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Transductional Construction of a Threonine-Hyperproducing Strain of *Serratia marcescens*: Lack of Feedback Controls of Three Aspartokinases and Two Homoserine Dehydrogenases

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To construct a threonine-hyperproducing strain of *Serratia marcescens* Sr41, the six regulatory mutations for three aspartokinases and two homoserine dehydrogenases were combined in a single strain by three transductional crosses. The constructed strain, T-1026, carried the *lysC1* mutation leading to lack of feedback inhibition and repression of aspartokinase III, the *thrA*$_1$*1* mutation desensitizing aspartokinase I to feedback inhibition, the *thrA*$_2$*1* mutation releasing feedback inhibition of homoserine dehydrogenase I, the two *hnr* mutations derepressing aspartokinase I and homoserine dehydrogenase I, and the *etr-1* mutation derepressing aspartokinase II and homoserine dehydrogenase II. The strain produced ca. 40 mg of threonine per ml of medium containing sucrose and urea. Furthermore, the productivity of strain T-1026 was compared with those of strains devoid of more than one of the six regulatory mutations.

Serratia marcescens is similar to *Escherichia coli* K-12 and *Salmonella typhimurium* in the regulatory mechanisms and genetic background for biosynthesis of amino acids of the aspartate family (Fig. 1) (22; S. Komatsubara, M. Kisumi, and I. Chibata, submitted for publication); i.e., *S. marcescens* has three aspartokinases (EC 2.7.2.4) and two homoserine dehydrogenases (EC 1.1.1.3). Aspartokinase I (*thrA*$_1$ product) and homoserine dehydrogenase I (*thrA*$_2$ product) are feedback inhibited by threonine and repressed by threonine plus isoleucine (see Table 2 for explanations of genotype designations). Aspartokinase II (*metL* product) and homoserine dehydrogenase II (*metM* product) are repressed by methionine but not feedback inhibited by the related end products. Aspartokinase III (*lysC* product) is subject to both feedback inhibition and repression by lysine. We have previously selected the following regulatory mutations for these enzymes, using *S. marcescens* Sr41. The *thrA*$_1$*1*(Fr [feedback-resistant]) and *thrA*$_2$*1*(Fr) mutations lead to lack of feedback inhibition of aspartokinase I and homoserine dehydrogenase I, respectively (13, 15). The two *hnr* mutations cause derepressed synthesis of the above two enzymes (13, 15). The *lysC1*(Fr) mutation codes for lack of both feedback inhibition and repression of aspartokinase III (12, 13). These mutations have been further combined in a single strain by two transductional crosses (13). The constructed strain produces about 25 mg of threonine per ml of medium containing sucrose and urea.

Recently, we have observed that the *etr-1* mutation selected for ethionine resistance causes derepressed synthesis of aspartokinase II and homoserine dehydrogenase II (14a). This mutation also contributes to threonine production more efficiently that we had expected and cotransduces with the Arg$^+$ (*argE*$^+$) selective marker at a high frequency. Therefore, we planned to use this regulatory mutation in the construction of a threonine-producing strain. This paper deals with the process for transductional construction of a threonine-hyperproducing strain having the six regulatory mutations for three aspartokinases and two homoserine dehydrogenases.

MATERIALS AND METHODS

Bacterial strains. Derivatives of *S. marcescens* Sr41 (18) were used (Table 1). Table 2 shows genotype designations, which are generally based on those for *E. coli* K-12 (3).

Media. The medium of Davis and Mingioli (5), modified by omitting the citrate and increasing the glucose to 0.5%, was used as a minimal medium. The medium for threonine production contained 15% sucrose, 1.5% urea, 0.05% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% corn steep liquor, and 1% $CaCO_3$. For strains other than P-200, the medium was supplemented with 0.13% L-isoleucine and 0.14% L-

