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Proline-Hyperproducing Strains of *Serratia marcescens*: Enhancement of Proline Analog-Mediated Growth Inhibition by Increasing Osmotic Stress

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Proline-producing strains of *Serratia marcescens* were more osmotolerant than wild-type strains. Growth inhibition by proline analogs was significantly enhanced by increasing the osmotic stress of the medium. Mutants resistant to azetidine-2-carboxylate were derived from a proline-producing strain, SP126, under a high osmotic condition. One of the mutants, strain SP187, produced 56 mg of L-proline per ml of medium containing sucrose and urea. This amount was ca. 3 times larger than that produced by strain SP126. The intracellular glutamate content which decreased in strain SP126 was restored in strain SP187. The glutamate dehydrogenase level of strain SP187 was 5 times higher than that of strain SP126.

Proline is synthesized from glutamate by glutamate kinase, glutamate-γ-semialdehyde dehydrogenase, and pyrroline-5-carboxylate reductase in bacteria (1, 23). Genes *proB* and *proA*, which specify glutamate kinase and glutamate-γ-semialdehyde dehydrogenase, respectively, were found to comprise an operon in *Salmonella typhimurium* (13) and *Escherichia coli* (9). The pyrroline-5-carboxylate reductase encoded in the *proC* region was not repressed by proline in *E. coli* (19). We found that feedback controls of proline biosynthesis in *Serratia marcescens* were similar to those in *E. coli*, and that feedback inhibition of glutamate kinase acted as the primary mechanism for the control of proline biosynthesis (M. Sugiura, T. Takagi, and M. Kisumi, Appl. Microbiol. Biotechnol., in press). Meanwhile, wild-type strains of *Serratia marcescens* rapidly degraded proline by proline oxidase (proline dehydrogenase), encoded in the *putA* gene. We have constructed a proline-overproducing strain, SP126, as a double mutant resistant to 3,4-dehydroproline and thiazolidine-4-carboxylate derived from a proline oxidase-deficient mutant. Strain SP126 produced ca. 20 mg of proline per ml in the fermentation medium (Sugiura et al., in press).

In many organisms from bacteria (16, 21) to plants (11, 24), the intracellular proline content increases in response to environmental osmotic stress. Csonka found that proline-overproducing strains of *Salmonella typhimurium* acquired osmotolerance (5). In this communication, we report the osmotolerance of proline-overproducing strains of *Serratia marcescens* and the enhancement of proline analog-mediated growth inhibition during osmotic stress. As a consequence of the mutation that caused the resistance to proline analogs during high osmotic stress, we derived proline-hyperproducing strains from strain SP126.

MATERIALS AND METHODS

Microorganisms. The strains of *Serratia marcescens* Sr41 used in this study are listed in Table 1.

Media. Minimal medium (M1 medium) was that of Davis and Mingioli (7), modified by omitting the citrate and increasing the glucose to 0.5%. Nutrient medium (N1 medium) was nutrient broth supplemented with 0.3% yeast extract (both from Difco Laboratories, Detroit, Mich.). Fermentation medium (F1 medium) contained 15% sucrose, 2% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% corn steep liquor, and 3% $CaCO_3$ (pH 7.0). Sucrose and urea were increased if necessary.

Growth studies. Growth experiments were performed with a Hitachi automated recording incubator system as reported previously (20). L-Azetidine-2-carboxylate (AZC), 3,4-dehydro-DL-proline (DHP), and L-thiazolidine-4-carboxylate (TAC) were added asceptically after filter sterilization.

Mutagenesis and isolation of mutants. Exponentially growing cells of strain SP126 were mutagenized with 250 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in N1 medium at 30°C for 15 min. The cells were spread on the M1 agar medium containing either (i) 200 mM NaCl–2 mM AZC–0.5% sodium succinate instead of glucose or (ii) 400 mM NaCl–4 mM DHP, and were incubated at 30°C for 4 days. The resulting colonies were picked up and tested for proline productivity in F1 medium. The represented strains were strain SP187 obtained in medium (i) and strain SP190 in medium (ii).

