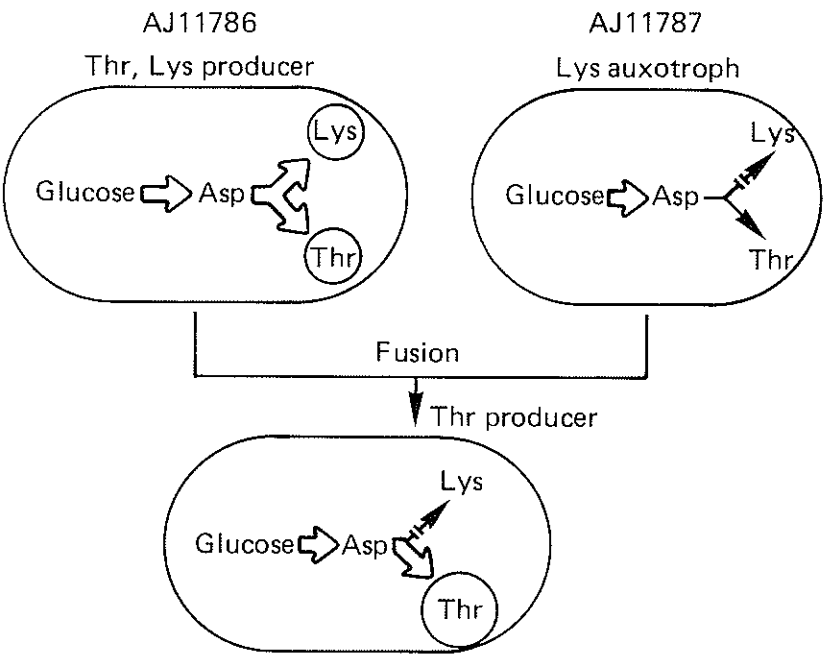


Figure 3. Breeding a threonine producer in *Brevibacterium lactofermentum* by the cell-fusion technique. Tosaka *et al.* (1982).

APPLICATION OF TRANSDUCTION TECHNIQUE

Kisumi (1982) applied the technique of transduction to *Serratia marcescens* in order to construct a new class of producers of histidine, arginine, threonine and isoleucine.

Histidine-producing strain

Histidine is synthesized from phosphoribosylpyrophosphate (PRPP) and ATP as shown in *Figure 4*. The nine structural genes coding for all the biosynthetic

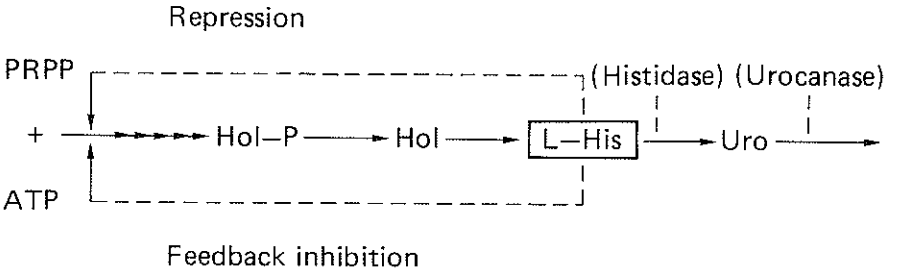


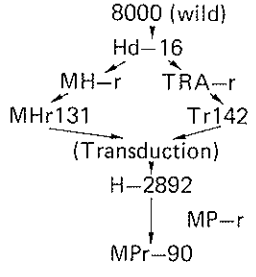
Figure 4. Biosynthetic pathway of histidine in *Serratia marcescens*. PRPP: phosphoribosylpyrophosphate; Hol-P: L-histidinol phosphate; Hol: L-histidinol; Uro: urocanate. (Kisumi, 1982).

enzymes belong to a single operon in *Escherichia coli*. Histidine biosynthesis is controlled by feedback inhibition of phosphoribosyltransferase (ATP phosphoribosyltransferase, EC 2.4.2.17), as the first enzyme, and by feedback repression of the nine enzymes. Histidine is degraded to urocanic acid by histidase (histidine ammonia-lyase, EC 4.3.1.3) while urocanic acid in turn is converted into glutamic acid. Kisumi *et al.* (1977) obtained strains of *Serratia marcescens* which produce histidine, by destroying the feedback control mechanism and removing histidase formation in the parent strain.

