APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1979, p. 1045-1051
0099-2240/79/12-1045/07$02.00/0
Vol. 38, No. 6

Transductional Construction of a Threonine-Producing Strain of *Serratia marcescens*

SABURO KOMATSUBARA,* MASAHIKO KISUMI, AND ICHIRO CHIBATA

Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co. Ltd., Kashima, Yodogawa-ku, Osaka, 532, Japan

Received for publication 17 September 1979

A threonine-producing strain of *Serratia marcescens* Sr41 was constructed according to the following process. Thr$^-$ strain E-60 was derived from strain HNr59 having constitutive levels of threonine-sensitive aspartokinase and homoserine dehydrogenase. Thr$^+$ transductant T-570 was constructed from strain E-60 and phage grown on strain HNr21 having feedback-resistant threonine-sensitive aspartokinase and homoserine dehydrogenase. This transductant lacked both feedback inhibition and repression for the two enzymes. Thr$^-$ strain N-11 was derived from strain AECr174 lacking feedback inhibition and repression of lysine-sensitive aspartokinase. Subsequently, the threonine region of strain T-570 was transduced into strain N-11. One of the Thr$^+$ transductants, strain T-693, produced markedly high levels of the two aspartokinases and homoserine dehydrogenase, which were insensitive to feedback inhibition. This strain produced about 25 mg of threonine per ml in the medium containing sucrose and urea.

In *Serratia marcescens*, threonine biosynthesis is regulated primarily by feedback controls of threonine-sensitive aspartokinase (*thrA*$_1$ product; EC 2.7.2.4) and homoserine dehydrogenase (*thrA*$_2$ product; EC 1.1.1.3) (Fig. 1). In other words, both enzymes are feedback inhibited by threonine and multivalently repressed by threonine and isoleucine (Komatsubara et al., unpublished data). The structural genes specifying the two enzymes probably belong to a single operon as described below. *S. marcescens* has another aspartokinase (*lysC* product) subject to feedback controls by lysine (22). This enzyme is involved not only in lysine biosynthesis but also in threonine biosynthesis (15). Thus, *S. marcescens* is very similar to *Escherichia coli* K-12 in the biosynthesis of amino acids of the aspartate family. Moreover, Matsumoto et al. revealed the close similarities in the gene arrangement of the *leu-thr* region between the two enteric bacteria (20). Therefore, we apply genotype designations for *E. coli* K-12 (2, 24, 25) to those for *S. marcescens* in this paper.

We previously derived β-hydroxynorvaline-resistant mutants from an *S. marcescens* mutant deficient in threonine-degrading enzymes (15, 16). One of them, strain HNr21, had feedback-resistant threonine-sensitive aspartokinase and homoserine dehydrogenase [*thrA*$_1$*1*(Fr) and *thrA*$_2$*1*(Fr); Fr implies a feedback-resistant mutation]. The other mutant, strain HNr59, had two mutations; feedback resistance of homoserine dehydrogenase [*thrA*$_2$*2*(Fr)] and constitutive synthesis of threonine biosynthetic enzymes (*hnr-1*; a mutation causing the constitutive synthesis is tentatively designated *hnr*). Furthermore, to obtain regulatory mutants for lysine-sensitive aspartokinase, we isolated *S*-2-aminoethyl cysteine-resistant mutants. One of them, strain AECr174, lacked both feedback inhibition and repression for aspartokinase [*lysC1*(Fr)] (14). The three mutants described above produced small amounts of threonine in a medium containing glucose and urea because at least one of the three enzymes in each strain was subject to feedback control.

The availability of transduction for the construction of *S. marcescens* strains producing amino acids and the related compounds was described by Kisumi et al. (10-13). Transductional cross is useful for combining several mutations of feedback controls. To obtain an *S. marcescens* strain that would produce larger amounts of threonine, we constructed, by transduction, multiple regulatory mutants for lysine-sensitive aspartokinase, threonine-sensitive aspartokinase, and homoserine dehydrogenase (*lysC thrA*$_1$ *thrA*$_2$ *hnr*). This paper deals with the process for transductional construction and the properties of the strain obtained.

MATERIALS AND METHODS

Bacterial strains. Derivatives of *S. marcescens* Sr41 (18) were used (Table 1). Figure 2 shows the genealogy of the main strains used.