Fermentation experiments. A loopful of cells (ca. 10^8 cells) grown on the N1 medium was inoculated into 15 ml of F1 medium in a 500-ml shaking flask. Cultivation was carried out on a reciprocal shaker (140 rpm, 7-cm stroke) in a room maintained at 30°C and 80% humidity. The evaporation of medium was less than 5% for the 7-day cultivation. Growth was estimated by measuring the optical density at 660 nm of fermentation broth diluted with 0.1 N HCl. It was expressed as dry cell weight calculated from a standard curve. The amount of proline was determined by microbioassay, using *Leuconostoc mesenteroides*.

Transduction experiments. Transduction was carried out by the method of Matsumoto et al. (15). N1 medium was used for cultivation of bacteria. The lysate of PS20 phage propagated on strain SP187 was added to the cultures of strain SP139 (*proB*$^-$/*A*$^-$) or SGD-11 (*gdhA*$^-$ *gltB*$^-$) to give a multiplicity of infection of 50. Pro$^+$ (*proB*$^+$/*A*$^+$) transductants were selected on M1 agar medium after incubation at 30°C for 2 days. Glu$^+$ (*gdhA*$^+$ *gltB*$^-$) transductants were selected as Glu$^+$ strains which exhibited glutamate auxotrophies only on low-ammonia medium [decreased $(NH_4)_2SO_4$ concentration of M1 medium to 0.0007%].

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TABLE 1. Strains of *Serratia marcescens* Sr41 used

Strain	Properties[a]	Reference
8000	Wild type	14
SP103	*putA*$^-$; proline oxidase-deficient	Sugiura et al., in press
SP105	SP103 *dpr-1*; resistant to 1 mM 3,4-dehydro-DL-proline	Sugiura et al., in press
SP126	SP105 *tcr-1*; resistant to 10 mM L-thiazolidine-4-carboxylate in succinate M1 medium	Sugiura et al., in press
SP139	SP103 *proB*$^-$/*A*$^-$; proline auxotroph	Sugiura et al., in press
SP187	SP126 *acr-2*; resistant to 2 mM L-azetidine-2-carboxylate and 200 mM Nacl in succinate M1 medium	This paper
SP190	SP126 *dpr-2*; resistant to 4 mM 3,4-dehydro-DL-proline and 400 mM NaCl	This paper
SGD11[b]	*gdhA*$^-$ *gltB*$^-$; glutamate auxotroph deficient in both GDH and GOGAT	This paper
SP457	SGD11 *gdhA*$^+$ by PS20 transduction from SP187	This paper
SP555	SP139 *proB*$^+$/*A*$^+$ by PS20 transduction from SP187	This paper

[a] Symbols other than *dpr-1*, *tcr-1*, *acr-2*, and *dpr-2* are described by Bachmann (3).
[b] Strain SGD11 was obtained by N. Nishimura in our laboratory.

Enzyme assays. Cells were cultured at 30°C with shaking in 500 ml-flasks containing 150 ml of M1 medium or 15 ml of F1 medium. Exponentially growing cells in M1 medium or 72-h cells in F1 medium were harvested by centrifugation, washed twice with cooled saline, suspended with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 30% glycerol and 1 mM dithiothreitol, disrupted with a sonic oscillator for 3 min below 5°C, and centrifuged at 27,000 × *g* for 30 min at 0°C. The supernatant fluids were used as cell-free extracts for enzyme assay. Glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) activities were determined by measuring the reduction of NADPH by the method of Dendinger et al. (8). Protein was measured by the procedure of Lowry et al. (12). Specific activities were expressed as micromoles of product per minute per milligram of protein.