First, a histidase-less mutant Hd-16 was isolated from the wild strain. Then, two types of regulatory mutants were independently derived from strain Hd-16. Strain MHr131, a mutant resistant to 2-methylhistidine (MH), carried a mutation leading to densensitization of the first enzyme to feedback inhibition. Strain Tr142, a mutant resistant to 1,2,4-triazole-3-alanine (TRA), had a mutation causing derepression of histidine biosynthetic enzymes. These single regulatory mutants produced only a small amount of histidine. Subsequently, the transductional cross was performed between strain Tr142 (donor) and MHr131 (recipient). TRA-resistant transductants were selected and strain H-2892 was found to carry the two regulatory mutations. This strain produced 20 g/ℓ of histidine, but was unstable in the production cycle, as shown by subculture. Strain H-2892 grew more slowly than the other strains. This slow growth was thought to result from low intracellular ATP concentration, because the growth was accelerated by adenine. It was therefore surmised that the acceleration of ATP formation would allow rapid growth, resulting in stabilization of the ability to produce histidine. Strain MPr-90, isolated as a 6-methylpurine-resistant mutant, in fact showed more rapid growth and was stable during histidine production (Kisumi, 1982) (*Table 2*).

Arginine-producing strain

Arginine biosynthesis is also regulated by both feedback inhibition and repression as shown in *Figure 5*. The two regulatory mutations were independently selected. A mutation leading to densitization of *N*-acetylglutamate synthetase (amino-acid acetyltransferase, EC 2.3.1.1) to feedback inhibition



Strain	Biosynthesis			Generation time (h)	L-Histidase produced (mg/ml)
	Repression	Inhibition	Histidase		
8000	+	+	+	1·0	0
Hd–16	+	+	–	1·0	0
MHr131	+	–	–	1·2	1·0
Tr142	–	+	–	1·3	1·5
H–2892	–	–	–	2·6	20·0
MPr–90	–	–	–	1·9	22·3

Table 2. Construction of a histidine-producing strain. From Kisumi (1982)

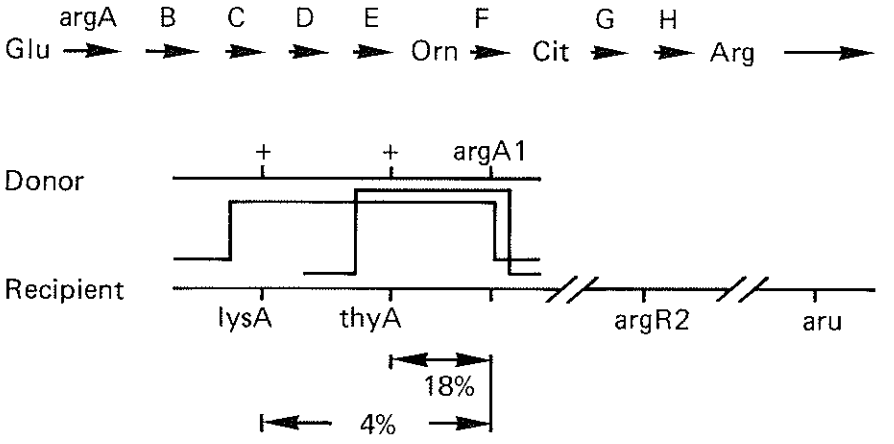


Figure 5. Construction of an arginine-producing strain. argA1: lack of feedback inhibition of *N*-acetylglutamate synthetase; argR2: lack of repression of Arg biosynthetic enzymes; aru: inability to degrade arginine; Glu: L-glutamic acid; Orn: ornithine; Cit: citrulline; Arg: arginine (Kisumi, 1982).

was denoted as argA1. Another mutation causing derepression of arginine biosynthetic enzymes was denoted as argR2. To combine these two mutations, argA1 was co-transduced with $thyA^+$ or $lysA^+$ as a selective marker into the strain which carried argR2 and was incapable of degrading arginine (Kisumi, Takagi and Chibata, 1978). Strain AT419, constructed thus, produced about 30 g/ℓ of arginine HCl, whereas the parent strains produced only a small amount of this amino acid. However, the production of strain AT419 was not stable, when this strain was subjected to subculture. This instability was assumed to be due to slow growth and high mutability of the argA1 allele. To stabilize productivity, Takagi, Kisumi and Chibata (1979) isolated derivatives, growing rapidly on minimum nutrient agar medium, from strain AT419 (*Table*

Table 3. Arginine-producing strain of *Serratia marcescens*. From Takagi, Kisumi and Chibata (1979)