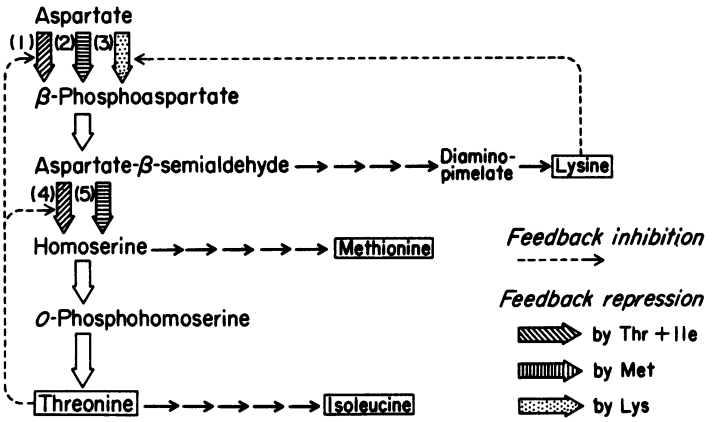

FIG. 1. Pathway and regulation of threonine biosynthesis in *S. marcescens*. (1) Aspartokinase I (*thrA*$_1$ product); (2) aspartokinase II (*metL* product); (3) aspartokinase III (*lysC* product); (4) homoserine dehydrogenase I (*thrA*$_2$ product); (5) homoserine dehydrogenase II (*metM* product).

methionine. The nutrient agar slant used was that described previously (13).

Isolation of auxotrophs. Cells of a parent strain were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1), modified as described previously (10). To enrich for auxotrophs, mutagenized cells were further treated with nalidixic acid by the procedure of Weiner et al. (23). The survivors were grown on nutrient agar plates overnight at 30°C. Resulting colonies were replica plated onto minimal agar plates with or without necessary supplements. Colonies showing the expected phenotype were purified by single-colony isolation and used for further studies.

Isolation of transductants. Phage PS20-mediated transduction was carried out by the method of Matsumoto et al. (19), modified as described previously (13). All agar plates described below were incubated at 30°C for 4 days. In the cross between strains AECr174 (donor) and HNr53 (recipient), *S*-2-aminoethylcysteine-resistant transductants were selected on minimal agar plates containing 20 mM *S*-2-aminoethyl-L-cysteine, 1 mM L-isoleucine, 1 mM L-methionine, and 1 mM L-threonine. In the cross between strains T-570 (do-

TABLE 1. *S. marcescens* Sr41 strains used

Strain[a]	Relevant genotype[b]	Origin	Reference
D-60	Wild type	Mutagenesis of Mu-910	16
HNr53	*hnrB2*	Mutagenesis of D-60	15
AECr174	*lysC1* (Fr)	Mutagenesis of HNr31	12
T-570	*thrA*$_1$*1* (Fr) *thrA*$_2$*1* (Fr) *hnrA1*	HNr21 phage × E-60	13
D-316	*thrA*$_2$*3*	Mutagenesis of D-315	Komatsubara et al., submitted
ETr17	*thrA*$_2$*3 etr-1*	Mutagenesis of D-316	14a
T-693	*lysC1* (Fr) *thrA*$_1$*1* (Fr) *thrA*$_2$*1* (Fr) *hnrA1*	T-570 phage × N-11	13
T-904	*lysC1* (Fr) *hnrB2*	AECr174 phage × HNr53	This paper
N-15	*lysC1* (Fr) *hnrB2 argE21*	Mutagenesis of T-904	This paper
N-16	*lysC1* (Fr) *hnrB2 argE21 thrB/C31*	Mutagenesis of N-15	This paper
T-1021	*lysC1* (Fr) *hnrB2 etr-1*	ETr17 phage × N-15	This paper
T-1022	*lysC1* (Fr) *thrA*$_1$*1* (Fr) *thrA*$_2$*1* (Fr) *hnrA1 hnrB2 argE21*	T-570 phage × N-16	This paper
T-1025	*lysC1* (Fr) *thrA*$_1$*1* (Fr) *thrA*$_2$*1* (Fr) *hnrA1 hnrB2*	ETr17 phage × T-1022	This paper
T-1026	*lysC1* (Fr) *thrA*$_1$*1* (Fr) *thrA*$_2$*1* (Fr) *hnrA1 hnrB2 etr-1*	ETr17 phage × T-1022	This paper
P-200	*lysC1* (Fr) *thrA*$_1$*1* (Fr) *thrA*$_1$*1* (Fr) *hnrA1 hnrB2 etr-1*	Mutagenesis of T-1026	This paper

[a] Except for strain P-200, all strains were defective in both threonine dehydrogenase (EC 1.1.1.103) and threonine deaminase (EC 4.2.1.16) and showed isoleucine auxotrophy. Strain P-200 was derived from strain T-1026 by adding isoleucine bradytrophy and methionine bradytrophy through three mutational events.