Intracellular contents of proline and glutamate. Exponentially growing cells in 150 ml of M1 medium were harvested by centrifugation and washed rapidly with 20 ml of cooled M1 medium. The cells were suspended with 2 ml of water, and amino acids were extracted by boiling for 10 min. After centrifugation at 8,000 × *g* for 3 min, proline or glutamate content in the supernatant was determined by microbioassay, using *L. mesenteroides* P-60. In this procedure, duplicates agreed usually to less than 10% from the mean. The content was expressed as millimolar concentration by assuming the cell-bound water contents to be four times the bacteria dry cell weight (21).

RESULTS

Growth of proline-producing strains during osmotic stress. In nonhalophilic bacteria, the intracellular content of proline is changed in response to osmotic stress (4, 10, 16, 21). We examined the growth of proline-producing strains during high osmotic stress imposed by NaCl (Fig. 1). Proline-nonproducing strain SP103, a proline oxidase-deficient mutant, was sensitive to high concentrations of NaCl, whereas proline-producing strains SP105 and SP126 were more resistant to the growth inhibition. The higher-producing strain SP126 was more resistant to the osmotic stress. The growth inhibition by NaCl was reversed by glutamate and proline (Fig. 2). Other common amino acids showed no effects (data not shown). In strain SP103, 1 mM L-proline reversed the growth inhibition caused by 400 mM NaCl, and glutamate showed a little effect. On the contrary, glutamate as well as proline reversed the growth inhibition in strain SP105. However, both amino acids did not reverse the inhibition completely in both strains.

Sensitivity to proline analogs during high osmotic stress. Since the intracellular content of proline may increase

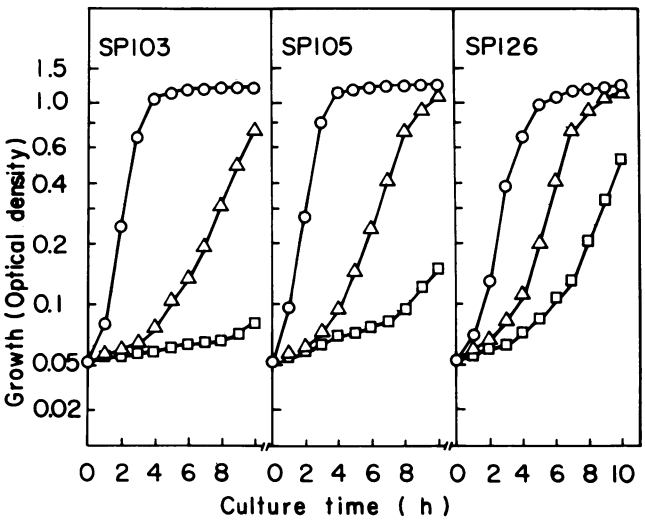

FIG. 1. Growth of *Serratia marcescens* SP103 (wild-type) and proline-producing strains SP105 and SP126 during high osmotic stress. Late-log-phase cells were inoculated into M1 medium to give an optical density at 660 nm of 0.05. Symbols: ○, no NaCl; △, 400 mM NaCl; □, 600 mM NaCl.

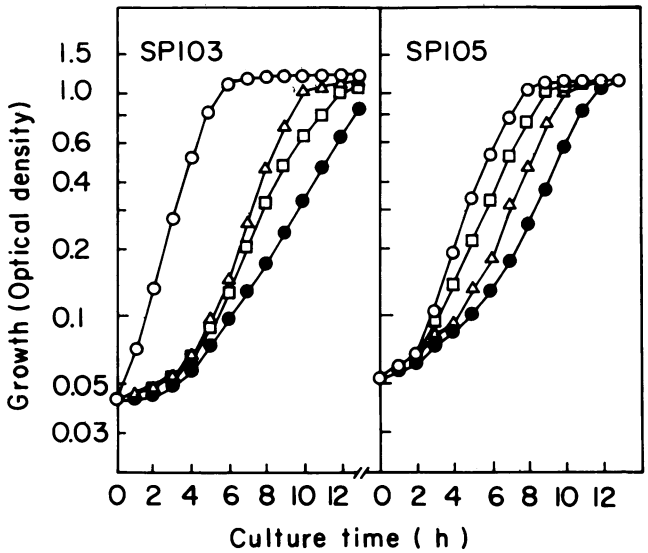

FIG. 2. Reversal of growth inhibition induced by 400 mM NaCl in *Serratia marcescens* SP103 and SP105. Symbols: ○, no addition; ●, 400 mM NaCl; △, 400 mM NaCl plus 1 mM L-proline; □, 400 mM NaCl plus 1 mM L-glutamate · sodium.

