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Threonine Production by Regulatory Mutants of *Serratia marcescens*

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β-Hydroxynorvaline (α-amino-β-hydroxyvaleric acid)-resistant mutants of *Serratia marcescens* deficient in both threonine dehydrogenase and threonine deaminase were isolated and characterized. One of the mutants, strain HNr21, lacked feedback inhibition of threonine-sensitive aspartokinase and homoserine dehydrogenase, was repressed for the two enzymes, and produced 11 mg of threonine per ml of medium containing a limiting amount of isoleucine. The other mutant, strain HNr59, was constitutively derepressed for aspartokinase and homoserine dehydrogenase. Its kinase was sensitive to feedback inhibition, but its dehydrogenase was insensitive to feedback inhibition. This strain produced 5 mg of threonine per ml of medium containing either a limiting or an excess amount of isoleucine. Diaminopimelate auxotrophs derived from strain HNr59 produced more threonine (13 mg/ml) than the parent strain. However, similar auxotrophs derived from strain HNr21 produced the same amount of threonine as that produced by the parent strain.

We have been studying isoleucine production by regulatory mutants of *Serratia marcescens* (11, 15, 16). These mutants lacked both feedback inhibition of threonine deaminase (EC 4.2.1.16) and repression of the isoleucine biosynthetic enzymes and produced large amounts of isoleucine in the medium containing glucose and urea (13). These observations indicated that strains of *S. marcescens* might possess high potential activity for threonine production, since isoleucine is formed from threonine. However, we found that the wild strain of *S. marcescens* rapidly degraded threonine added to the medium (10). This degradation is catalyzed by both threonine dehydrogenase (EC 1.1.1.103) and "biosynthetic" threonine deaminase (Komatsubara et al., manuscript in preparation). Therefore, we expected that the combination of threonine biosynthesis release from feedback controls and the deficiency of threonine-degrading enzymes would induce mutants of *S. marcescens* to produce large amounts of threonine.

The isolation of a mutant deficient in both threonine dehydrogenase and threonine deaminase will be described in a related paper (Komatsubara et al., manuscript in preparation). The present paper deals with threonine production by regulatory mutants derived from a mutant deficient in threonine-degrading enzymes.

MATERIALS AND METHODS

Bacterial strains. Derivatives of *S. marcescens* Sr41 (20) were used (Table 1).

Growth experiments. Growth experiments were performed by the methods described previously (14). Growth was estimated as optical density at 660 nm, using a Hitachi electric photometer (type EPO-B). An optical density of 0.10 corresponds to 5×10^8 cells per ml.

Isolation of β-hydroxynorvaline (α-amino-β-hydroxyvaleric acid)-resistant mutants. Cells of strain D-60 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1), modified by replacing the buffer for mutagenesis with nutrient broth. Nutrient broth contained 0.5% glucose, 1.0% peptone, 0.3% meat extract, 1.0% yeast extract, and 0.5% NaCl. Treated cells were spread on minimal agar plates containing DL-β-hydroxynorvaline (10 and 20 mg/ml), L-isoleucine (10 mg/ml), L-methionine (10 mg/ml), L-lysine (10 mg/ml), and 0.5% glycerol as a carbon source. After 2 days of incubation at 30°C, colonies appeared on the plates. These colonies were tested for threonine excretion by streaking on agar plates seeded with a threonine auxotroph. Colonies forming a halo were further purified by single-colony isolation and were used as β-hydroxynorvaline-resistant mutants.

Isolation of diaminopimelate and lysine auxotrophs. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated cells of strains HNr21 and HNr59 were further treated with nalidixic acid by the method of Weiner et al. (30) to enrich amino acid-requiring mutants. Auxotrophy was determined by auxanography.

Enzyme activities. A 500-ml Sakaguchi shaking flask contained 150 ml of minimal medium (14) supplemented with 0.02% glucose as a carbon source. The medium was inoculated with a loopful of cells grown on a nutrient agar slant overnight and incubated at 30°C with reciprocal shaking (7-cm stroke, 140 rpm).