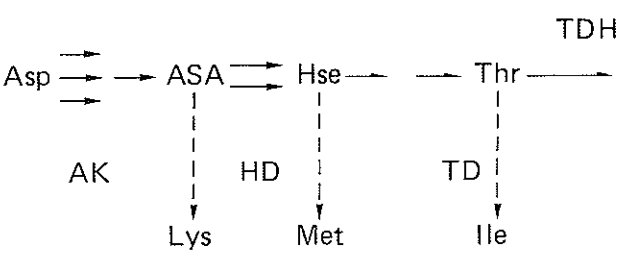
Strain	Genotype*	L-Arginine (mg/ml)
8000	wild type	0
PA1012	aru	0
PA2028	aru, lysA	0
PA3179	aru, lysA, argR1	3·4
PA8146	aru, argR2	3·6
PA8172	aru, argR2, thyA	3·6
RA3236	proA/B, argD, argR3	0
RA4240	proA/B, argD, argR3, argA,	0
AT404	aru, argR1, argA1	25·3
AT419	aru, argR1, argA1	25·4
AT428	aru, argR2, argA2	25·0

* Genetic symbols for chromosomal markers are those of Bachmann *et al.* (1976) *Bacteriol. Rev.*, **40**, 116. The symbol aru denotes utilization of L-arginine as a sole nitrogen source.

3). One of these derivatives, strain AT428, produced about 30 g/ℓ of arginine HCl even after the third subculture, and was therefore considered to be stable during arginine production. Furthermore, culture conditions for industrial production of this amino acid were studied using strain AT428. About 65 g/ℓ of arginine HCl was produced after 96 h of incubation (Kisumi, 1982).

Threonine-producing strain

Aspartokinase (AK) (EC 2.7.2.4) and homoserine dehydrogenase (HD) (EC 1.1.1.3) are rate-limiting for threonine biosynthesis. *Serratia marcescens* has three AKs and two HDs, all of which are feedback-controlled by threonine, isoleucine, methionine and lysine (singly or in combination) (Komatsubara *et al.* 1978a, b). Six regulatory mutations were independently selected by isolating mutants resistant to analogues of threonine, methionine and lysine (*Figure 6*). The $thrA_1$1 and $thrA_2$1 mutations led to lack of feedback inhibition of AKI and HDI: the hnrA1 and hnrB2 mutations led to derepression of these two enzymes. The hnrA1 mutation was linked to the *thr* locus but the hnrB2 mutation was not. A mutation causing lack of both feedback inhibition and repression of AKIII was denoted as lysC1. The etr-1 mutation caused derepression of AKII and HDII. By four transductional crosses, these mutations were combined in a single strain D-60 defective in threonine-



Enzyme	Gene	Inhibition	Repression
AK I	*$thrA_1$*	Thr	Thr + Ile
HD I	*$thrA_2$*	Thr	Thr + Ile
AK II	*metL*	–	Met
HD II	*metM*	–	Met
AK III	*lysC*	Lys	Lys

Figure 6. Threonine biosynthesis and degradation in *Serratia marcescens*. AK: aspartokinase; HD: homoserine dehydrogenase; TD: threonine deaminase; *$thrA_1$*: threonine-sensitive aspartokinase; *$thrA_2$*: threonine-sensitive homoserine dehydrogenase; ASA: aspartic semialdehyde; Hse: homoserine; Thr: threonine; Ile: isoleucine; Lys: lysine; Met: methionine; Asp: aspartate. (Komatsubara *et al.*, 1979).

degrading enzymes (threonine dehydrogenase (L-threonine 3-dehydrogenase, EC 1.1.1.103) and threonine deaminase (threonine dehydratase, EC 4.2.1.16)). Selective markers were *S*-(β-aminoethyl)-L-cysteine resistance for transfer of lysC1 mutation, Thr^+ ($thrB^+/C^+$) phenotype for construction of the $thrA_1$1, $thrA_2$1, and hnrA1 mutation, and Arg^+ ($argE^+$) phenotype for co-transduction of the etr-1 mutation (Komatsubara, Kisumi and Chibata, 1979a, b). In strain T-1026, three AKs and two HDs were released from both feedback inhibition and repression by means of the six regulatory mutations. This strain produced about 40 g/ℓ of threonine, whereas the other strains, lacking more than one of the six mutations, produced less than 30 g g/ℓ of threonine. Strain T-1026 was further improved to strain P–200, which had both isoleucine and methionine bradytrophies. Strain P–200 produced more than 40 g/ℓ of threonine in a simple fermentation medium (*Table 4*) (Kisumi, 1982).