Media. The medium of Davis and Mingioli (4) was modified by omitting the citrate and increasing the glucose to 0.5%, and used as the minimal medium. Nutrient broth contained 0.5% glucose, 1.0% peptone, 0.3% meat extract, 1.0% yeast extract, and 0.5% NaCl. The complete medium used for transduction was nutrient broth (Difco Laboratories) supplemented with 0.3% yeast extract (Difco). The medium for threonine production contained 15% sucrose, 1.5% urea, 0.05% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.04% L-methionine, 0.03% D-threonine (an isoleucine source), 0.2% corn steep liquor, and 1% $CaCO_3$. Sucrose was autoclaved separately and mixed with the other components.

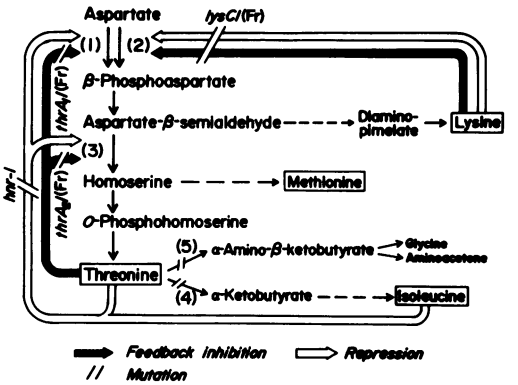

FIG. 1. *Regulation of biosynthesis and degradation of threonine in S. marcescens. The presence of methionine-repressible aspartokinase and homoserine dehydrogenase, as found for Escherichia coli, is not clear. For genotype designations, see footnote to Table 1. Enzymes: (1) threonine-sensitive aspartokinase ($thrA_1$); (2) lysine-sensitive aspartokinase (lysC); (3) homoserine dehydrogenase ($thrA_2$); (4) threonine deaminase (ilvA); and (5) threonine dehydrogenase.*

Isolation of Thr⁻ strains. Cells of strains HNr59 and AECr174 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1), modified as described previously (15). Mutagenized cells were further treated with nalidixic acid by the procedure of Weiner et al. (27) to enrich auxotrophs, and Thr⁻ strains were selected.

Isolation of transductants. Phage PS20, which is a generalized transducing phage of *S. marcescens* Sr41 (19), was used. Lysates of the phage grown on strain HNr21 were prepared by the method of Matsumoto et al. (19), except that the complete medium was used. Lysates of the phage grown on lysogenic strain T-570 were prepared by inducing prophages by ultraviolet irradiation and centrifuging the culture after lysis of cells. Lysates were added to 1 ml of the culture broth of recipient strains (1×10^9 cells) to give a multiplicity

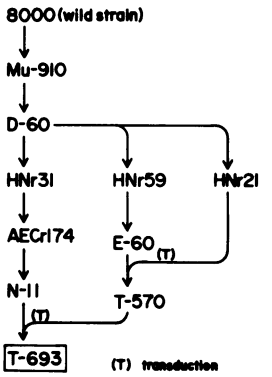

FIG. 2. *Genealogy of main strains used. For further details see Table 1.*

TABLE 1. *S. marcescens Sr41 strains used*

Strain[a]	Relevant genotype[b]					Origin	Reference
	lysC	$thrA_1$	$thrA_2$	*thrB/C*	*hnr*		
D-60	+	+	+	+	+	Mutagenesis of Mu-910	15, 16
HNr21	+	1(Fr)	1(Fr)	+	+	Mutagenesis of D-60	15
HNr31	+	+	+	+	+	Mutagenesis of D-60	14
HNr59	+	+	2(Fr)	+	1	Mutagenesis of D-60	15
E-60	+	+	2(Fr)	11	1	Mutagenesis of HNr59	This paper
AECr174	1(Fr)	+	+	+	+	Mutagenesis of HNr31	14
N-11	1(Fr)	+	+	13	+	Mutagenesis of AECr174	This paper
T-570	+	1(Fr)	1(Fr)	+	1	HNr21 phage × E-60	This paper
T-676	1(Fr)	+	+	+	1	T-570 phage × N-11	This paper
T-693	1(Fr)	1(Fr)	1(Fr)	+	1	T-570 phage × N-11	This paper
T-720	1(Fr)	1(Fr)	1(Fr)	+	+	T-570 phage × N-11	This paper

[a] Strain D-60 was isolated from strain Mu-910 as an isoleucine auxotroph, lacking threonine deaminase (EC 4.2.1.16). Strain Mu-910 was isolated from strain 8000 (wild-type strain) and lacks threonine dehydrogenase (EC 1.1.1.103). Therefore, all strains listed here lack both threonine-degrading enzymes. Strain HNr31, selected for β-hydroxynorvaline resistance, excretes a small amount of threonine and exhibits a methionine bradytrophy. Strains AECr174, N-11, T-676, T-693, and T-720 are also methionine bradytrophs.