TABLE 1. *Strains of S. marcescens Sr41 used*

Strain	Parent	Property			
		Threonine dehydrogenase	Threonine deaminase	Auxotrophy	β-Hydroxynorvaline resistance
8000	Wild	+	+	None	Sensitive
Mu-910	8000	−	+	None	Sensitive
D-60	Mu-910	−	−	Isoleucine	Sensitive
HNr21	D-60	−	−	Isoleucine	Resistant
HNr53	D-60	−	−	Isoleucine	Resistant
HNr59	D-60	−	−	Isoleucine	Resistant
E-74	HNr59	−	−	Isoleucine, lysine	Resistant
E-84	HNr59	−	−	Isoleucine, diaminopimelate	Resistant
J-6	HNr21	−	−	Isoleucine, diaminopimelate	Resistant

After 16 h the growth ceased at an optical density of 0.05 to 0.08 because of glucose limitation. The glucose concentration was increased to 0.5%, and the incubation was continued until the optical density reached approximately 0.5 (late log phase). The medium for isoleucine auxotrophs contained 0.05 mM L-isoleucine initially. After 16 h of incubation, the medium was supplemented with 1 mM L-threonine plus 1 mM L-isoleucine or 1 mM L-threonine plus 0.5 mM D-threonine for repression or derepression of threonine biosynthetic enzymes, respectively. D-Threonine was used as a limiting source of isoleucine (14). Late-log-phase cells were harvested, washed, and suspended in 50 mM potassium phosphate buffer (pH 8.0) containing 30% glycerol for enzyme stabilization. Cell-free extracts were prepared by the methods described previously (14).

Aspartokinase and homoserine dehydrogenase activities were determined by measuring aspartylhydroxamate formation and nicotinamide adenine dinucleotide phosphate reduction, respectively, at 30°C. The reaction conditions were described previously (13). Protein concentration was measured by the procedure of Lowry et al. (19). Specific activities are expressed as micromoles of products per milligram of protein per minute.

Threonine production. The medium for threonine production contained 10% glucose, 1% urea, 0.05% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_2O$, and 4% $CaCO_3$. Glucose was autoclaved separately and mixed with the other components. A loopful of cells grown on nutrient slants overnight was inoculated into 15 ml of the medium in 500-ml Sakaguchi shaking flasks and incubated at 30°C with reciprocal shaking (7-cm stroke, 140 rpm). The growth was estimated as described previously (12) and is expressed as dry cell weight. Threonine was measured by bioassay with *Leuconostoc mesenteroides* p-60.

Chemicals. DL-β-Hydroxynorvaline (*threo* form) was prepared from methylisocyanoacetate and propionaldehyde by the synthetic method for *threo*-threonine (21), modified by replacing acetaldehyde with propionaldehyde. Methylisocyanoacetate was kindly given by K. Matsumoto. The other chemicals for the synthesis were reagent grade. The preparation contained less than 1% DL-β-allo-hydroxynorvaline.

α,ε-Diaminopimelate was a mixture of the LL, DD, and *meso* isomers obtained from Sigma Chemical Co., St. Louis, Mo. The other reagents were of the highest quality commercially available.

RESULTS

Antagonism between β-hydroxynorvaline and threonine in the growth of *S. marcescens*. Isolation of threonine analog-resistant mutants was necessary for obtaining regulatory mutants of threonine biosynthesis. Therefore, the growth of *S. marcescens* was tested for the inhibition by β-hydroxynorvaline (2, 5, 24). This analog inhibited the growth of strain D-60, deficient in threonine dehydrogenase and threonine deaminase. When glucose was used as a carbon source, the inhibition was not sufficiently strong to isolate resistant mutants. To enhance the growth inhibition, glucose was replaced with several other carbon sources, and glycerol was selected as the best carbon source. When glycerol was used, high concentrations of the analog were required for strong inhibition (Fig. 1). This inhibition was reversed completely by threonine (Fig. 2). Methionine and lysine had little effect on the inhibition. Isoleucine showed only minimal antagonism toward β-hydroxynorvaline, using strain Mu-910, in contrast to the above experiments with strain D-60, which requires isoleucine.