[b] For explanations of genotype designations, see Table 2.

TABLE 2. Genotype designations for mutations

Genotype[a]	Phenotype[b]	Alteration of enzymes[c]
lysC1 (Fr)	AEC resistance	Lack of feedback inhibition and repression of AK III
thrA$_1$*1* (Fr)	HN resistance	Lack of feedback inhibition of AK I
thrA$_2$*1* (Fr)	HN resistance	Lack of feedback inhibition of HD I
thrA$_2$*3*	Met sensitivity	Defect of HD
thrB1C31	Thr auxotrophy	Defect of HD or TS
hnrA1	HN resistance	Derepression of AK I, HD I, and *ilv* enzymes
hnrB2	HN resistance	Derepression of AK I and HD I
etr-1	Eth resistance	Derepression of AK II and HD II
argE21	Arg auxotrophy	Defect of AD

[a] In *E. coli* K-12, the *thrA* gene specifies a single polypeptide chain but is composed of two cistrons, *thrA*$_1$ and *thrA*$_2$, which correspond to aspartokinase I and homoserine dehydrogenase I, respectively (21).

[b] AEC, *S*-2-Aminoethylcysteine; HN, β-hydroxynorvaline; Met, methionine; Thr, threonine; Eth, ethionine; Arg, arginine.

[c] AK, Aspartokinase; HD, homoserine dehydrogenase; TS, threonine synthase; AD, acetylornithine deacetylase.

nor) and N-16 (recipient), lysates of phage grown in lysogenic strain T-570 were prepared by prophage induction (treatment with mitomycin C) and centrifugation of the culture broth after lysis of cells. Thr$^+$ transductants were selected on minimal agar plates containing 1 mM L-isoleucine and 1 mM L-arginine. In the cross between strains ETr17 (donor) and T-1022 or N-15 (recipient), Arg$^+$ transductants were selected on minimal agar plates containing 1 mM L-isoleucine.

Auxanographic feeding tests. To test for threonine production, cells were collected with toothpicks and placed onto minimal agar plates supplemented with 1 mM L-isoleucine. The plates were seeded with an indicator threonine auxotroph at 2.5×10^7 cells per ml. After incubation at 30°C overnight, the sizes of the halos were recorded.

Growth study. Cells were grown at 30°C in an automated recording incubator system (Hitachi, Ltd., Tokyo) (4) as described previously (16). Growth was measured turbidimetrically (660 nm) at 60-min intervals. Specific growth rate was calculated from optical densities of 0.1 to 0.5 (exponential growth phase).

Enzyme assay. Cells were cultured in minimal medium at 30°C with shaking, and cell extracts were prepared from exponentially growing cells by the method described previously (13). The activities of aspartokinase, homoserine dehydrogenase, threonine deaminase, and acetohydroxy acid synthase were determined at 30°C as described previously (10, 11). Protein was measured by the procedure of Lowry et al. (17). Specific activities are expressed as micromoles of products per milligram of protein per minute.

Threonine production. Unless otherwise noted, cells were cultured in shaking flasks containing the medium for threonine production under conditions described previously (13). For threonine production by strain P-200, a 3-liter jar fermentor (type MB-W; Iwashiya K. Sawada Co. Ltd., Tokyo, Japan) was used. Each medium component except sucrose and sucrose was separately autoclaved at 120°C for 20 min. For preparation of the inoculum, cells were grown at 30°C for 28 h in shaking flasks containing the same medium under conditions described previously (13). We transferred 75 ml of the inoculum culture into the fermentor, which contained 1.5 liters of medium. Incubation was carried out at 30°C with aeration (0.75 liters of air per min) and agitation (1,200 rpm).

Growth was estimated as stated previously (13). L-Threonine was determined by bioassay with *Leuconostoc mesenteroides* P-60. The other amino acids were determined with an amino acid analyzer (model 835; Hitachi). Sucrose was measured by the method of Dubois et al. (6).