DNA recombination technology

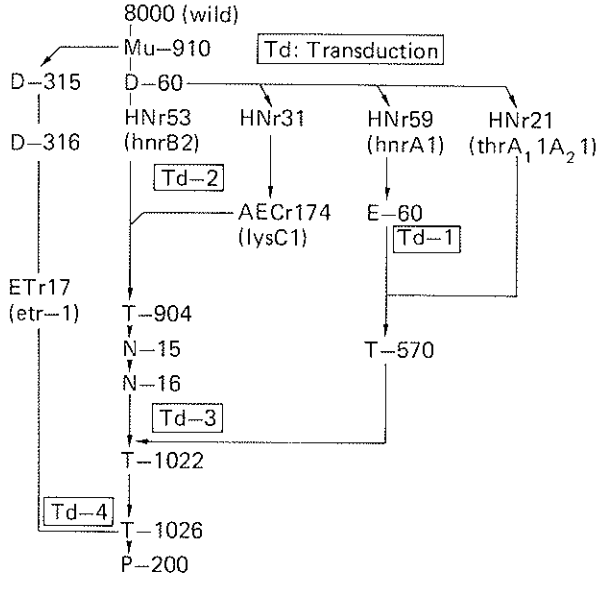
Of late, DNA recombination technology has been widely used in microbial genetics, and its application to the development of micro-organisms for amino-acid production is one important example. One possible approach is to think of increasing the activity of an enzyme of this biosynthetic pathway by amplifying the gene copy number encoding the enzyme. Such an increase in the gene copy number would lead to overproduction of amino acid if the synthesis of the enzyme varies in correspondence with the gene copy number and if the step involved is one of the limiting steps in the synthesis of the amino acid. Such experiments can already be performed using DNA recombinant techniques by cloning the relevant gene(s) on a plasmid present at a high copy number. These techniques have been employed successfully in genetic breeding programmes for threonine, tryptophan, lysine and proline producers.

THREONINE-PRODUCING STRAIN

Miwa *et al.* (1981) successfully applied DNA recombination technology to the genetic manipulation of a threonine producer from *Escherichia coli* βIM4 using a recombinant plasmid, pAJ294 which contained the genes *thr A, B, C* relating to threonine biosynthesis.

In *Escherichia coli* K-12, it has been shown that the threonine operon is mapped nearly zero min. of the chromosome and is composed of four structural genes (Cossart *et al.*, 1979; Cossart, Katinka and Yaniv, 1981).

The wild strain does not overproduce threonine, because of the metabolic regulatory processes which include feedback inhibition and multivalent repression not only by threonine but also by lysine, methionine and isoleucine. As the first step in the breeding of an overproducer, Shiio and Nakamori (1969) obtained mutants resistant to a threonine analogue, α-amino-β-hydroxyvaleric acid (AHV): next, some auxotrophic mutants were derived from these strains. One of them, βIM4 (AHV^r, Ile^-, Met^-, Pro^-, $thiamine^-$) produced 3–6 g/ℓ of threonine. In this strain, aspartokinase and homoserine dehydrogenase were released from the repression caused by threonine. This implies that the



Strain	Genotype						L-Threonine produced (mg/ml)
	lys	thr		hnr			
	C	A_1	A_2	A	B	etr	
D–60	+	+	+	+	+	+	0·1
HNr21	+	–	–	+	+	+	4·2
HNr53	+	+	+	+	–	+	0·7
HNr59	+	+	–	–	+	+	4·3
AECr174	–	+	+	+	+	+	7·4
Etr17	+	+	–	+	+	–	7·5
T–570	+	–	–	–	+	+	8·8
T–904	–	+	+	+	–	+	10·2
T–1021	–	+	+	+	–	–	28·9
T–1025	–	–	–	–	–	+	25·8
T–1026	–	–	–	–	–	–	40·3

Table 4. Process for transductional construction of threonine-producing strains. From Kisumi (1982). (–): lack, (+): positive, lysC: lysine-sensitive aspartokinase, $thrA_1$: threonine-sensitive aspartokinase, $thrA_2$: homoserine dehydrogenase, hnr: repression of threonine operon, etr: repression of aspartokinase and homoserine dehydrogenase.

productivity of threonine in *Escherichia coli* K-12 depends on the activities of these two enzymes regulated by the various amino acids mentioned above.

On the basis of these investigations, Miwa *et al.* (1981) chose βIM4 as the DNA donor and pBR322 as a vector plasmid. Chromosomal DNA fragments from βIM4 were combined with pBR322 DNA using T-4 ligase and then these hybrid plasmids were transformed to a threonine auxotrophic mutant derived from βIM4 and the transformants not requiring threonine were selected. One recombinant plasmid (pAJ294) containing the threonine operon was extracted from the transformants and then was retransformed to βIM4. The transformant No. 294 showed the highest productivity. The accumulation was 12 g/ℓ and the conversion yield from glucose was 40% in a shake-flask-scale fermentation.

Analysis of plasmid pAJ294 showed that the molecular weight was about 7·8 Md and the copy number was about 12. The amount of homoserine dehydrogenase coded by the *thrA* gene increased fivefold when the operon was amplified in the pAJ294 plasmid (*Figures 7 and 8*).

