Transductional Construction of an Isoleucine-producing Strain of *Serratia marcescens*

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Strain GIHVLr6426 was isolated as an isoleucine hydroxamate-resistant mutant from the wild-type strain of *Serratia marcescens* Sr41. This mutant had high activities of threonine deaminase and acetohydroxyacid synthase, both of which were insensitive to feedback inhibition. Genetic analysis revealed that strain GIHVLr6426 carried two regulatory mutations located in the *ilv* region. Strain T-693, a threonine-producing strain which was previously reported to carry four regulatory mutations for threonine biosynthesis, had an increased activity of acetohydroxyacid synthase. Transductional analysis revealed that one of the four mutations carried by strain T-693 was responsible for constitutive synthesis of both isoleucine and threonine biosynthetic enzymes. Strain T-803 was constructed by transferring the two mutations carried by strain GIHVLr6426 into strain T-693. The constructed strain had the six regulatory mutations for threonine and isoleucine biosyntheses, and produced about 25 mg isoleucine ml⁻¹ in a medium containing sucrose and urea.

INTRODUCTION

In micro-organisms isoleucine is synthesized from aspartate via threonine (Fig. 1). The regulation of isoleucine biosynthesis in enteric bacteria is complicated, since (i) valine and leucine are related to feedback controls of isoleucine formation; (ii) lysine, methionine and isoleucine participate in the regulation of the biosynthesis of threonine, a precursor of isoleucine; (iii) there are several isoenzymes of aspartokinase (EC 2.7.2.4), homoserine dehydrogenase (EC 1.1.1.3) and acetohydroxyacid synthase (EC 4.1.3.18) and (iv) four isoleucine biosynthetic enzymes are functional for valine biosynthesis (Umberger, 1978).

We have previously reported transductional construction of a threonine-producing strain of *Serratia marcescens* (Komatsubara *et al.*, 1979*b*). The constructed strain was shown to carry four regulatory mutations for threonine production: *lysC1*(Fr), lack of both feedback inhibition and repression of lysine-sensitive aspartokinase; *thrA₁1*(Fr), lack of feedback inhibition of threonine-sensitive aspartokinase; *thrA₂1*(Fr), lack of feedback inhibition of homoserine dehydrogenase; *hnr-1*, constitutive synthesis of the *thrA₁* and *thrA₂* products. Such a strain produced very large amounts of threonine in a medium containing sucrose and urea. We have also been studying isoleucine production by regulatory mutants of *S. marcescens* (Kisumi *et al.*, 1972, 1977). These mutants lacked feedback controls of isoleucine biosynthetic enzymes but were not genetically altered in the regulation of threonine biosynthesis. They therefore produced only a small amount of isoleucine in a medium without the addition of an isoleucine precursor.

To obtain an *S. marcescens* mutant producing larger amounts of isoleucine, we planned to combine regulatory mutations for threonine biosynthesis with those for isoleucine biosynthesis. This paper deals with the transductional construction of an isoleucine-producing strain and, in addition, genetic analysis of regulatory mutations for isoleucine biosynthesis.

Fig. 1. Pathway and regulation of isoleucine biosynthesis in *S. marcescens*. It is temporarily assumed that *S. marcescens* has two isoenzymes of acetohydroxyacid synthase. The presence of methionine-repressible aspartokinase and homoserine dehydrogenase, as found for *E. coli*, is not clear. Enzymes: (1) threonine-sensitive aspartokinase, (2) lysine-sensitive aspartokinase, (3) threonine-sensitive homoserine dehydrogenase, (4) threonine deaminase, (5) valine-insensitive acetohydroxyacid synthase, (6) valine-sensitive acetohydroxyacid synthase, (7) transaminase B.

METHODS

Bacterial strains. The wild-type strain and derivatives of *S. marcescens* Sr41 (Matsumoto *et al.*, 1975) were used (Table 1). Genotype designations are based on those for *Escherichia coli* K12 (Bachmann *et al.*, 1976).

Media. The minimal medium was that of Davis & Mingioli (1950) modified by omitting citrate and increasing glucose to a final concentration of 0.5% (w/v). Nutrient broth was described previously (Komatsubara *et al.*, 1978a). The medium for isoleucine production contained (% w/v): sucrose, 15; urea, 1.5; $(\text{NH}_4)_2\text{SO}_4$, 0.05; K_2HPO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; corn steep liquor (Nihonshokuhinkako Co., Japan), 0.1; CaCO_3 , 1. Sucrose was autoclaved separately and then mixed with the other components.