RESULTS

Strategy for construction of a threonine-hyperproducing strain. We intended to construct a strain in which all feedback controls of three aspartokinases and two homoserine dehydrogenases were removed so as to gain higher production of threonine and, in addition, higher stability in an industrial production operation. Therefore, we planned to combine the six regulatory mutations related to threonine production in a single strain (Fig. 2).

Both *hnrA1* and *hnrB2* mutations, selected for β-hydroxynorvaline resistance, caused derepressed synthesis of aspartokinase I and homoserine dehydrogenase I (13, 15). Because a strain carrying both mutations might have higher activities of the two enzymes and a higher threonine production stability, compared with those observed for strains carrying either mutation alone, we intended to combine the *hnrA1* mutation with the *hnrB2* mutation. The former muta-

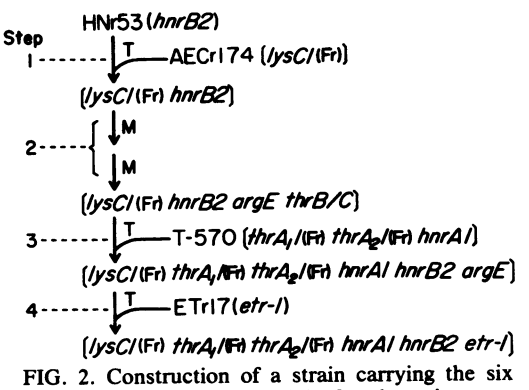

FIG. 2. Construction of a strain carrying the six regulatory mutations responsible for threonine production. For explanation of genotype designations, see Table 2. T, Transduction; M, mutagenesis.

tion derepressed the enzymes specified by the *ilv* genes as well as those specified by the *thr* genes, but the latter mutation had no effect on the formation of the *ilv* enzymes (13, 14). The *hnrA1* mutation was cotransduced with the Thr^+ ($thrB^+/C^+$) marker, but the genetic locus of the *hnrB2* mutation was unclear (13). Because of weak growth inhibition, it seemed difficult to transfer the *hnrB2* mutation carried by strain HNr53 to the other strain by selecting for β-hydroxynorvaline resistance. So we decided to transfer the other five mutations, including *hnrA1*, to strain HNr53 by sequential transductional crosses.

The *lysC1*(Fr) mutation, selected for *S*-2-aminoethylcysteine resistance, causes the lack of feedback inhibition and repression of aspartokinase III and participates in threonine production (12). Although we did not demonstrate the selective marker for cotransduction of this mutation, it was deemed possible to isolate transductants that would receive the *lysC1*(Fr) mutation by selecting for both *S*-2-aminoethylcysteine resistance and threonine production. Such a mutation transfer was considered to be difficult when a strain overproducing threonine was used as the recipient for the transduction. Considering these factors, we planned the first transductional cross whereby the *lysC1*(Fr) mutation would be transferred into strain HNr53 (step 1).

The $thrA_1$*1*(Fr), $thrA_2$*1*(Fr), and *hnrA1* mutations are cotransduced with the Thr^+ ($thrB^+/C^+$) marker (13), and the *etr-1* mutation is linked very closely to the *argE* gene (14a). Therefore, we intended to add Arg^- (*argE*) and Thr^- (*thrB/C*) markers to a strain carrying both *lysC1*(Fr) and *hnrB2* mutations (step 2). Thereafter, we planned to transfer the three regulatory mutations carried by strain T-570 (step 3). After step 3, we expected to readily identify transductants receiving all three mutations on the basis of both colony size and threonine production, since the *hnrA1* mutation led to slow growth on a nutrient agar plate and the other mutations led to enhancement of threonine production. As the final step, we planned to cotransduce the *etr-1* mutation with the Arg^+ marker to the strain constructed by prior manipulations (step 4).