[b] Genotype designations are as follows: *lysC1*(Fr), lack of both feedback inhibition and repression of lysine-sensitive aspartokinase; $thrA_11$(Fr), lack of feedback inhibition of threonine-sensitive aspartokinase; $thrA_21$(Fr) and $thrA_22$(Fr), lack of feedback inhibition of homoserine dehydrogenase; *thrB/C11* and *thrB/C13*, lack of homoserine kinase (EC 2.7.1.39) or threonine synthase (EC 4.2.99.2); *hnr-1*, lack of repression of threonine operon.

of infection of about 20. The mixture was kept at 30°C for 30 min to allow phage absorption. After centrifugation at 2,500 × g for 5 min, the cells were spread on the minimal agar plates containing 10 mM L-lysine, 10 mM L-isoleucine, and 10 mM L-methionine. Thr^+ colonies found after 4 days of incubation at 30°C were examined for threonine excretion by an auxanographic feeding test. Some of them were purified by single-colony isolation and used for further studies. Frequencies of spontaneous Thr^+ colonies from recipient strains E-60 and N-11 were less than 3×10^{-8}. Frequencies of Thr^+ transductants ranged from 2×10^{-7} to 5×10^{-7}.

Auxanographic feeding test. Cells of the strains to be tested were spotted on a minimal agar plate seeded with a Thr^- strain. Agar plates were supplemented with 10 mM L-isoleucine with or without 10 mM L-lysine and 10 mM L-methionine. After 24 h at 30°C, threonine excretion was scored by visual inspection of the halos formed.

Growth study. Cells were grown in test tubes (15 by 150 mm) containing 3 ml of medium with a Hitachi automated recording incubator system (3). Incubation was carried out at 30°C with shaking (140 rpm, 4-cm stroke). Growth was turbidimetrically measured at 660 nm at 45-min intervals by the automated method. An optical density of 0.10 corresponded to 85 μg of dry cells per ml.

Enzyme assay. To a 500-ml Sakaguchi shaking flask was added 150 ml of the minimal medium, modified by decreasing the glucose to 0.02% and adding 0.05 mM L-isoleucine with or without 0.05 mM L-methionine. The medium was inoculated with a loopful of cells grown on nutrient agar slants overnight. After 16 h of incubation at 30°C with shaking (140 rpm, 7-cm stroke), the growth ceased at 80 to 120 μg (dry weight of cells) per ml. Then the glucose was increased to 0.5%. Furthermore, 1 mM L-isoleucine was added for the repression of threonine biosynthetic enzymes or 0.5 mM D-threonine was added as a limiting source of isoleucine for the derepression (15). When strains carrying methionine bradytrophy were used, the methionine was also increased to 1 mM. After these supplements, the incubation was continued until the culture reached 700 to 900 μg/ml (late log phase). The cells were centrifuged at 10,000 × g for 10 min, washed, and suspended in 50 mM potassium phosphate buffer (pH 8.0) containing 30% glycerol for enzyme stabilization. Cell-free extracts were prepared by sonication as described previously (7).

The activities of aspartokinase and homoserine dehydrogenase were determined at 30°C as described previously (6). For threonine-sensitive aspartokinase assay, the reaction mixture containing 50 mM L-lysine to inhibit lysine-sensitive aspartokinase activity was used. 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. A loopful of cells grown on nutrient agar slants overnight was inoculated into 15 ml of medium in 500-ml Sakaguchi shaking flasks and incubated at 30°C with reciprocal shaking (140 rpm, 7-cm stroke). Growth was estimated by measuring the optical density of culture broth diluted with 0.1 N HCl to dissolve $CaCO_3$ in the broth and is expressed as dry cell weight calculated from a standard curve. L-Threonine was determined by bioassay with *Leuconostoc mesenteroides* P-60.