Isolation of isoleucine hydroxamate-resistant mutants. Strain 8000 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the procedure of Adelberg *et al.* (1965), modified as described previously (Komatsubara *et al.*, 1978a). Isoleucine hydroxamate-resistant colonies were selected under conditions where growth inhibition was enhanced by adding valine and leucine, and using glycerol as a carbon source (Kisumi *et al.*, 1977). Colonies were tested for isoleucine formation from L-threonine in the presence of glycyl-valine and leucine by auxanographic feeding tests. Isoleucine-excreting colonies were purified by single colony isolation.

Transduction. Phage PS20-mediated transduction was carried out by the method of Matsumoto *et al.* (1973), modified as described previously (Komatsubara *et al.*, 1979b). When strains D-60 and D-43 were used as recipients, Ile⁺ and Thr⁺ transductants were selected on minimal agar plates. In the cross between strains GIHVLr6426 and T-693, isoleucine hydroxamate-resistant transductants were selected on minimal agar plates containing 10 mM-L-isoleucine hydroxamate, 1 mM-L-valine, 1 mM-L-leucine and 1 mM-L-methionine. Colonies of transductants were tested for the excretion of isoleucine and/or threonine by auxanographic feeding tests. Some of them were purified by single-colony isolation and used for further characterization.

Table 1. *Serratia marcescens* Sr41 strains used

Strain*	Relevant genotype†							Origin	Reference
	<i>lysC</i>	<i>thrA₁</i>	<i>thrA₂</i>	<i>thrB/C</i>	<i>hnr</i>	<i>ihr</i>	<i>ilvA</i>		
8000	+	+	+	+	+	+	+	Wild-type strain of Sr41	Matsumoto <i>et al.</i> (1975)
Mu-910	+	+	+	+	+	+	+	Mutagenesis of 8000	Komatsubara <i>et al.</i> (1978b)
D-43	+	+	+	21	+	+	+	Mutagenesis of Mu-910	Komatsubara <i>et al.</i> (1979b)
D-60	+	+	+	+	+	+	<i>I</i>	Mutagenesis of Mu-910	Komatsubara <i>et al.</i> (1978b)
HNr21	+	<i>I</i> (Fr)	<i>I</i> (Fr)	+	+	+	<i>I</i>	Mutagenesis of D-60	Komatsubara <i>et al.</i> (1978a)
HNr59	+	+	2(Fr)	+	<i>I</i>	+	<i>I</i>	Mutagenesis of D-60	Komatsubara <i>et al.</i> (1978a)
AECr174	<i>I</i> (Fr)	+	+	+	+	+	<i>I</i>	Mutagenesis of HNr31	Komatsubara <i>et al.</i> (1979a)
TA-21	+	+	2(Fr)	+	<i>I</i>	+	+	HNr59 phage × D-43	This paper
TA-49	+	+	+	+	<i>I</i>	+	+	HNr59 phage × D-43	This paper
GIHVLr6426	+	+	+	+	+	<i>I</i>	2(Fr)	Mutagenesis of 8000	This paper
TA-103	+	+	+	+	+	<i>I</i>	2(Fr)	GIHVLr6426 phage × D-60	This paper
TA-116	+	+	+	+	+	+	2(Fr)	GIHVLr6426 phage × D-60	This paper
T-693	<i>I</i> (Fr)	<i>I</i> (Fr)	<i>I</i> (Fr)	+	<i>I</i>	+	<i>I</i>	T-570 phage × N-11	Komatsubara <i>et al.</i> (1979b)
T-803	<i>I</i> (Fr)	<i>I</i> (Fr)	<i>I</i> (Fr)	+	<i>I</i>	<i>I</i>	2(Fr)	GIHVLr6426 phage × T-693	This paper

* Except strains 8000 and GIHVLr6426, all strains are deficient in threonine dehydrogenase. Strains AECr174, T-693 and T-803 are methionine bradytrophs.