Step 1: transfer of the *lysC1*(Fr) mutation into strain HNr53 (*hnrB2*). A transductional cross was performed between strain HNr53 carrying the *hnrB2* mutation and phage grown on strain AECr174 carrying the *lysC1*(Fr) mutation. Large colonies found on minimal agar plates containing *S*-2-aminoethylcysteine were tested for threonine production by auxanographic feeding tests. Of 44 *S*-2-aminoethylcysteine-resistant colonies, 43 formed large halos, indicating abundant threonine production. One colony forming a small halo was considered to have acquired resistance by spontaneous mutation. Five colonies producing threonine in large amounts were purified and examined for aspartokinase and homoserine dehydrogenase. All five strains, including strain T-904, had levels of aspartokinase that were higher than those observed for strains AECr174 and HNr53 (Table 3). The aspartokinase activities of the five strains were insensitive to lysine, as was that of strain AECr174. The aspartokinase activity in strain AECr174 was hardly inhibited by threonine, but the activity in strain T-904 was partially inhibited. These results appeared to be due to the derepression of latter strain for aspartokinase I and the repression of former strain for the enzyme. The inhibition of the aspartokinase activity in strain HNr53 by threonine was greater than the inhibition observed for wild-type strain D-60. Strain T-904 had a higher activity of homoserine dehydrogenase, which was sensitive to threonine-mediated inhibition, indicating that this strain was similar to strain HNr-53 in homoserine dehydrogenase. These results indicated that, as expected, strain T-904 carried both *lysC1*(Fr) and *hnrB2* mutations.

Step 2: addition of selective markers to strain T-904 [*lysC1*(Fr) *hnrB2*]. Two markers for cotransduction of the $thrA_1$*1*(Fr), $thrA_2$*1*(Fr), *hnrA1*, and *etr-1* mutations were added to strain T-904 by two sequential mutageneses. First, Arg^- strains were isolated from strain T-904 cultures and strain N-15 was identified as an *argE* mutant by testing for growth on ornithine

TABLE 3. Aspartokinase and homoserine dehydrogenase in strains D-60, AECr174, HNr53, and T-904

Strain	Aspartokinase				Homoserine dehydrogenase	
	Sp act	Inhibition (%) by[a]:			Sp act	Inhibition (%) by Thr[b]
		Thr	Lys	Both		
D-60 (wild-type)	0.05	15	50	63	0.007	58
AECr174 (donor)	0.21	6	3	5	0.010	58
HNr53 (recipient)	0.19	65	21	83	0.072	75
T-904 (transductant)	0.39	41	0	45	0.061	73

[a] L-Threonine (Thr) and L-lysine (Lys) were added at 50 mM.
[b] Added at 10 mM.

TABLE 4. Aspartokinase, homoserine dehydrogenase, and acetohydroxy acid synthase in strains T-570, N-16, and T-1022

Strain	Aspartokinase				Homoserine dehydrogenase		Acetohydroxy acid synthase	
	Sp act	Inhibition (%) by[a]:			Sp act	Inhibition (%) by Thr[b]	Sp act	Inhibition (%) by Val[c]
		Thr	Lys	Both				
T-570 (donor)	0.16	5	32	43	0.061	20	0.087	18
N-16 (recipient)	0.42	43	3	48	0.054	65	0.022	51
T-1022 (transductant)	0.40	6	4	4	0.054	22	0.091	15

[a] L-Threonine (Thr) and L-lysine (Lys) were added at 50 mM.
[b] Added at 10 mM.
[c] L-Valine (Val) was added at 10 mM.

or *N*-acetylornithine. Thereafter, strain N-16 was isolated from strain N-15 cultures as a Thr$^-$ strain which carried the *thrB/C* mutation.

Step 3: transfer of the *thrA*$_1$*1*(Fr), *thrA*$_2$*1*(Fr), and *hnrA1* mutations into strain N-16 (*lysC1*(Fr) *hnrB2*). The *thrA*$_1$*1*(Fr), *thrA*$_2$*1*(Fr), and *hnrA1* mutations are simultaneously cotransduced with the *thrB*$^+$/*C*$^+$ marker (13). A transductional cross was performed with strain T-570 carrying the above three mutations as a donor and strain N-16 as a recipient. We examined 100 Thr$^+$ recombinants for colony size on nutrient agar plates and for threonine halo size on agar plates in an auxanographic feeding test. Three recombinants formed small colonies, as did strain T-570, and larger halos than those observed for strains T-570 and T-904 (strain N-16 was derived from the latter). These Thr$^+$ recombinants were examined for related enzymes (Table 4). A representative strain, T-1022, had an increased level of valine-insensitive acetohydroxy acid synthase, indicating that it received the *hnrA1* mutation from strain T-570 (14). In strain T-1022, the activities of aspartokinase and homoserine dehydrogenase were not inhibited by threonine, indicating that it received the *thrA*$_1$*1*(Fr) and *thrA*$_2$*1*(Fr) mutations from strain T-570.