Inhibition of aspartokinase and homoserine dehydrogenase activities by β-hydroxynorvaline. We examined whether β-hydroxynorvaline inhibited the activities of threonine-sensitive aspartokinase and homoserine dehydrogenase. Cell-free extracts were prepared from cells of strain D-60 cultured under conditions where the two enzymes are derepressed by limiting the source of isoleucine. The inhibition of threonine-sensitive aspartokinase activity was examined in the presence of lysine to avoid the effect of lysine-sensitive aspartokinase activity (Table 2). As expected, β-hydroxynorvaline inhibited the activities of aspartokinase and homoserine dehydrogenase to the same extent as threonine. Thus, β-hydroxynorvaline was found to be a false feedback inhibitor of threonine-sensitive kinase and dehydrogenase and a favorable antagonist in selecting for feedback inhibition-insensitive mutants.

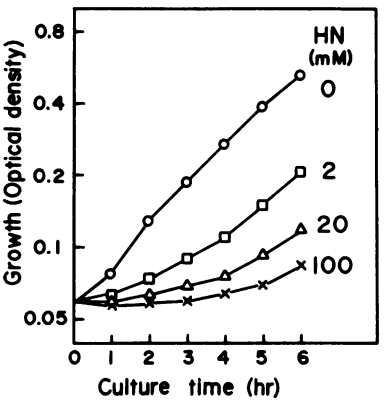

FIG. 1. *Growth inhibition of strain D-60 by β-hydroxynorvaline.* DL-*β-Hydroxynorvaline (HN) was added to the minimal medium containing glycerol as a carbon source and 0.3 mM* L-*isoleucine.*

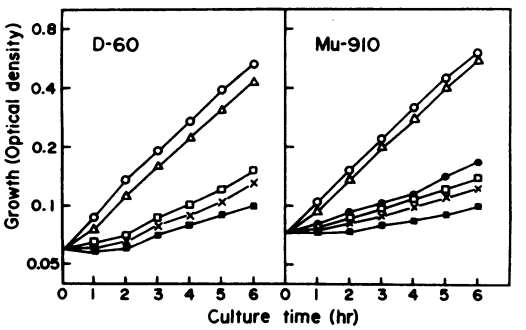

FIG. 2. *Antagonism betweeen β-hydroxynorvaline and threonine in strains D-60 and Mu-910. The medium for strain D-60 was supplemented with 0.3 mM* L-*isoleucine. Addition to the minimal medium containing glycerol as a carbon source:* (○), *none;* (×), *20 mM* DL-*β-hydroxynorvaline (HN):* (△), *20 mM HN + 1 mM* L-*threonine;* (□), *20 mM HN + 1 mM* L-*methionine;* (■), *20 mM HN + mM* L-*lysine;* (●), *20 mM HN + 1 mM* L-*isoleucine.*

Derepression of aspartokinase and homoserine dehydrogenase by β-hydroxynorvaline. If β-hydroxynorvaline acts as a false feedback inhibitor in vivo, this antagonist would decrease threonine formation and probably derepress threonine biosynthetic enzymes. Therefore, the effect of β-hydroxynorvaline on the formation of threonine-sensitive aspartokinase and homoserine dehydrogenase was examined (Table 3). Prototrophic strain Mu-910 was used to exclude the possibility of interaction between the analog and isoleucine in strain D-60. The addition of β-hydroxynorvaline derepressed aspartokinase and homoserine dehydrogenase. The derepressed aspartokinase activity was markedly inhibited by threonine and only slightly by lysine, suggesting the derepression of threonine-sensitive aspartokinase. This derepression was overcome by threonine. These results indicate that mutants in which threonine biosynthetic enzymes are constitutive would also be found among β-hydroxynorvaline-resistant mutants.

Isolation of β-hydroxynorvaline-resistant mutants. Mutated cells of strain D-60 were spread on minimal agar plates containing glycerol as a carbon source and high concentrations of β-hydroxynorvaline, isoleucine, methionine, and lysine. The three natural amino acids were added to select mutants lacking feedback con-

TABLE 2. *Effect of β-hydroxynorvaline on the activities of aspartokinase and homoserine dehydrogenase*[a]

Addition to the reaction mixture	Inhibition (%)	
	Aspartokinase[b]	Homoserine dehydrogenase
None	0 (0.13)[c]	0 (0.051)
L-Threonine		
0.1 mM	0	0
1 mM	4	70
10 mM	65	79
DL-β-Hydroxynorvaline		
0.2 mM	0	0
2 mM	3	55
20 mM	72	81

[a] Cell-free extracts were prepared from cells of strain D-60 grown under conditions where threonine biosynthetic enzymes were derepressed by limiting the isoleucine source.