RESULTS

Construction of strains lacking both feedback inhibition and repression of threonine-sensitive aspartokinase and homoserine dehydrogenase [$thrA_1$1(Fr) $thrA_2$1-(Fr) hnr-1]. We previously isolated mutants lacking either feedback inhibition or repression of threonine-sensitive aspartokinase and homoserine dehydrogenase (15). Therefore, we intended to construct strains lacking both feedback inhibition and repression. Thr^- strain E-60, carrying the *thrB/C* mutation, was derived from strain HNr59 [*thrA*$_2$2(Fr) *hnr-1*]. With PS20-mediated transduction, we transferred the *thrA*$_1$*1*(Fr) and *thrA*$_2$*1*(Fr) mutations of strain HNr21 into strain E-60 by selecting Thr^+ strains (Fig. 3). Of 25 Thr^+ strains, 11 formed larger threonine halos than those of the other Thr^+ strains on the minimal agar plates containing excess isoleucine. These 11 strains showed small colony sizes, as did strain HNr59. All the strains forming larger halos were tested for aspartokinase and homoserine dehydrogenase. Nine isolates, including strain T-570, had the activities as high as those of strain HNr59 (Table 2). Their aspartokinase activities were insensitive to threonine to the same extent as that of strain HNr21. Homoserine dehydrogenases of these transductants were less sensitive to feedback inhibition by threonine than that of strain HNr59. This indicates that the *thrA*$_2$ genes of the transductants are of donor type. Accordingly, we infer that strain T-570 carries *thrA*$_1$*1*(Fr), *thrA*$_2$*1*(Fr), and *hnr-1*.

Of 25 Thr^+ transductants, 14 formed smaller threonine halos than that of strain T-570. Several of the 14 strains were tested for threonine-sensitive aspartokinase and homoserine dehydrogenase. In these strains, the two enzymes were found to be repressible but to be insensitive to feedback inhibition. Therefore, these Thr^+ strains were concluded to be recombinants carrying *thrA*$_1$*1*(Fr), *thrA*$_2$*1*(Fr), and *hnr*$^+$. Moreover, these results indicate that *hnr-1* is closely linked to the threonine operon.

Construction of strains lacking both

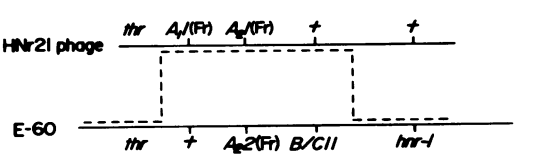

FIG. 3. *Diagramatic representation of the cross to give strain T-570 [thrA*$_1$*1(Fr) thrA*$_2$*1(Fr) hnr-1].*

TABLE 2. *Threonine-sensitive aspartokinase and homoserine dehydrogenase in strains D-60, HNr21, HNr59, and T-570*

Strain	Addition to minimal medium (mM)[a]	Threonine-sensitive aspartokinase		Homoserine dehydrogenase	
		Sp act	Inhibition (%)[b]	Sp act	Inhibition (%)[c]
D-60	L-Ile (1)	0.03	NT[d]	0.006	NT
	D-Thr (0.5)	0.12	60	0.047	75
HNr21	L-Ile (1)	0.03	NT	0.008	NT
	D-Thr (0.5)	0.11	11	0.045	23
HNr59	L-Ile (1)	0.12	67	0.078	35
T-570	L-Ile (1)	0.15	14	0.066	17

[a] Excess L-isoleucine was added to the medium for repression. D-Threonine was added as a limiting source of isoleucine for phenotypic derepression.
[b] By 50 mM L-threonine.
[c] By 10 mM L-threonine.
[d] NT, Not tested.