† Genotype designations: *lysC**I*(Fr), lack of both feedback inhibition and repression of lysine-sensitive aspartokinase; *thrA₁**I*(Fr), lack of feedback inhibition of threonine-sensitive aspartokinase; *thrA₂**I*(Fr) and *thrA₂*2(Fr), lack of feedback inhibition of homoserine dehydrogenase; *thrB/C*21, defect of homoserine kinase or threonine synthase; *hnr*-1, lack of repression of both isoleucine and threonine biosynthetic enzymes (β -hydroxynorvaline-resistance); *ihr*-1, lack of repression of isoleucine biosynthetic enzymes (isoleucine hydroxamate-resistance); *ilvA*1, defect of threonine deaminase; *ilvA*2(Fr), lack of feedback inhibition of threonine deaminase. Fr denotes feedback-resistant mutations.

Table 2. Conditions for auxanographic feeding tests

Excretion	Indicator strain	Addition(s) to minimal agar plate* (mm)	Genotype identified
Isoleucine	Ile ⁻ (<i>ilvA</i>)	L-Thr (3)	<i>ilvA</i> 2(Fr)
		L-Thr (3)+Gly-Val (1)+Leu (1)	<i>ihr</i> -1 + <i>ilvA</i> 2(Fr)
	Ile ⁻ Val ⁻ (<i>ilvC/D</i>)	D-Thr (3)+Gly-Val (1)+Leu (1)	<i>hnr</i> -1
Threonine	Thr ⁻ (<i>thrB/C</i>)	Ile (10)+Met (10)+Lys (10)	<i>thrA₂</i> 1(Fr) + <i>hnr</i> -1 <i>thrA₂</i> 2(Fr) + <i>hnr</i> -1

* *Serratia marcescens* strains form isoleucine from D-threonine in the presence of valine and leucine when they have derepressed levels of isoleucine biosynthetic enzymes (Kisumi *et al.*, 1970). Glycyl-L-valine was used as a valine source. When an Ile-Val⁻ strain was used as an indicator, 0.01 mM-calcium pantothenate was also added to the agar plates.

Auxanographic feeding tests. To identify regulatory mutations in the colonies obtained, auxanographic feeding tests were performed. Cells from the colonies were streaked on to supplemented minimal agar plates seeded with an indicator strain at 2.5×10^7 cells ml⁻¹ (Table 2). After incubation at 30 °C for 1 or 2 d, the size of halos was scored.

Growth study. Cells were grown at 30 °C using a Hitachi automated recording incubator system (Chibata *et al.*, 1977) as described previously (Komatsubara *et al.*, 1978b).

Enzyme assay. To a 500 ml Sakaguchi shaking flask was added 150 ml minimal medium modified by decreasing the glucose concentration to 0.02 % (w/v). Where required for growth, amino acids were added to the medium at 0.05 mM. The medium was inoculated with a loopful of cells grown on a nutrient agar slant overnight, and incubated at 30 °C on a reciprocal shaker (140 rev. min⁻¹, 7 cm stroke). After 16 h,

growth ceased [80 to 120 μg dry wt cells (ml medium) $^{-1}$]. More glucose and amino acids were then added to give final concentrations of 0.5 % (w/v) and 1 mM, respectively. For repression of isoleucine biosynthetic enzymes, 1 mM-L-isoleucine, 1 mM-glycyl-L-valine and 1 mM-L-leucine were added after 16 h incubation. Glycyl-valine was used as a valine source to avoid isoleucine-mediated interference with the entry of valine into the cells (Kisumi *et al.*, 1971*a*). Incubation was continued until growth reached about 900 μg dry wt cells ml $^{-1}$ (late exponential phase). Cultures were then centrifuged, and the cells were washed and suspended in 50 mM-potassium phosphate buffer (pH 8.0) containing 30 % (w/v) glycerol for enzyme stabilization. Cell extracts were prepared by sonication (Kisumi *et al.*, 1971*b*).

The activities of aspartokinase (EC 2.7.2.4), homoserine dehydrogenase (EC 1.1.1.3), threonine deaminase (EC 4.2.1.16) and acetohydroxyacid synthase (EC 4.1.3.18) were determined at 30 °C by the methods described previously (Kisumi *et al.*, 1971*b*, 1977). Although acetohydroxyacid synthase is generally believed to be unstable (Grimminger & Umbarger, 1979), we could determine activities and valine sensitivities of this enzyme in cell extracts with reproducibility since glycerol was used for enzyme stabilization. Specific activities are expressed as μmol products formed (mg protein) $^{-1}$ min $^{-1}$. Protein was measured by Lowry's method.