[b] The inhibition was determined in the presence of 50 mM L-lysine to avoid the effect of lysine-sensitive aspartokinase activity.

[c] Numbers in parentheses represent specific activities.

TABLE 3. *Effect of β-hydroxynorvaline on the formation of aspartokinase and homoserine dehydrogenase*[a]

Addition to minimal medium[b]	Sp act		
	Aspartokinase[c]		Homoserine dehydrogenase
	None	L-Lysine	
None	0.06	0.03	0.009
L-Threonine	0.05	0.03	0.008
DL-β-Hydroxynorvaline	0.10	0.08	0.027
DL-β-Hydroxynorvaline + L-threonine	0.05	0.03	0.009

[a] Cell-free extracts were prepared from cells of strain Mu-910.

[b] L-Threonine and DL-β-hydroxynorvaline were added at 1 mM.

[c] The activities were determined in the presence and absence of 50 mM L-lysine.

trols to a greater extent. Large colonies found after 2 days were tested for threonine excretion. Threonine-excreting colonies were purified by single-colony isolation. Thus, 65 strains were selected as β-hydroxynorvaline-resistant mutants. The three resistant mutants discussed below are representatives of the alteration of feedback controls.

Growth of β-hydroxynorvaline-resistant mutants. The growth rate of strain HNr21 in a minimal medium containing glycerol or glucose as a carbon source was similar to that of the parental strain D-60 (Table 4). Strains HNr53 and HNr59 showed slower growth rates than strain D-60. These three mutants were resistant to a high concentration of the analog.

Homoserine inhibits the growth of *Escherichia coli* B/r by inhibiting glutamate dehydrogenase activity (18). The growth rate of *S. marcescens* D-60 was also decreased by homoserine. If β-hydroxynorvaline-resistant mutants have derepressed levels of homoserine kinase (EC 2.7.1.39) and threonine synthase (EC 4.2.99.2), homoserine added to the medium is rapidly converted to *O*-phosphohomoserine or threonine and does not inhibit their growth. The growth rate of strain HNr59 was not affected by homoserine and that of strain HNr53 was minimally decreased, whereas those of strains D-60 and HNr21 were strongly reduced. These results suggest that there are differences among β-hydroxynorvaline-resistant mutants in the alteration of feedback controls of threonine biosynthesis.

Alterations of aspartokinase and homoserine dehydrogenase in β-hydroxynorvaline-resistant mutants. We determined whether threonine-sensitive aspartokinase and homoserine dehydrogenase are released from feedback inhibition and repression in the β-hydroxynorvaline-resistant mutants (Table 5, Fig. 3). Both enzymes of strain HNr21 were significantly insensitive to feedback inhibition by threonine, whereas they were repressed by an excess of threonine plus isoleucine. Their sensitivities to threonine were decreased more than 100-fold as compared with those of the parent strain. Strain HNr53 had constitutively high levels of aspartokinase and homoserine dehydrogenase. The two enzymes were sensitive to feedback inhibition to the same extent as those of strain D-60. Strain HNr59 also showed constitutive formation of the two enzymes. Its aspartokinase was normally sensitive to feedback inhibition, but homoserine dehydrogenase was less sensitive to threonine. Strains HNr53 and HNr59 have not been tested for homoserine kinase and threonine synthase. However, the above-described weak, or lack of, growth inhibition by homoserine indicates that these strains have constitutive levels of both the kinase and the synthase. Thus, three types of regulatory mutants were found among β-hydroxynorvaline-resistant mutants derived from strain D-60.

TABLE 4. *Effects of β-hydroxynorvaline and homoserine on the growth rates of β-hydroxynorvaline-resistant mutants*

Strain	Specific growth rate[a] (*k*)			
	Glycerol-minimal medium		Glucose-minimal medium	
	None	DL-β-Hydroxynorvaline (100 mM)	None	L-Homoserine (10 mM)
Mu-910	0.39	<0.07	0.53	0.16
D-60[b]	0.39	<0.09	0.58	0.26
HNr21[b]	0.39	0.31	0.53	0.24
HNr53[b]	0.30	0.31	0.36	0.27
HNr59[b]	0.26	0.20	0.23	0.23

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

[b] The medium for these strains was supplemented with 0.3 mM L-isoleucine.