feedback inhibition and repression of lysine-sensitive aspartokinase, threonine-sensitive aspartokinase, and homoserine dehydrogenase [lysC1(Fr), thrA$_1$1(Fr), thrA$_2$1(Fr), hnr-1]. We previously observed that strain AECr174 lacked both feedback inhibition and repression of lysine-sensitive aspartokinase (14). The regulatory mutation carried by this strain is tentatively designated *lysC1*(Fr), although we are uncertain whether the two phenotypes depend on a single lesion or two separate lesions very closely linked to each other. However, the *thrA$_1$1*(Fr), *thrA$_2$1*(Fr), and *hnr-1* mutations of strain T-570 were expected to be simultaneously cotransduced with *thrB/C*. Therefore, multiple regulatory mutants for aspartokinases and homoserine dehydrogenase were constructed (Fig. 4). Thr$^-$ strain N-11, carrying *thrB/C* and *lysC1*(Fr), was derived from strain AECr174 and used as recipient for transduction. Strain T-570 was used as donor. Before the transduction, the number of plaque-forming units of lysates was decreased by ultraviolet irradiation to isolate nonlysogenic transductants easily. Sixty-three Thr$^+$ strains obtained were examined for colony size on nutrient agar plates and for threonine excretion on minimal agar plates containing excess isoleucine, lysine, and methionine (Table 3). Thr$^+$ transductants were classified into three groups. Strains of group 1 formed small colonies as strain T-570 did and formed larger threonine halos than those of the strains of the other groups. A representative strain T-693 had a higher activity of aspartokinase than that of strain T-570 or AECr174 (Table 4). The aspartokinase activity was not inhibited by lysine or threonine, separately or together. Its homoserine dehydrogenase was constitutively derepressed and insensitive to threonine-mediated feedback inhibition. These data indicate that strain T-693 carries the expected genotype: *lysC1*(Fr), *thrA$_1$1*(Fr), *thrA$_2$1*(Fr), *hnr-1*. Strain T-693 and the other three strains of group 1 were nonlysogenic for phage PS20.

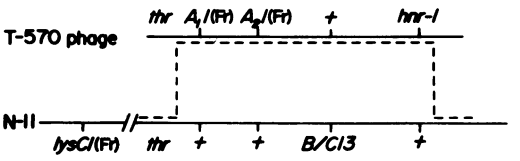

FIG. 4. *Diagramatic representation of the cross to give strain T-693 [lysC1(Fr) thrA$_1$1(Fr) thrA$_2$1(Fr) hnr-1].*

TABLE 3. *Groups of Thr$^+$ transductants found in the cross between strains T-570 and N-11*

Strain or group of transductant	Colony size[a]	Threonine halo size[b]	No. of transductants found
T-570	Small	Small	—
AECr174	Normal	Small	—
Transductant			
Group 1	Small	Larger	14
Group 2	Small	Large	3
Group 3	Normal	Large[c]	46

[a] Cells were streaked on nutrient agar plates and incubated for 24 h.
[b] Cells were picked up onto the minimal agar plates seeded with cells of a Thr$^-$ strain and incubated for 24 h.
[c] A few strains of group 3 formed small halos like that of strain AECr174.

Of 63 Thr$^+$ transductants, 3 formed small colonies and large halos as compared with those of strain AECr174. One of the three strains, strain T-676, had constitutive level of homoserine dehydrogenase that was feedback-inhibited by threonine. This indicates that transductants of group 2 possess *lysC1*(Fr), *thrA$_1$*$^+$, *thrA$_2$*$^+$, and *hnr-1*.

Thr$^+$ transductants of group 3 showed the same colony sizes as that of strain AECr174 and

TABLE 4. *Aspartokinase and homoserine dehydrogenase in transductants T-676, T-693, and T-720*

Strain	Addition to minimal medium (mM)	Total aspartokinase				Homoserine dehydrogenase		
		Sp act	Inhibition (%) by:[a]			Sp act	Inhibition (%) by:	
			L-Thr	L-Lys	Both		1 mM L-Thr	10 mM L-Thr
D-60	L-Ile (1)	0.06	13	51	54	0.008	45	56
	D-Thr (0.5)	0.15	66	22	86	0.047	68	75
T-570	L-Ile (1)	0.18	7	26	40	0.066	2	18
AECr174	L-Ile (1) + L-Met (1)	0.21	5	3	7	0.010	50	58
T-676	L-Ile (1) + L-Met (1)	0.33	32	9	38	0.063	52	66
T-693	L-Ile (1) + L-Met (1)	0.31	0	2	5	0.061	4	21
T-720	L-Ile (1) + L-Met (1)	0.22	4	3	5	0.012	0	9

[a] L-Threonine and L-lysine were at 50 mM.

formed smaller threonine halos than strains of group 1 did. One of them, strain T-720 had a feedback-resistant homoserine dehydrogenase, and its level was low in the presence of excess isoleucine, indicating that the genotype is *lysC1*(Fr) *thrA*$_1$*1*(Fr) *thrA*$_2$*1*(Fr) *hnr*$^+$.

Growth rates and cell yields of various strains. The growth rate of strain T-693 was compared with those of the other strains (Table 5). Strain T-693 required methionine for the maximum growth rate in the minimal medium. This phenotype was inherited from strain HNr31. Strains T-693, T-570, and HNr59, all of which carry *hnr-1*, grew more slowly than the other strains in minimal medium containing isoleucine and methionine, and also in nutrient broth. The cell yield of strain T-693 was reduced to about 60% of that of the wild-type strain D-60 in the minimal medium containing the required amino acids.