Further investigations were carried out under culture conditions using a mini-jar fermenter (Shimizu *et al.*, 1983). Of various culture conditions examined, the optimization of oxygen and substrate supply gave rise to a remarkable increase in productivity. In particular it was found that the dissolved oxygen (DO) level affected productivity, with the maximum productivity occurring at a DO level of over 15 kPa under these conditions (DO was in equilibrium with air). The growth of cells was partly inhibited; nevertheless the copy number of the plasmid seemed to be increased. The accumulation of threonine was 65 g/ℓ with 48% of the conversion yield from glucose.

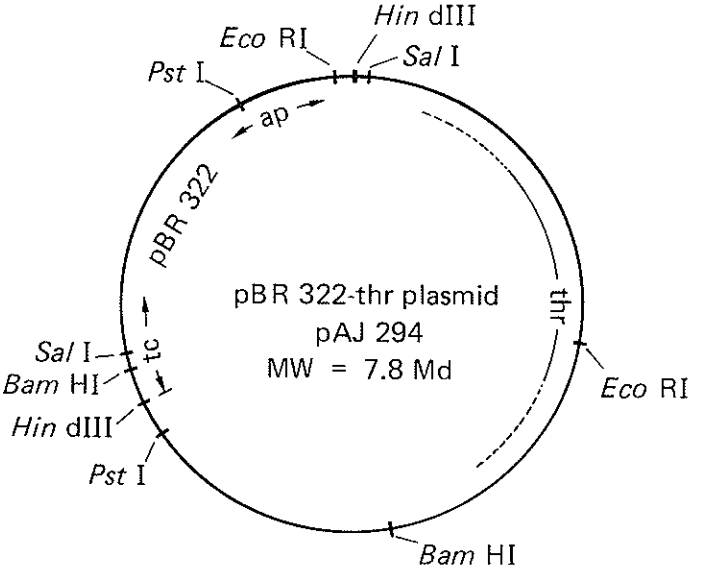


Figure 7. Plasmid pAJ 294 (Miwa *et al.*, 1981).

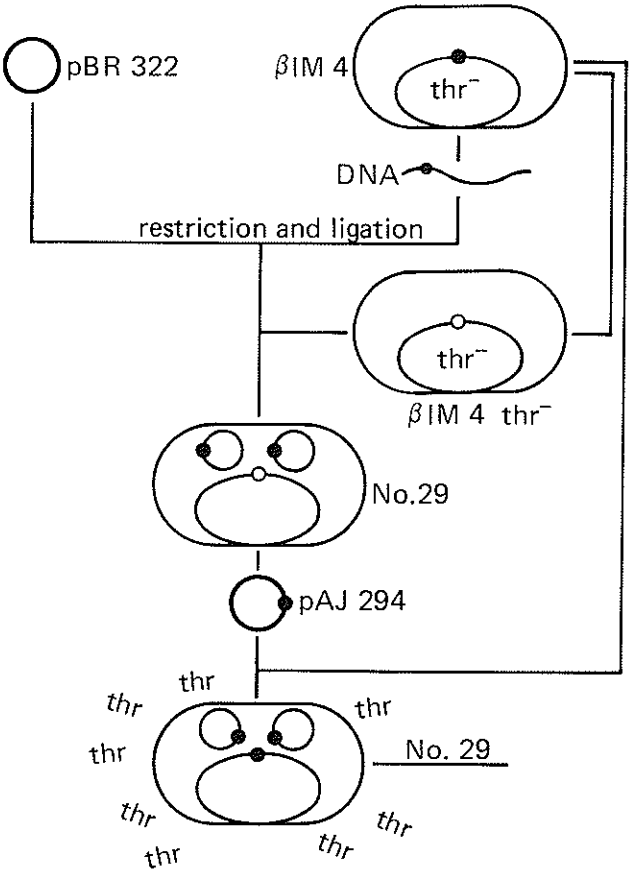


Figure 8. Construction of a threonine-producing strain by a DNA recombinant technique (Miwa *et al.*, 1981).

Plasmids are sometimes lost during cultivation so that the microbial characteristics controlled by such plasmids become unstable. This phenomenon restricts the commercial application of the plasmid-containing micro-organisms. Indeed, the plasmid-free cells of the threonine producer dominated during sequential subculturing. If the cells are cultured in a medium containing the antibiotics corresponding to the resistant factors conferred by the plasmid, only those cells which contain the plasmid can survive. This method, however, suffers from the drawback that relatively large amounts of antibiotic must be used, and therefore it is not well adapted to industrial exploitation.