TABLE 5. *Feedback inhibition and repression of aspartokinase and homoserine dehydrogenase in β-hydroxynorvaline-resistant mutants*

Strain	Medium[a]	Sp act		
		Aspartokinase[b]		Homoserine dehydrogenase
		None	L-Lysine	
Mu-910	Minimal	0.05	0.03	0.007
D-60	Excess	0.05	0.03	0.010
	Limiting	0.13	0.11 (58)[c]	0.044 (78)
HNr21	Excess	0.05	0.03	0.006
	Limiting	0.12	0.10 (2)	0.049 (22)
HNr53	Excess	0.17	0.14	0.081
	Limiting	0.15	0.12 (55)	0.061 (80)
HNr59	Excess	0.14	0.13	0.067
	Limiting	0.15	0.13 (57)	0.067 (38)

[a] The excess medium contained 1 mM L-threonine and 1 mM L-isoleucine for repression. The limiting medium contained 1 mM L-threonine and 0.5 mM D-threonine as a limiting source of isoleucine.

[b] The activities were determined in the presence and absence of 50 mM L-lysine.

[c] Numbers in parentheses are percent inhibition by 10 mM L-threonine.

Threonine production by β-hydroxynorvaline-resistant mutants. The three representative β-hydroxynorvaline-resistant mutants were tested for threonine production (Table 6). The parent strain D-60 produced minimal levels of threonine in the medium containing a limiting or an excess amount of isoleucine. Strain HNr21 produced approximately 11 mg of threonine per ml on addition of a limiting amount of isoleucine. This production was decreased with a higher

concentration of isoleucine, owing to repression of threonine biosynthetic enzymes. Strain HNr59 also produced threonine, but its productivity was lower than that of strain HNr21, because of feedback inhibition of aspartokinase. In this strain, a higher concentration of isoleucine had no effect on the threonine production, owing to the constitutive synthesis of threonine biosynthetic enzymes. Strain HNr53 produced a trace amount of threonine because of feedback inhibition of aspartokinase and homoserine dehydrogenase.

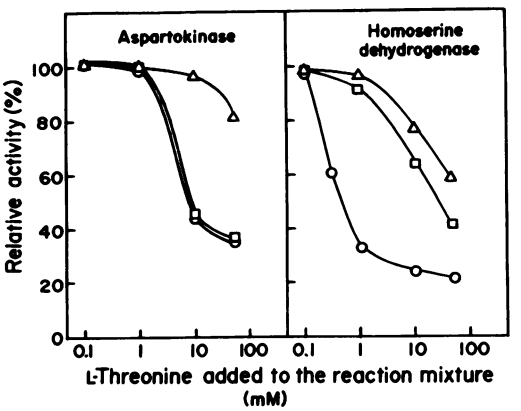

FIG. 3. *Lack of feedback inhibition of aspartokinase and homoserine dehydrogenase in β-hydroxynorvaline-resistant mutants. Cell-free extracts were prepared from cells grown in isoleucine-limiting minimal medium. The inhibition of aspartokinase activity was determined in the presence of 50 mM* L-*lysine. Strains:* (○), *D-60;* (△), *HNr21;* (□), *HNr59.*

Threonine production by diaminopimelate and lysine auxotrophs derived from β-hydroxynorvaline-resistant mutants. In *S. marcescens,* one of the aspartokinases is feedback controlled by lysine. Lysine is synthesized from aspartate-β-semialdehyde, an intermediate of threonine, via diaminopimelate. The lack of feedback controls of lysine-sensitive aspartokinase and the block of aspartate-β-semialdehyde flow to the lysine biosynthetic pathway might increase threonine production in β-hydroxynorvaline-resistant mutants. Therefore, diaminopimelate and lysine auxotrophs were derived from strain HNr59. A representative lysine auxotroph, E-74, produced almost the same amount of threonine as that produced by strain HNr59 (Table 7). Strain E-84, a diaminopimelate auxotroph, produced an increased amount of threonine with the addition of appropriate amounts