Isoleucine and threonine production. Isoleucine and threonine production were examined by the methods previously described (Kisumi *et al.*, 1970; Komatsubara *et al.*, 1978*a*).

Chemicals. L-Isoleucine hydroxamate and glycyl-L-valine were obtained from Sigma.

RESULTS

Selection of isoleucine regulatory mutations

The selection of both feedback-resistant and constitutive mutants for isoleucine biosynthesis was required for the construction of an isoleucine-producing strain from a threonine-producing strain. Although we previously reported isoleucine regulatory mutations carried by isoleucine hydroxamate-resistant mutants of *S. marcescens* no. 1 (Kisumi *et al.*, 1971*c*, 1977), a transductional cross between strains of *S. marcescens* no. 1 and those of *S. marcescens* Sr41 has not been achieved. Therefore, we intended to select isoleucine regulatory mutations by isolating isoleucine hydroxamate-resistant mutants of *S. marcescens* Sr41. The resistant mutants were isolated under conditions where growth inhibition was enhanced by adding valine and leucine, and using glycerol as a carbon source (Kisumi *et al.*, 1977). Strain GIHVLR6426 was found among the resistant colonies excreting isoleucine in the presence of L-threonine, glycyl-valine and leucine. The mutations in this strain were further characterized.

Table 3. *Threonine deaminase and acetohydroxyacid synthase in strain GIHVLR6426 and related strains*

Strain	Additions to minimal medium*	Specific activity†	
		Threonine deaminase	Acetohydroxyacid synthase
8000 (wild-type)	None	0.15 (95)	0.021 (46)
	Ile+ Gly-Val+ Leu	0.05	0.009
GIHVLR6426	None	0.83 (5)	0.063 (4)
	Ile+ Gly-Val+ Leu	0.71	0.059
TA-103 (GIHVLR6426 phage $\times$ D-60)	None	0.84 (6)	0.063 (5)
	Ile+ Gly-Val+ Leu	0.72	0.054
TA-116 (GIHVLR6426 phage $\times$ D-60)	None	0.14 (7)	0.022 (53)
	Ile+ Gly-Val+ Leu	0.05	0.009

* L-Isoleucine, glycyl-L-valine and L-leucine were added at 1 mM.

† Specific activities are expressed as μmol product (mg protein) $^{-1}$ min $^{-1}$. Values in parentheses are the percentage inhibition by 10 mM-L-isoleucine for threonine deaminase and 10 mM-L-valine for acetohydroxyacid synthase.

Fig. 2. Lack of feedback inhibition of threonine deaminase and acetohydroxyacid synthase in strain GIHVLr6426. Extracts were prepared from cells grown in minimal medium. Activities are expressed relative to the specific activities determined in the absence of isoleucine or valine (= 100 %). Strains: ○, 8000 (wild-type); ●, GIHVLr6426.

Table 4. *Isoleucine excretion by Ile⁺ transductants*
(GIHVLr6426 phage × D-60)

Strain*	Isoleucine excretion†		
	L-Thr	L-Thr + Gly-Val + Leu	D-Thr + Gly-Val + Leu
Mu-910 (wild-type)	—	—	—
GIHVLr6426 (donor)	Heavy	+	+
Ile ⁺ transductants (130)			
Group 1 (114)	Heavy	+	+
Group 2 (13)	Heavy	—	—
Group 3 (3)	—	—	—

* Values in parentheses are the numbers of colonies tested or found.

† Tested in the presence of L-threonine, L-threonine + glycyl-L-valine + L-leucine, or D-threonine + glycyl-L-valine + L-leucine.

Regulatory mutations for isoleucine biosynthesis in strain GIHVLr6426

Strain GIHVLr6426 had high activities of threonine deaminase and acetohydroxyacid synthase when cultured in minimal medium (Table 3). The activities were not affected by the addition of isoleucine, glycyl-valine and leucine to the growth medium. The threonine deaminase of this strain was not feedback-inhibited by isoleucine even at high concentration (Fig. 2). In addition, its acetohydroxyacid synthase activity was not inhibited by valine (Fig. 2).