Miwa, Nakamori and Momose (1982) have therefore developed a new method for stabilizing the characteristics of the strain containing a plasmid. They have genetically constructed the strain by introducing a new hybrid plasmid into a streptomycin-dependent mutant of the threonine producer. This plasmid incorporates a chromosomal DNA fragment which controls depend-

ence on streptomycin (streptomycin-independent gene). The growth of such a strain is not dependent on streptomycin, and therefore it can grow in a culture medium devoid of streptomycin. However, if the plasmid should be eliminated from the cell, it becomes dependent on streptomycin and cannot grow in a culture medium devoid of streptomycin.

The transformant thus obtained maintained a higher productivity of threonine during fermentation and the characteristics controlled by the plasmid were stably maintained in the medium devoid of streptomycin. It was stable for about two years on an agar slant not containing streptomycin.

TRYPTOPHAN-PRODUCING STRAIN

Aiba (1982) and Aiba, Tsunekawa and Imanaka (1982) derived a tryptophan producer from *Escherichia coli* by DNA recombinant techniques. They introduced a hybrid plasmid (pSC101-trp) carrying the *trp* operon into the strain AE1, which was the deletion mutant of the *trp* operon (trpAE1). They then obtained a 5-methyltryptophan-resistant mutant strain (M7) from transformant AE1/pSC101-*trp* by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; the activity of anthranilate synthase (EC 4.1.3.27) in strain M7 was not inhibited by tryptophan. Furthermore, they transformed the plasmid pSC101-trp115, which was extracted from strain M7, into the strain Tna, which was a *trp* repressor amber mutant and which was also deficient in tryptophanase (EC 4.1.99.1) and was derived from strain AE1. The activity of anthranilate synthase of this transformant Tna/pSC101-trp115-14 was also only slightly inhibited by tryptophan. *Figure 9* demonstrates the production of tryptophan in Tna/pSC101-trp115-14 cells in batch culture, feeding anthranilic acid continuously at a rate of 50 mg/ℓ/h after 7 h incubation; the yield of tryptophan was 6·2 g/ℓ.

Similarly, Tribe and Pittard (1979) described the production of tryptophan by *Escherichia coli*, using plasmid ColV *trp* or F′ *trp* (copy number, 1–3 per chromosome). Their data showed that 1 g of tryptophan per litre accumulated after 12·5 h incubation, the production rate being 0·08 g of tryptophan/ℓ/h. Their principal concern was with the use of various mutants of the host strain rather than plasmids.

Anderson and Spencer (1982) reported work with genes for the enzyme tryptophan synthase (EC 4.2.1.20) from *Escherichia coli*. Two of the genes, *trpA* and *trpB*, encode two subunits, α and β, which combine to form a very stable α2β2 complex. The α2 portion of the complex catalyses the conversion of indoleglycerol-P to indole. The β2 part combines serine with indole bound to the enzyme and catalyses the synthesis of tryptophan.

The first task was to splice the *trpAB* genes, together with hybrid *trp/lac* promoter, that is a promoter capable of activating both the tryptophan and the *lac* operons (Boer, Comstock and Vasser, 1983) into a plasmid. By adding compounds that induce higher production, the output of the enzymes was increased 230-fold. The enzymes made up approximately 20% of the total cell proteins. The next step was to use the extracted enzyme preparation to synthesize tryptophan. Indole and serine were added to the enzyme extract and