Threonine production by various strains. Threonine production by strains having various genotypes was examined by using a medium containing sucrose and urea, and supplemented with a limiting source of isoleucine (Table 6). As expected, strain T-693, carrying the four regulatory mutations for threonine biosynthesis, produced the largest amounts of threonine (about 25 mg/ml) among the nine strains tested. The other strains, lacking more than one of the four mutations, produced less than 14 mg of threonine per ml. These data indicate that each regulatory mutation contributes to the high threonine production by this strain.

Several lysogenic transductants carrying the same genotype as that of strain T-693 were tested for threonine productivity. They produced the same amount of threonine as that produced by nonlysogenic strain T-693. This indicates that the lysogeny for phage PS20 does not affect threonine productivity of *S. marcescens* strains.

TABLE 5. *Growth rates and cell yield of various strains in the minimal medium and nutrient broth*

Strain	Specific growth rate[a] (*k*)				Cell yield in the minimal medium[c]
	Minimal medium containing:[b]			Nutrient broth	
	L-Met	L-Ile	L-Met + L-Ile		
D-60	<0.02	0.66	0.71	1.19	1.60
HNr21	<0.02	0.63	0.66	1.19	1.66
HNr31	<0.02	0.43	0.73	1.33	1.52
HNr59	<0.02	0.44	0.45	0.69	1.48
AECr174	<0.02	0.30	0.66	0.93	1.35
T-570	<0.02	0.44	0.49	0.63	1.26
T-693	<0.02	0.34	0.44	0.56	0.99

[a] The specific growth rate constant, *k*, is defined as: k (h^{-1}) = ln 2/mass-doubling time (hours).

[b] L-Methionine and L-isoleucine were at 1 mM.

[c] Values indicate milligrams (dry weight) per milliliter. The minimal medium contained L-methionine and L-isoleucine.

TABLE 6. *Threonine production by various strains in a medium containing sucrose and urea*

Strain[a]	L-Threonine produced (mg/ml) at:		
	72 h	96 h	120 h
D-60	<0.1	<0.1	<0.1
HNr21	6.9	8.1	8.7
HNr31	0.7	0.7	0.5
HNr59	3.6	4.1	3.9
AECr174	4.1	6.0	6.2
T-570	5.5	8.7	8.3
T-676	7.5	8.6	8.5
T-693	19.5	23.5	24.7
T-720	7.5	12.9	14.0

[a] All strains produced similar cell yields (30 to 36 mg of dry cells per ml) at 96 to 120 h.

DISCUSSION

In recent years, a variety of amino acid-producing strains have been developed by selecting

regulatory mutants for resistance to amino acid analogs (5, 23). When the bacteria had complex regulatory mechanisms, multiple regulatory mutants were constructed by adding sequentially several analog resistances to a single strain (21). However, this method did not always provide mutants having as high productivities as expected. This is probably due to the failure to obtain multiple mutants that completely lack individual regulatory mechanisms because of cross-resistance or reversion of prior mutations.

More recently, we reported transductional construction of strains producing norvaline, histidine, urocanate, and arginine (10–13). The present paper deals with a threonine-producing strain constructed by a similar method. Since these *S. marcescens* mutants produce very large amounts of amino acids and urocanate, they can be used for industrial production.

By using a transductional cross, we can readily construct multiple mutants whose regulatory mechanisms are completely destroyed. In this case, individual mutations causing complete defects of various regulatory mechanisms can be selected independently, because the most suitable strains including wild-type strains can be used as parents. The mutations selected are combined by cotransducing with appropriate selective markers. Therefore, strains producing the expected metabolites in large amounts are readily obtained. Transduction is especially useful for the construction of strains producing metabolites whose biosynthesis is subject to complex regulation.