To gain a better understanding of the regulatory mutations carried by strain GIHVLr6426, the *ilv* region of this strain was transferred into an Ile[−](*ilvA*) strain by transduction and Ile⁺ recombinants were characterized by testing for isoleucine excretion on minimal agar plates containing L-threonine or D-threonine, with or without glycyl-valine plus leucine (Table 4). Thus, we tested 130 recombinants, which could be classified into three groups. Most of the recombinants (group 1) excreted isoleucine in the presence or absence of glycyl-valine and leucine. Enzyme activity was measured in ten strains of this group, which were identified as having feedback-resistant threonine deaminase and constitutively high levels of the two isoleucine biosynthetic enzymes, as shown for strain TA-103 in Table 3. Thirteen of the recombinants (group 2) formed isoleucine from L-threonine only in the absence of glycyl-valine and leucine. Five strains of group 2 were tested and found to be Ile⁺ recombinants

Table 5. *Threonine deaminase, acetohydroxyacid synthase and homoserine dehydrogenase in strain T-693 and related strains*

Strain	Addition(s) to minimal medium*	Specific activity†		
		Threonine deaminase	Acetohydroxyacid synthase	Homoserine dehydrogenase
Mu-910	None	0.15 (97)	0.027 (46)	0.007 (50)
D-60	Ile	ND	0.021 (51)	0.006 (52)
T-693	Ile	ND	0.085 (10)	0.064 (12)
HNr21	Ile	ND	0.025 (49)	0.008 (7)
HNr59	Ile	ND	0.078 (13)	0.078 (25)
AECr174	Ile	ND	0.021 (48)	0.010 (58)
TA-21 (HNr59 phage × D-43)	None	1.62 (96)	0.093 (11)	0.085 (15)
	Ile + Gly-Val + Leu	1.45 (97)	0.080 (6)	NT
TA-49 (HNr59 phage × D-43)	None	2.02 (95)	0.10 (11)	0.056 (65)
	Ile + Gly-Val + Leu	1.85 (95)	0.11 (5)	NT

ND, Not detected; NT, not tested.

* L-Isoleucine, glycyl-L-valine and L-leucine were added at 1 mM. The medium for strains T-693 and AECr174 contained 1 mM-L-methionine.

† Specific activities are expressed as $\mu\text{mol product (mg protein)}^{-1} \text{ min}^{-1}$. Values in parentheses are the percentage inhibition by 10 mM-L-isoleucine for threonine deaminase, 10 mM-L-valine for acetohydroxyacid synthase and 10 mM-L-threonine for homoserine dehydrogenase.

which had received only the mutation responsible for desensitization of threonine deaminase, as shown for strain TA-116 in Table 3. Three recombinants (group 3) excreted no isoleucine and were similar to the wild-type strain in feedback inhibition and repression of threonine deaminase (results not shown).

On the basis of these results we inferred that strain GIHVLR6426 carried two regulatory mutations in the *ilv* region. One, designated *ilvA2*(Fr), leads to desensitization of threonine deaminase, and the other, designated *ihr-1*, leads to constitutive synthesis of threonine deaminase and acetohydroxyacid synthase.

Derepression of both isoleucine and threonine biosynthetic enzymes by the hnr-1 mutation

To construct an isoleucine-producing strain, strain T-693 was used as a recipient in transduction. This strain was previously reported to have four regulatory mutations responsible for threonine production and to lack threonine deaminase activity (Komatsubara *et al.*, 1979b). However, it was not known whether it was altered in the regulation of isoleucine biosynthesis. Therefore, we examined isoleucine biosynthetic enzymes in strain T-693 and in the strains used in its construction.

The acetohydroxyacid synthase activity of strain T-693 was higher and less sensitive to valine-mediated inhibition than that of the wild-type strain Mu-910 (Table 5). Among strains D-60, HNr21, HNr53, HNr59 and AECr174, all of which were used in the construction of strain T-693, strain HNr59 showed a similar profile of acetohydroxyacid synthase to that observed for strain T-693. Strain HNr59 was previously confirmed to carry the *hnr-1* mutation, responsible for constitutive synthesis of threonine biosynthetic enzymes, and the *thrA₂*(Fr) mutation, responsible for desensitization of homoserine dehydrogenase. It was possible that the *hnr-1* mutation might affect repression of isoleucine biosynthetic enzymes as well. To examine this possibility, the *hnr-1* mutation, located in the *thr* region of strain HNr59, was transferred into a Thr⁻ strain by transduction and Thr⁺ recombinants were characterized (Table 6). Of 61 Thr⁺ strains, 20 formed isoleucine from D-threonine under conditions where isoleucine biosynthetic enzymes were repressible. Most of these isoleucine excretors (group 1) formed threonine in the presence of excess isoleucine,