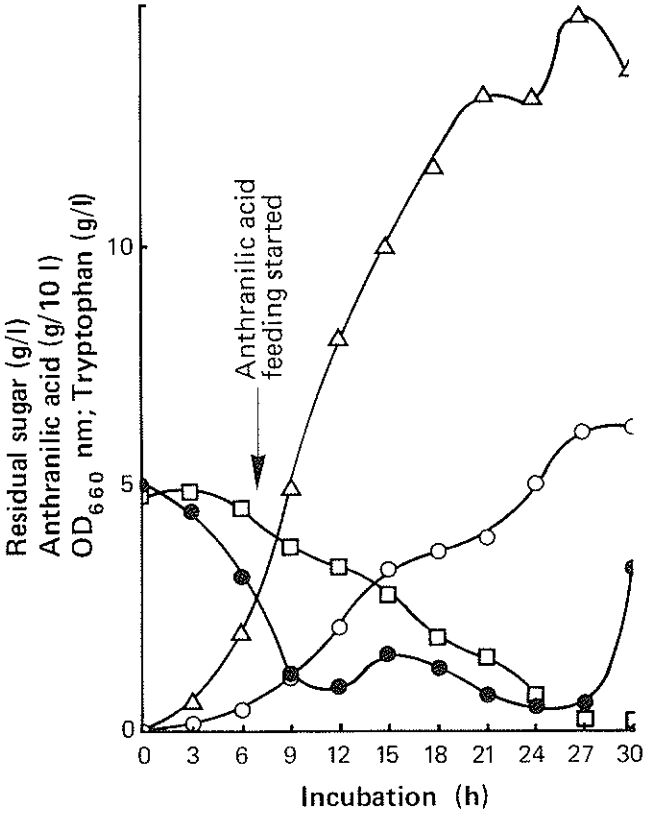


Figure 9. Batch culture of Tna/pSC101-trp115-14 in a mini-fermenter. Medium contained (per litre): glucose, 50 g; casamino acid, 10 g; anthranilic acid, 0·5 g; tetracycline, 10 mg; KH_2PO_4, 3 g; K_2HPO_4, 7 g; NH_4Cl, 3 g; $MgSO_47H_2O$, 0·2 g. The initial pH of the medium was adjusted to 7·0 with 2N NaOH. The total amount of anthranilic acid added to the medium was: 0·5 g (initial) + 50 mg/ℓ per hour for 20 h (feeding period) = 1·5 g. (Aiba, Tsunekawa and Imanaka, 1982).
□—□ Residual sugar; ●—● anthranilic acid; ○—○ tryptophan; △—△ OD_{660nm}

97–100% of the indole was converted into tryptophan at concentrations up to 78·2 g/ℓ, so much so that part of the tryptophan precipitated out.

LYSINE-PRODUCING STRAIN

Reverend *et al.* (1982) cloned separately several genes of the lysine biosynthetic pathway on the high copy number plasmid pBR322, and then introduced various plasmids each carrying one of the wild-type genes, *asd*, *dapA*, *dapB*, *dapD* and *lysA*, into strain TOC R 21 that overproduces lysine due to mutations altering the aspartokinase (EC 2.7.2.4.) reaction (*Figure 10*). The synthesis of lysine was studied in these different strains. It appears that only plasmids containing the *dapA* gene (encoding dihydrodipicolinate synthase, EC 4.2.1.52) lead to an increase in lysine production. This result allowed Reverend