The loci of regulatory mutations on the genetic map are important for transductional construction of multiple regulatory mutants. During the initial stage of this study, we assumed that the constitutive mutation for threonine biosynthetic enzymes in strain HNr59 was located in the operator locus of threonine operon (*thrO*). If so, Thr^+ transductants carrying both $thrA_1$ and constitutive mutations would be very rare in the cross between strain HNr21 having the $thrA_1$ mutation and strain E-60 having the constitutive mutation. However, about 30% of Thr^+ transductants tested had both mutations. This indicated that the constitutive mutation carried by strain HNr59 might be located in the locus distant from the operator-promoter region, although the locus is linked to the threonine operon. Therefore, we tentatively denoted the mutation as *hnr-1*. Recently, we found that *hnr-1* causes constitutive synthesis of isoleucine-valine biosynthetic enzymes as well as that of threonine biosynthetic enzymes (submitted for publication). In *E. coli* K-12, isoleucyl transfer ribonucleic acid is thought to act as a co-repressor for the regulation of both threonine and isoleucine-valine biosyntheses (26). Accordingly, the *hnr-1* mutation may alter the formation of isoleucyl transfer ribonucleic acid.

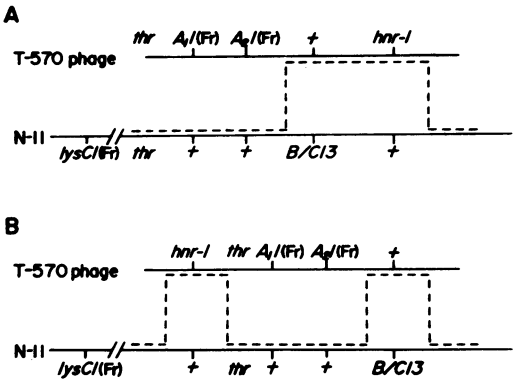

FIG. 5. *Diagramatic representation of the cross to give strain T-676 [lysC1(Fr) $thrA_1^+$ $thrA_2^+$ hnr-1].*

The results described above indicate that $thrA_1$, $thrA_2$, *thrB*, and *thrC* belong to a single operon, and that the *hnr-1* mutation is closely linked to *thr* operon. In the cross between strains T-570 and N-11, recombinants of group 1 (T-693) and group 3 (T-720) can be constructed independently from the order of *hnr-1* and the other *thr* genes, because these recombinants require only two cross-overs in any order (Fig. 4). On the other hand, recombinants of group 2 (T-676) must be constructed only in the order of $thrA_1$-$thrA_2$-*thrB*-*thrC*-*hnr-1* after two cross-overs (Fig. 5A). However, if *hnr-1* is located to the left of $thrA_1$, these recombinants require four crossovers and would be very rare (Fig. 5B). Therefore, we conclude that the order is $thrA_1$-$thrA_2$-*thrB*-*thrC*-*hnr-1*.

Our threonine-producing strain has not only multiple regulatory mutations but also defects of threonine-degrading enzymes; threonine dehydrogenase and threonine deaminase. These defects constitute one of the reasons why the constructed strain exhibits the highest threonine productivity, because the wild-type strain of *S. marcescens* rapidly degrades threonine added to the medium (16). Furthermore, the defect of threonine deaminase (*ilvA* product) is favorable for constructing an isoleucine-producing strain from the threonine-producing strain. Although we previously reported isoleucine production by regulatory mutants (6, 8, 9), these mutants were not altered in the regulation of threonine biosynthesis. Therefore, we introduced the *ilvA* mutation causing lack of feedback inhibition of threonine deaminase into strain T-693. The transductional construction of an isoleucine-producing strain of *S. marcescens* will be described in a separate paper (submitted for publication).