TABLE 6. *Threonine production by β-hydroxynorvaline-resistant mutants*

Strain	Addition of L-isoleucine to the medium (mM)	Growth (mg/ml, dry wt) at:				L-Threonine produced (mg/ml) at:			
		48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
D-60	2	5.8	7.4	12.8	23.8	0.1	0.1	0.3	0.3
	10	17.2	27.3	25.2	19.4	0.1	0.1	0.1	0.1
HNr21	2	7.0	11.2	19.0	18.1	1.9	7.4	8.9	10.9
	10	16.8	22.7	24.2	20.0	0.9	3.6	4.2	3.7
HNr53	2	1.6	7.3	11.3	18.3	0.1	0.3	0.3	0.3
	10	12.8	18.3	19.4	18.3	0.3	0.3	0.3	0.3
HNr59	2	3.2	13.3	26.1	28.9	1.2	2.9	4.8	5.0
	10	8.4	25.3	27.4	26.0	1.0	3.0	4.6	5.1

TABLE 7. *Threonine production by diaminopimelate and lysine auxotrophs derived from strains HNr59 and HNr21*

Strain	Parent	Addition to medium[a] (mM)	Growth (mg/ml, dry wt) at:			L-Threonine produced (mg/ml) at:		
			72 h	96 h	120 h	72 h	96 h	120 h
HNr59	Parent	None	16.3	24.3	23.3	3.1	4.9	4.7
E-74	HNr59	Lys, 5	4.7	5.5	14.3	1.5	1.8	2.6
		Lys, 10	11.8	14.3	15.0	3.7	4.8	5.8
E-84	HNr59	Dap, 5, + Lys, 5	4.5	5.0	7.1	2.8	3.8	4.8
		Dap, 5, + Lys, 10	13.0	15.0	16.7	3.8	7.7	12.7
		Dap, 10, + Lys, 5	8.6	13.6	14.7	2.3	5.1	7.0
		Dap, 10, + Lys, 10	15.0	19.0	21.0	1.6	7.4	12.7
HNr21	Parent	None	14.3	21.3	22.7	4.9	9.1	10.0
J-6	HNr21	Dap, 5, + Lys, 5	6.3	10.0	12.3	2.7	4.7	6.0
		Dap, 5, + Lys, 10	9.2	11.8	13.9	6.2	8.8	10.3
		Dap, 10, + Lys, 5	7.1	11.3	11.3	2.6	4.4	4.4
		Dap, 10, + Lys, 10	12.3	16.3	14.1	5.9	8.8	10.1

[a] In addition to DL-α,ε-diaminopimelate (Dap) and L-lysine (Lys), all media contained 2 mM L-isoleucine.

of diaminopimelate and lysine. On the other hand, diaminopimelate auxotroph J-6, which was derived from strain HNr21, did not produce a larger amount of threonine than the parent strain under the test conditions used.

DISCUSSION

Auxotrophic and analog-resistant mutants of various bacteria have been used for the microbial production of threonine from simple carbon sources (6–9, 27, 28). In these mutants, the threonine biosynthetic enzymes are released from feedback controls "phenotypically" and genetically, but the threonine-degrading enzymes are not altered. On the other hand, our threonine-producing strains of *S. marcescens* not only lack the feedback controls but also are deficient in the degrading enzymes. The deficiency of threonine-degrading enzymes was essential for the construction of threonine-producing strains of *S. marcescens*, since the wild strain rapidly degraded threonine. Thus, the combination of a deficiency in degradation and lack of feedback controls makes it possible to construct excellent amino acid-producing strains. Histidine- and arginine-producing strains of *S. marcescens* were also constructed by similar methods (17; Kisumi et al., manuscript in preparation).