Step 4: transfer of the *etr-1* mutation into strain T-1022 (*lysC1*(Fr) *thrA*$_1$*1*(Fr) *thrA*$_2$*1*(Fr) *hnrA1 hnrB2*). Finally, the *etr-1* mutation was transductionally transferred into strain T-1022 by selecting Arg$^+$ colonies. Such colonies were tested for ethionine resistance. Of 20 Arg$^+$ strains, 15, including strain T-1026, were resistant to ethionine-mediated growth inhibition, and 5, including strain T-1025, were sensitive (Table 5). The homoserine dehydrogenase level of strain T-1026 was only slightly higher than that of strain T-1025, and the aspartokinase levels of the two strains hardly differed (Table 6). However, this was reasonable, considering that the levels of aspartokinase II and homoserine dehydrogenase II were low, compared with those of aspartokinase I and homoserine dehydrogenase I, even when the former isoenzymes were derepressed by the *etr-1* mutation. From the above

TABLE 5. Growth of *etr-1* strains

Strain	*k* in medium containing[a]:				Relevant genotype
	No amino acid	Met	Eth	Eth + Met	
D-316	0.48	0.15	<0.01[b]	<0.01[b]	*thrA*$_2$*3 etr*$^+$
ETr17	0.50	0.45	0.36	0.46	*thrA*$_2$*3 etr-1*
D-60	0.69	0.66	0.05	0.73	*thrA*$_2$$^+$ *etr*$^+$
T-1025	0.26	0.32	<0.01[b]	0.41	*thrA*$_2$*1* (Fr) *etr*$^+$
T-1026	0.22	0.27	0.32	0.33	*thrA*$_2$*1* (Fr) *etr-1*

[a] Cells were inoculated into minimal medium containing 1 mM L-isoleucine to give an optical density (660 nm) of ca. 0.005 and incubated for 24 h. The medium contained 1 mM L-methionine (Met), 20 mM DL-ethionine (Eth), or both. Mass doubling times were calculated from optical density values of 0.1 to 0.5 (exponential growth phase). Specific growth rate (*k*) was defined as: *k* (per hour) = ln 2/mass doubling time (hours).
[b] No detectable growth was observed for 24 h.

TABLE 6. Aspartokinase and homoserine dehydrogenase in various strains

Strain	Aspartokinase		Homoserine dehydrogenase	
	Sp act	Inhibition (%) by Thr + Lys[a]	Sp act	Inhibition (%) by Thr[b]
D-316	0.05	55	0.001	0
ETr17	0.11	42	0.014	3
D-60	0.05	63	0.007	58
T-1025	0.39	4	0.058	20
T-1026	0.42	6	0.067	15

[a] L-Threonine (Thr) and L-lysine (Lys) were added at 50 mM.
[b] Added at 10 mM.

TABLE 7. Comparison of threonine (Thr) production by various strains having different genotypes

Strain[a]	Growth (dry cell wt, mg/ml)		Thr produced (mg/ml)	
	96 h	120 h	96 h	120 h
D-60	38.2	40.6	0.1	0.1
HNr53	24.6	29.6	0.7	0.6
AECr174	26.4	35.8	6.0	7.4
T-570	42.2	42.2	8.2	8.8
ETr17	29.6	30.8	7.1	7.5
T-904	30.8	32.8	8.8	10.2
T-1021	28.0	30.8	25.4	28.9
T-693	29.6	39.0	21.7	24.6
T-1025	30.0	29.4	21.7	25.8
T-1026	26.4	30.8	33.1	40.3

[a] All strains were grown in medium containing L-isoleucine and L-methionine. For relevant genotypes and explanations of designations, see Tables 1 and 2.

results, we inferred that strain T-1026 carried the six regulatory mutations: *lysC1*(Fr), *thrA*$_1$*1*(Fr), *thrA*$_2$*1*(Fr), *hnrA1*, *hnrB2*, and *etr-1*.