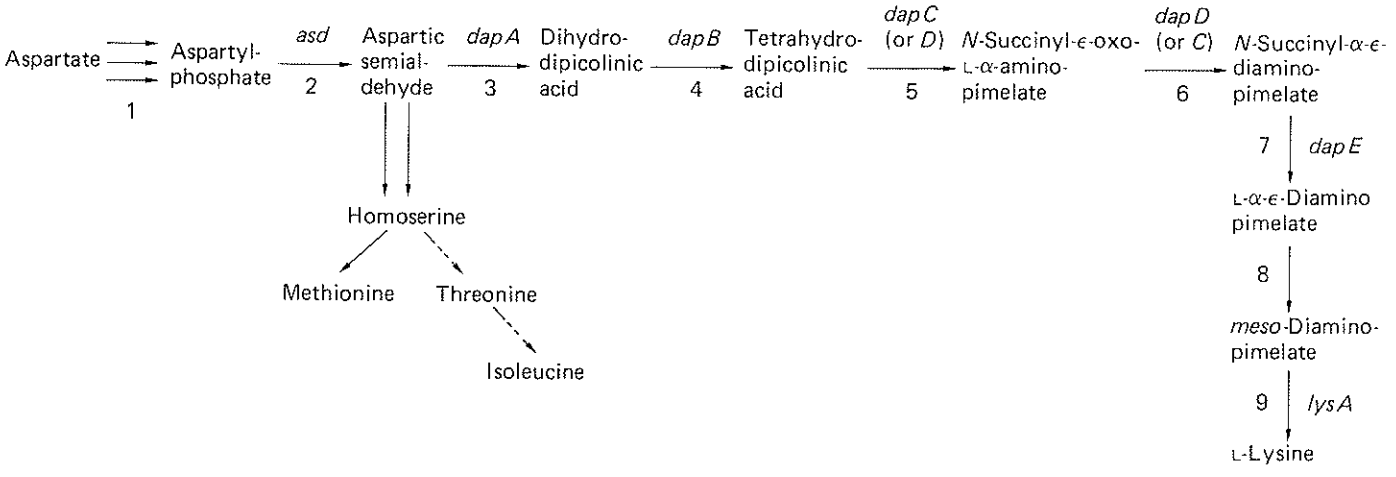


Figure 10. Lysine biosynthetic pathway. The numbers indicate enzymes and the corresponding genes.
1: aspartokinase I, *thrA*; aspartokinase II *metL*; aspartokinase III *lysC*; (EC 2.7.2.4)
2: aspartic semialdehyde dehydrogenase (ASA-dehydrogenase), *asd*; (EC 1.2.1.11)
3: dihydrodipicolinate synthase (DHDP-synthetase), *dapA*: (EC 4.2.1.52)
4: dihydrodipicolinate reductase (DHDP-reductase), *dapB*; (EC 1.3.1.26)
5: tetrahydrodipicolinate succinylase (THDP-succinylase), *dapC* or *D*;
6: *N*-succinyl diaminopimelate aminotransferase, *dapD* or *C*; (EC 2.6.1.17)
7: *N*-succinyl diaminopimelate desuccinylase (deacylase) *dapE*; (EC 3.5.1.18)
8: diaminopimelate epimerase (DAP-epimerase); (EC 5.1.1.7)
9: diaminopimelate decarboxylase (DAP-decarboxylase) *lysA*. (EC 4.1.1.20); (Reverend *et al.*, 1982).

et al. (1982) to identify this reaction as the limiting biosynthetic step in strain TOC R 21 and indicates that such a method of gene amplification can be used to improve strains overproducing amino acids (*Table 5*).

PROLINE-PRODUCING STRAIN

In *Escherichia coli*, proline is synthesized from glutamate via a series of three enzymic steps, γ-glutamyl kinase (EC 2.7.2.11), γ-glutamyl phosphate reductase (glutamate-semialdehyde dehydrogenase, EC 1.2.1.41) and 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) with an intervening spontaneous conversion of intermediates. Hayzer and Leisinger (1980) demonstrated that gene *proA* codes for γ-glutamyl phosphate reductase and *proB* for γ-glutamyl kinase, and showed that introduction of a ColE1 hybrid plasmid containing the *proA,B* region into a strain with a chromosomal deletion of *proA,B* led to threefold and 17-fold increases in the specific activities of γ-glutamyl kinase and γ-glutamyl phosphate reductase, respectively.

A dehydroproline-resistant mutant was derived from *Escherichia coli*, in which γ-glutamyl kinase, the first enzyme of the pathway, was no longer allosterically inhibited by proline. This mutant produced small amounts of proline. Then, by cloning the closely linked genes *proA* and *proB* from this mutant, Siewert and Boidol (1982) obtained recombinant plasmids which cause secretion of proline in *Escherichia coli* wild-type cells or increase the productivity of the mutants mentioned above. Improvement of the plasmids was achieved by adding the gene *proC*. High yields of proline could only be obtained with these plasmids in appropriate host cells. Most important is the use, as host cells, of mutants unable to metabolize proline. The productivity could be further increased if growth of the host cells was limited by employing an auxotrophic mutation together with reduced amounts of the corresponding nutrient in the culture medium. The yields of proline in these strains were about 16.1 g/ℓ in culture broths.

Conclusion

Nowadays, most types of amino acids are available in large quantities at reasonable prices, because of the industrial application of microbial processes for their production. These compounds supplied in large quantities are utilized in such diverse fields as food and feed additives, pharmaceuticals and specialty chemicals.

Industrial-scale microbial production of amino acids, which has been carried out successfully over the past twenty years, has relied upon progress in basic research in microbiology. Biosynthetic pathways and regulatory mechanisms of such primary metabolites as amino acids have been extensively studied, and most of the basic steps were clarified by about 1970. This knowledge was of great value in genetic manipulation of amino-acid producers.

Recent biotechnological innovations will be relevant to further improvement of the production processes. Technologies of DNA recombination and gene manipulation *in vivo*, such as cell fusion and transduction, are most promising.

Table 5. Specific activities of enzymes and lysine excretion in strains harbouring different plasmids. From Reverend *et al.* (1982)

Strain	Plasmids	Enzymatic specific activities					Approximate plasmid copy number per chromosome	Lysine production (g/ℓ)
		AK III	ASA-dehydrogenase	DHDP-synthetase	DHDP-reductase	DAP-decarboxylase		
RM 4102	—	17	520	150	30	8·9	—	
RM 4102	pDA1	16·5	7000				25	
TOC R 21	—	210	270	145	10	2·5	—	4
TOC R 21	pBR322	—					30	3·8
TOC R 21	pAD1	195	4500				25	
TOC R 21	pDA1	190		4550			30	6·5
TOC R 21	pDB2	211			220		30	
TOC R 21	pDD1	209					30	
TOC R 21	pLA17	198				11·0	150	

AK: aspartokinase; ASA: aspartic semialdehyde; DHDP: dihydrodipicolinate; DAP: diaminopimelate.

Microbial breeding based on DNA recombination, cell fusion and transduction will not only improve the established processes but also will bring about new ways of producing useful compounds. Utilization of less expensive natural and synthetic raw materials will also be widely investigated for future application by using these techniques.

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