Table 6. *Isoleucine and threonine excretion by Thr⁺ transductants (HNR59 phage × D-43)*

Strain*	Isoleucine excretion†	Threonine excretion
Mu-910 (wild-type)	—	—
HNR59 (donor)	+	+
Thr ⁺ transductants (61)		
Group 1 (18)	+	+
Group 2 (2)	+	—
Group 3 (41)	—	—

* Values in parentheses are the numbers of colonies tested or found.

† Tested in the presence of D-threonine + glycyl-L-valine + L-leucine.

Table 7. *Aspartokinase, homoserine dehydrogenase, threonine deaminase and aceto-hydroxyacid synthase in strain T-803 and related strains*

Strain*	Specific activity†			
	Aspartokinase	Homoserine dehydrogenase	Threonine deaminase	Aceto-hydroxyacid synthase
Mu-910 (wild-type)	0.06 (55)	0.007 (50)	0.15 (95)	0.026 (45)
GIHVLr6426 (donor)	0.08 (60)	0.012 (62)	0.83 (22)	0.065 (14)
T-693 (recipient)	0.28 (7)	0.067 (10)	ND	0.085 (12)
T-803 (transductant)	0.25 (6)	0.049 (5)	1.65 (23)	0.125 (10)

ND, Not detected.

* Strains Mu-910 and GIHVLr6426 were grown in minimal medium. Strains T-693 and T-803 were grown in the minimal medium supplemented with 1 mM-L-methionine + 1 mM-L-isoleucine or 1 mM-L-methionine.

† Specific activities are expressed as $\mu\text{mol product (mg protein)}^{-1} \text{ min}^{-1}$. Values in parentheses are the percentage inhibition by 50 mM-L-threonine plus 50 mM-L-lysine for aspartokinase, 10 mM-L-threonine for homoserine dehydrogenase, 10 mM-L-isoleucine for threonine deaminase and 10 mM-L-valine for aceto-hydroxyacid synthase.

methionine and lysine, but two of them (group 2) excreted no threonine. Strains TA-21 and TA-49, shown in Table 5, are, respectively, representative strains of groups 1 and 2; they had high activities of homoserine dehydrogenase, threonine deaminase and aceto-hydroxyacid synthase. These results indicated that the *hnr-1* mutation carried by strains HNR59 and T-693 caused constitutive synthesis of both isoleucine and threonine biosynthetic enzymes.

Transfer of the ihr-1 and ilvA2(Fr) mutations into strain T-693

To construct an isoleucine-producing strain, we transferred the two regulatory mutations carried by strain GIHVLr6426 into strain T-693 by transduction. Thirty isoleucine hydroxamate-resistant recombinants were tested for isoleucine excretion in the presence of excess lysine and methionine. Most of them formed extremely large isoleucine halos in an auxanographic feeding test. One such recombinant, strain T-803, was examined for the enzymes related to threonine and isoleucine biosyntheses (Table 7). There was no marked difference between strains T-803 and T-693 in aspartokinase and homoserine dehydrogenase. This indicated that strain T-803 carried the *lysC1*(Fr), *thrA11*(Fr), *thrA21*(Fr) and *hnr-1* mutations. Strain T-803 had a higher activity of feedback-resistant threonine deaminase and aceto-hydroxyacid synthase than strain T-693 or GIHVLr6426.

Two transductional crosses were performed to confirm the genotypes of strain T-803 (Table 8). In the cross between strain D-60 (Ile⁻) and phage grown on strain T-803, 66 of 74 Ile⁺ recombinants excreted isoleucine in the presence of L-threonine and glycyl-valine

Table 8. *Evidence for the presence of both hnr-1 and ihr-1 mutations in strain T-803*

Cross	Selected marker*	Unselected marker*		
		Threonine excretor	Isoleucine excretor†	
			L-Thr+GVL	D-Thr+GVL
T-803 phage × D-60	Ile ⁺ , 74	NT	66	NT
T-803 phage × D-43	Thr ⁺ , 72	29	NT	29

NT, Not tested.