ACKNOWLEDGMENTS

We thank F. Murakami and S. Sindo for technical assistance.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
3. **Chibata, I., H. Itoh, and T. Morimoto.** 1977. Automated recording incubator for tube culture of microorganism. Chem. Econ. Eng. Rev. **9**:11–15.
4. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
5. **Kisumi, M., J. Kato, S. Komatsubara, and I. Chibata.** 1973. Production of isoleucine, valine, and leucine by regulatory mutants of *Serratia marcescens*. p. 267–287. *In* Z. Vaněk, Z. Hošťálek, and J. Cudlín (ed.), Genetics of industrial microorganisms, vol. 1. Elsevier Publishing Co., Amsterdam.
6. **Kisumi, M., S. Komatsubara, and I. Chibata.** 1977. Enhancement of isoleucine hydroxamate-mediated growth inhibition and improvement of isoleucine-producing strains of *Serratia marcescens*. Appl. Environ. Microbiol. **34**:647–653.
7. **Kisumi, M., S. Komatsubara, M. Sugiura, and I. Chibata.** 1971. Isoleucine hydroxamate, an isoleucine antagonist. J. Bacteriol. **107**:741–745.
8. **Kisumi, M., S. Komatsubara, M. Sugiura, and I. Chibata.** 1971. Properties of isoleucine hydroxamate-resistant mutants of *Serratia marcescens*. J. Gen. Microbiol. **69**:291–297.
9. **Kisumi, M., S. Komatsubara, M. Sugiura, and I. Chibata.** 1972. Isoleucine accumulation by regulatory mutants of *Serratia marcescens*: lack of both feedback inhibition and repression. J. Bacteriol. **110**:761–763.
10. **Kisumi, M., N. Nakanishi, T. Takagi, and I. Chibata.** 1977. Histidine production by histidase-less regulatory mutants of *Serratia marcescens* constructed by transduction. Appl. Environ. Microbiol. **34**:465–472.
11. **Kisumi, M., N. Nakanishi, T. Takagi, and I. Chibata.** 1978. Construction of a urocanic acid-producing strain of *Serratia marcescens* by transduction. Appl. Environ. Microbiol. **35**:231–236.
12. **Kisumi, M., M. Sugiura, T. Takagi, and I. Chibata.** 1977. Norvaline accumulation by regulatory mutants of *Serratia marcescens*. J. Antibiot. **30**:111–117.
13. **Kisumi, M., T. Takagi, and I. Chibata.** 1978. Construction of an L-arginine-producing mutant in *Serratia marcescens*. Use of the wide substrate specificity of acetylornithinase. J. Biochem. (Tokyo) **84**:881–890.
14. **Komatsubara, S., M. Kisumi, and I. Chibata.** 1979. Participation of lysine-sensitive aspartokinase in threonine production by *S*-2-aminoethyl cysteine-resistant mutants of *Serratia marcescens*. Appl. Environ. Microbiol. **38**:777–782.
15. **Komatsubara, S., M. Kisumi, K. Murata, and I. Chibata.** 1978. Threonine production by regulatory mutants of *Serratia marcescens*. Appl. Environ. Microbiol. **35**:834–840.
16. **Komatsubara, S., K. Murata, M. Kisumi, and I. Chibata.** 1978. Threonine degradation by *Serratia marcescens*. J. Bacteriol. **135**:318–323.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Matsumoto, H., S. Hosogaya, K. Suzuki, and T. Tazaki.** 1975. Arginine gene cluster of *Serratia marcescens*. Jpn. J. Microbiol. **19**:35–44.
19. **Matsumoto, H., T. Tazaki, and S. Hosogaya.** 1973. A generalized transducing phage of *Serratia marcescens*. Jpn. J. Microbiol. **17**:473–479.
20. **Matsumoto, H., T. Tazaki, and S. Hosogaya.** 1974. Transductional analysis of the *leu-thr* region of chromosome of *Serratia marcescens*. J. Bacteriol. **117**: 1365–1367.
21. **Nakayama, K., K. Araki, H. Hagino, H. Kase, and H. Yoshida.** 1976. Amino acid fermentations using regulatory mutants of *Corynebacterium glutamicum*, p. 437–449. *In* K. D. MacDonald (ed.), Second international symposium on the genetics of industrial microorganisms. Academic Press Inc., New York.
22. **Shailaja, M. S., and M. R. Raghavendra Rao.** 1975. Lysine-sensitive aspartokinase of *Serratia marcescens*. Indian J. Biochem. Biophys. **12**:17–20.
23. **Shiio, I.** 1973. Lysine and threonine production by *Brevibacterium flavum* mutants and their regulatory mechanisms, p. 249–265. *In* Z. Veněk, Z. Hošťálek, and J. Cudlín (ed.), Genetics of industrial microorganisms, vol. 1. Elsevier Publishing Co., Amsterdam.
24. **Thèze, J., D. Margarita, G. N. Cohen, F. Borne, and J. C. Patte.** 1974. Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K-12. J. Bacteriol. **117**:133–143.
25. **Thèze, J., and I. Saint-Girons.** 1974. Threonine locus of *Escherichia coli* K-12: genetic structure and evidence for an operon. J. Bacteriol. **118**:990–998.
26. **Umbarger, H. E.** 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47**:533–606.
27. **Weiner, R. M., M. J. Voll, and T. M. Cook.** 1974. Nalidixic acid for enrichment of auxotrophs in cultures of *Salmonella typhimurium*. Appl. Microbiol. **28**:579–581.