To ascertain the contribution of the *etr-1* mutation to threonine production, the mutation was transferred to strain N-15 with the *argE*$^+$ marker. From growth and enzymatic data, we inferred that the constructed strain, T-1021, carried the three regulatory mutations: *lysC1*(Fr), *hnrB2*, and *etr-1*. This strain differed from strain T-904 in the *etr-1* mutation and from strain T-1026 in the *hnrA*, *thrA*$_1$, and *thrA*$_2$ genes.

Comparison of threonine production by strains having different genotypes. Threonine production by strains having different genotypes was examined by using the medium containing sucrose and urea (Table 7). As expected, strain T-1026, carrying all six regulatory mutations for three aspartokinases and two homoserine dehydrogenases, produced the largest amounts of threonine (ca. 40 mg/ml) of the 10 strains examined. The other strains, lacking more than one of the six mutations, produced <30 mg of threonine per ml. By comparing all 10 strains, we found that each regulatory mutation, singly or in combination, contributed to the increase in threonine production. The combination of the two *hnr* mutations was unlikely to significantly contribute to threonine production but likely to contribute to the stability of production (see below).

Further improvement of strain T-1026 and threonine production by strain P-200. The amount of isoleucine added to the production medium depended on the isoleucine requirement of the strain. The requirement resulted from the mutational defect in the gene coding for threonine deaminase; this defect prevented threonine degradation. Methionine was added to the medium so as to inhibit the activity of the first enzyme in the methionine-specific pathway. The omission of methionine from the medium decreased threonine production to ca. 30 mg/ml, suggesting that in this strain, appreciable amounts of homoserine overflow into methionine biosynthesis.

Therefore, strain T-1026 was further improved so that the two amino acids could be omitted from the production medium. First, a leaky isoleucine revertant was isolated from a culture of strain T-1026 after spontaneous mutation. In minimal medium containing no isoleucine, this revertant showed a growth rate that was half that in medium containing isoleucine. However, the threonine deaminase activity of this revertant was hardly recovered (<3% of the wild-type activity). Subsequently, methionine auxotrophs were derived from the revertant by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Finally, leaky methionine revertants were isolated from the culture of one of these auxotrophs after spontaneous mutation. The revertants were examined for threonine production. Strain P-200 was found to produce ca. 40 mg of threonine per ml of production medium lacking methionine. The strain grew very slowly in minimal medium containing no methionine (ca. 30% of the growth rate in medium containing methionine) but as fast as strain T-1026 in production medium lacking methionine.

Strain P-200 was examined for threonine production in a jar fermentor containing medium without isoleucine and methionine. Figure 3 shows typical changes during threonine produc-

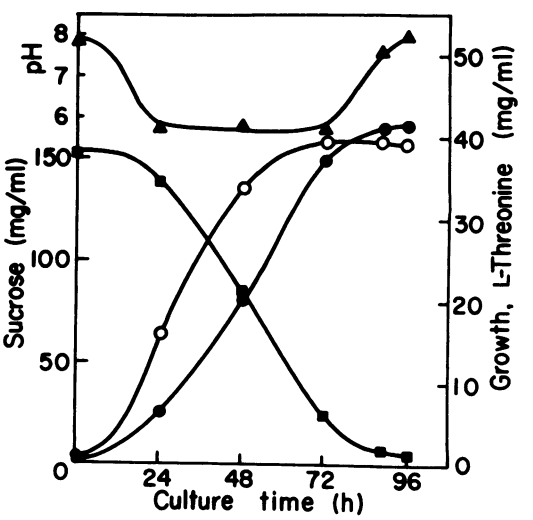

FIG. 3. Changes during threonine production by strain P-200 in a jar fermentor. Symbols: ○, growth (dry cell weight); ●, L-threonine produced; ■, sucrose found; ▲, pH of medium.

TABLE 8. Stability of threonine (Thr) production by strain P-200 during subculture

Inoculum history	Growth (dry cell wt, mg/ml)		Thr produced (mg/ml)	
	96 h	120 h	96 h	120 h
Primary culture on nutrient agar slant[a]	22.8	26.8	25.0	39.9
Fifth subculture on nutrient agar slant[b]	22.8	27.2	22.5	40.7
Fifth subculture in production medium[c]	25.2	27.6	25.3	40.9

[a] Cells of a single colony on a nutrient agar plate were grown on a nutrient agar slant for 24 h, and a loopful of cells was inoculated into production medium.