* Values are the numbers of colonies tested or found.

† Tested in the presence of L-threonine+glycyl-L-valine (GV)+L-leucine (L) or D-threonine+glycyl-L-valine+L-leucine.

Table 9. *Growth rates of various strains in minimal medium and nutrient broth*

Strain	Specific growth rate* (h ⁻¹)					Nutrient broth
	Addition(s) to minimal medium†					
	None	Met	Met + Gly-Val	Met + L-Thr	Met + L-Thr + Gly-Val	
Mu-910	0.66	0.67	0.66	0.63	0.63	1.26
GIHVLr6426	0.54	0.59	0.63	0.55	0.63	1.31
T-693	0.29	0.45	0.44	0.45	0.41	0.58
T-803	0.23	0.35	0.43	0.12	0.43	0.55

* Defined as $\ln 2$ /mass doubling time (h).

† L-Methionine, glycyl-L-valine and L-threonine were added at 1 mM. The minimal medium for strain T-693 contained 1 mM-L-isoleucine.

plus leucine. This result suggested that strain T-803 carried both *ihr-1* and *ilvA2*(Fr) mutations. In the cross between strain D-43 (Thr⁻) and phage grown on strain T-803, 29 of 72 Thr⁺ recombinants excreted threonine in the presence of excess isoleucine, methionine and lysine, and also formed isoleucine from D-threonine in the presence of glycyl-valine and leucine. This result suggested that strain T-803 carried the *hnr-1* mutation. From these enzymic and transductional results, we concluded that strain T-803 possessed the *lysC1*(Fr), *thrA₁1*(Fr), *thrA₂1*(Fr), *hnr-1*, *ihr-1* and *ilvA2*(Fr) mutations.

Growth rates of strain T-803 and related strains

Growth rates of strain T-803 were compared with those of three related strains (Table 9). In minimal medium, methionine stimulated the growth of strain T-803. This property was inherited from strain T-693, used as recipient in transduction to construct strain T-803. In the medium containing methionine, glycyl-valine slightly elevated the growth rate of strain T-803 but L-threonine markedly lowered it. Growth inhibition by L-threonine was reversed by glycyl-valine. On the other hand, the two compounds showed only a slight effect on the growth rates of the other three strains. There was no marked difference in growth rates between strain GIHVLR6426 (donor) and strain Mu-910 (wild-type). In nutrient broth, strain T-803 had the same growth rate as strain T-693, although this was less than half the growth rate of the wild-type.

Isoleucine production by strain T-803

Strain T-803 and the related strains were examined for isoleucine production in medium containing sucrose as a carbon source and urea as a nitrogen source (Table 10). Strain GIHVLR6426, carrying two regulatory mutations for isoleucine biosynthesis, produced only

Table 10. *Isoleucine production by strain T-803 and related strains grown in medium containing sucrose and urea for 72 or 96 h*

Strain	Growth (mg dry wt ml ⁻¹)		L-Isoleucine produced (mg ml ⁻¹)		L-Threonine produced (mg ml ⁻¹)	
	72 h	96 h	72 h	96 h	72 h	96 h
Mu-910	29.0	34.8	< 0.1	< 0.1	< 0.1	< 0.1
GIHVLr6426	29.7	36.2	3.0	3.5	< 0.1	< 0.1
T-693*	26.7	34.0	< 0.1	< 0.1	19.5	23.5
T-803†	27.4	36.0	19.0	24.5	< 0.1	< 0.1

* Cultured in medium supplemented with 6.6 mM-L-isoleucine and 3.3 mM-L-methionine.

† Cultured in medium supplemented with 3.3 mM-L-methionine.

3.5 mg isoleucine ml⁻¹, because its threonine biosynthesis was subject to feedback controls. Strain T-693 produced large amounts of threonine but no isoleucine because it lacked threonine deaminase activity. On the other hand, strain T-803, constructed from these two strains, produced large amounts of isoleucine. Isoleucine production by strain T-803 paralleled growth and reached a maximum of about 25 mg ml⁻¹ at 96 h. No detectable threonine was formed as a by-product during this period.