The regulation of threonine biosynthesis in enteric bacteria (4, 29) is complicated, since (i) aspartate-β-semialdehyde and homoserine are common intermediates in the biosynthesis of threonine, lysine, and methionine, (ii) threonine is a direct precursor of isoleucine, and (iii) three separate aspartokinases and two distinct homoserine dehydrogenases are subject to feedback controls by threonine, isoleucine, lysine, and methionine alone or in combination (Fig. 4). However, *Brevibacterium flavum* and *Corynebacterium glutamicum* possess more simple regulatory mechanisms, since they have no isozymes for the two enzymes and since the repression of their threonine biosynthesis is not strict (22, 23, 26). Therefore, the derivation of threonine-producing strains of these bacteria is easier than that of the strains of *S. marcescens*. However, the advantage of using *S. marcescens* for the construction of threonine-producing strains is the high possibility that biochemical and genetic data obtained from the other enteric bacteria apply to *S. marcescens*.

S. marcescens has aspartokinase and homoserine dehydrogenase that are feedback inhibited by threonine and multivalently repressed by threonine and isoleucine, as observed in *E. coli* and *Salmonella typhimurium*. β-Hydroxynorvaline-resistant mutants of *S. marcescens* lacked either feedback inhibition or repression of threonine-sensitive aspartokinase and homoserine dehydrogenase. Based on the correlation between threonine production and lack of feedback controls in the three types of mutants, we presume that the feedback inhibition of homoserine dehydrogenase is primarily rate limiting for threonine production and the feedback inhibition of aspartokinase is secondarily rate limiting.

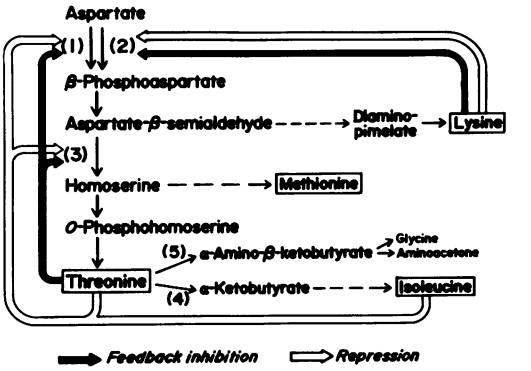

FIG. 4. *Pathways of biosynthesis and degradation of threonine in S. marcescens. The presence of methionine-repressible aspartokinase and homoserine dehydrogenase, as found for E. coli, is not clear. Enzymes: (1) threonine-sensitive aspartokinase; (2) lysine-sensitive aspartokinase; (3) homoserine dehydrogenase; (4) threonine deaminase; (5) threonine dehydrogenase.*

Lysine-sensitive aspartokinase relates not only to lysine biosynthesis but also to threonine biosynthesis in *E. coli* (4). *S. marcescens* also possesses an aspartokinase subject to feedback inhibition and repression by lysine (unpublished data). Derivation of diaminopimelate auxotrophs from strain HNr59 increased threonine production under conditions where lysine-sensitive aspartokinase might be released from feedback controls. These data indicate that in *S. marcescens* lysine-sensitive aspartokinase also participates in threonine formation, at least when threonine-sensitive aspartokinase is subject to feedback inhibition.

The third aspartokinase and the second homoserine dehydrogenase are present as minor components in *E. coli* and *S. typhimurium* (4, 29). In some cases, these enzymes are involved in threonine biosynthesis (3, 29). Nevertheless, we found no detectable activities of the two enzymes in *S. marcescens* Sr41 by the assay method we used. *S. marcescens* Sa-3 was recently reported to have methionine-repressible homoserine dehydrogenase, the activity of which was detected in cell-free extracts after aging for several days at a low temperature (25). In some β-hydroxynorvaline-resistant mutants,

which produced a small amount of threonine, no significant alteration was found in feedback controls of threonine-sensitive enzymes. However, the possibility is not excluded that methionine-repressible enzymes participate in threonine production in these mutants.

We are constructing threonine-producing strains suitable for the industrial production of threonine. The combination of genetic desensitization and genetic derepression of threonine-sensitive aspartokinase and homoserine dehydrogenase is expected to increase threonine productivity of *S. marcescens*. Moreover, genetic release of feedback controls of lysine-sensitive aspartokinase will also result in higher productivity. Therefore, to obtain strains having higher productivity, we plan to combine loss of feedback controls for threonine-sensitive aspartokinase, homoserine dehydrogenase, and lysine-sensitive aspartokinase by transduction.

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LITERATURE CITED

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