[b] The primary culture was subjected to subculture at five 24-h intervals with nutrient agar slants. A loopful of cells of the fifth subculture was inoculated into production medium.

[c] A loopful of cells of the primary culture was inoculated into production medium, and 0.5 ml of culture was subjected to subculture at five 24-h intervals with the same medium.

tion by strain P-200. Threonine production paralleled growth and reached a maximum of 41 mg/ml at 88 h. Sucrose was gradually utilized as fermentation proceeded, and only a small amount remained at 88 to 96 h. The pH of the medium was near 6 for 24 to 72 h and near 8 at 96 h.

Amino acids other than threonine in the production medium were measured with an amino acid analyzer after incubation for 96 h. All amino acids produced as by-products were found in only small amounts (≤0.1 mg/ml). Isoleucine was the most abundant of these amino acids. The amount of homoserine, an intermediate of threonine biosynthesis, was 0.01 mg/ml.

The stability of threonine production was examined (Table 8). Strain P-200 was subjected to subculture on nutrient agar slants or in threonine production medium. When the fifth subculture was used as the inoculum, strain P-200 produced ca. 40 mg of threonine per ml and no detectable amounts of the other amino acids. Therefore, we concluded that the productivity of strain P-200 was stable during subculture.

DISCUSSION

It is important that industrial strains produce a desired compound in large amounts. As stated above, we succeeded in the construction of *S. marcescens* P-200. This strain produced threonine in amounts as large as 40 to 41 mg/ml, which was 27% of the weight of the sucrose added to the medium. Threonine-producing strains of other bacteria have been reported, but their productivities are not very high (<20 mg/ml) in media containing a sugar as a carbon source (2, 7–9, 20). Our strain showed the highest threonine production of those reported.

It is also important that industrial strain production is stable. Generally, strains with multiple regulatory mutations produce a specific metabolite in large amounts but grow more slowly than wild-type strains, owing to unusual metabolic flow. Therefore, the productivities of such mutants are inclined to decrease in large-scale cultivation for industrial production because of the increase in the number of wild-type revertants arising after many generations. Our threonine-producing strain also showed slow growth, compared with growth of the wild-type strain. However, the productivity of our strain was stable, owing to the fact that reversion of any one of six regulatory mutations would not significantly decrease productivity. This aspect is supported by the finding that strains lacking one or two of the six mutations produced 25 to 29 mg of threonine per ml, whereas strain T-1026, which carries all six mutations, produced 40 mg/ml.

Finally, it is important that industrial strains produce few unusable by-products. Our threonine-producing strain produced only small amounts of amino acids other than threonine. Because high-quality threonine was obtained at a high yield by a simple process, we will use *S. marcescens* P-200 for industrial production.

We are interested in further enhancement of *S. marcescens* threonine production by genetic manipulations. The *thrA*$_1$*1*(Fr) and *thrA*$_2$*1*(Fr) mutations rendered aspartokinase I and homoserine dehydrogenase I insensitive to feedback inhibition. The levels of these two enzymes, which, owing to the *hnrA1* and *hnrB2* mutations, were constitutive, were much higher than those of aspartokinase II and homoserine dehydrogenase II, which, owing to the *etr-1* mutation, were also constitutive. However, on the basis of threonine production by strains having different genotypes (Table 7), we conclude that in strain T-1026 or P-200, aspartokinase I and homoserine dehydrogenase I do not efficiently contribute to threonine production, as do aspartokinase II and homoserine dehydrogenase II. This is possibly due to the fact that inhibition of the activities of the former enzymes by threonine is greater in vivo than in vitro. Therefore, we need to select mutations which confer aspartokinase I and homoserine dehydrogenase I feedback inhibition insensitivities that are greater than those conferred by the *thrA*$_1$*1*(Fr) and *thrA*$_2$*1*(Fr) mutations. Utilization of such mutations for construction of threonine-producing strains will enhance productivity.

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