Strain T-803 was non-lysogenic for phage PS20. Several lysogenic transductants carrying the same genotypes as those of strain T-803 also produced 23 to 25 mg isoleucine ml⁻¹. This indicated that lysogeny for phage PS20 did not affect the isoleucine productivity of *S. marcescens*.

DISCUSSION

In *S. marcescens*, isoleucine biosynthesis is regulated primarily by both feedback inhibition and repression of threonine deaminase and acetohydroxyacid synthase (Kisumi *et al.*, 1970, 1971*a*). However, the two regulatory mutations for these enzymes allowed strain GIHVLr6426 [*ihr-1*, *ilvA2*(Fr)] to produce only a small amount of isoleucine. This strain had about twice the homoserine dehydrogenase activity of the wild-type. These results suggested that in strain GIHVLr6426 threonine biosynthesis was partially derepressed by the reduction of the intracellular threonine pool. It could therefore be concluded that multiple regulatory mutations not only for isoleucine biosynthesis but also for threonine biosynthesis were required for higher isoleucine production. This was confirmed by the transductional construction of strain T-803 having such mutations. This strain is useful for industrial production of isoleucine because of its high productivity.

Strain T-803, constructed in this experiment, produced 1 to 2 mg α -aminobutyric acid ml⁻¹ together with 25 mg isoleucine ml⁻¹. Generally, α -aminobutyric acid is formed from α -ketobutyrate by transamination when acetohydroxyacid synthase-catalysed conversion of α -ketobutyrate to α -aceto- α -hydroxybutyrate is limited (Rudman & Meister, 1953; Kisumi *et al.*, 1970). In strain T-803, α -ketobutyrate is considered to be formed in large amounts since both the threonine biosynthetic enzymes and threonine deaminase are released from feedback controls. Therefore, large amounts of α -ketobutyrate are converted to isoleucine. However, the appreciable amounts of α -ketobutyrate formed seem not to flow into isoleucine biosynthesis but to overflow into α -aminobutyric acid formation. Therefore, the activity of acetohydroxyacid synthase in strain T-803 is likely to be insufficient for maximum isoleucine production. Accordingly, a mutation increasing the activity of acetohydroxyacid synthase is expected to result in higher isoleucine production. An isoleucine-producing strain carrying such a mutation is being constructed.

Isoleucine is implicated in the repression of both *ilv* and *thr* operons in *E. coli* K12 (Umbarger, 1978). Isoleucyl-tRNA is formed from isoleucine by isoleucyl-tRNA synthetase

and is supposed to act as a signal for the repression in *E. coli* K12 (Dwyer & Umbarger, 1968; Iaccarino & Berg, 1971; Lawther & Hatfield, 1977). The *hnr-1* mutation described in this paper caused constitutive synthesis of enzymes responsible for isoleucine and threonine biosyntheses and was located in the *thr* region. In *E. coli* K12, the *ileS* gene, coding for isoleucyl-tRNA synthetase, is located between the *leu* and *thr* operons, and the *ileS* mutation derepresses the enzymes of *ilv* and *thr* operons (Dwyer & Umbarger, 1968; Iaccarino & Berg, 1971). Therefore, the *hnr-1* mutation of *S. marcescens* could be analogous to the *ileS* mutation of *E. coli* K12.

We have described another mutation, *ihr-1*, constitutive for isoleucine biosynthesis. This mutation was found to derepress threonine deaminase and acetohydroxyacid synthase and to be closely linked to the *ilvA* gene. These characteristics indicate that this regulatory mutation is similar to the *ilvO* mutation described for *E. coli* K12 by Ramakrishnan & Adelberg (1964, 1965*a, b*), Favre *et al.* (1976) and Smith *et al.* (1976).

Escherichia coli and *Salmonella typhimurium* have at least two isoenzymes of acetohydroxyacid synthase: valine-sensitive and valine-insensitive isoenzymes (Umbarger, 1978; De Felice *et al.*, 1979). *Serratia marcescens* mutants carrying the *hnr-1* or *ihr-1* mutations showed an increased activity of acetohydroxyacid synthase, which was insensitive to valine. Assuming that *S. marcescens* has both isoenzymes, it is possible that the above regulatory mutations lead to constitutive synthesis of the valine-insensitive isoenzyme along with the other isoleucine biosynthetic enzymes. Genetic study of the two isoenzymes of acetohydroxyacid synthase in *S. marcescens* will be described in a separate paper.

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