during high osmotic stress, the sensitivity to proline analogs must decrease in that condition. Accordingly, the sensitivities to AZC, TAC, and DHP were examined under a high osmotic stress. Contrary to expectation, the sensitivity to proline analogs increased significantly (Fig. 3). In M1 medium, 1 mM AZC and TAC did not inhibit the growth of strain SP103. In the presence of 500 mM NaCl, however, AZC or TAC inhibited the growth significantly. More surprisingly, sensitivity to proline analogs was enhanced even in the proline-overproducing strain SP126. We found that strain SP126 was completely resistant to 10 mM AZC, TAC, and DHP in M1 medium, as was strain SP105 (Sugiura et al., in press). However, 1 mM AZC or DHP significantly inhibited the growth of strain SP126 during a high osmotic stress (Fig. 3). The reversal of growth inhibition by AZC was examined during the high osmotic stress (Fig. 4). The AZC inhibition in strain SP103 was reversed by proline and partially by glutamate. On the other hand, the inhibition in strain SP126 was reversed by glutamate as well as by proline. Reversal of growth inhibition induced by DHP was similar to the inhibition by AZC (data not shown). These results indicated that mutants having higher productivities would be isolated from strain SP126 as AZC- or DHP-resistant mutants during a high osmotic stress.

Proline analog-resistant mutants obtained during high osmotic stress. AZC- or DHP-resistant mutants were isolated from strain SP126 in the presence of NaCl and tested for proline productivities in F1 medium. About 2% of AZC- and 3% of DHP-resistant mutants were enhanced in proline

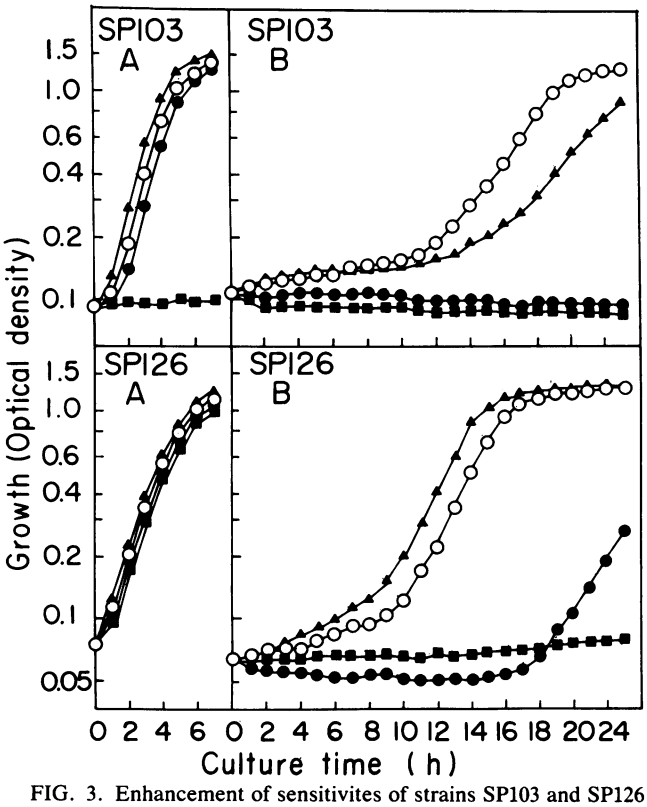

FIG. 3. Enhancement of sensitivites of strains SP103 and SP126 to proline analogs during high osmotic stress. Late-log-phase cells were inoculated into M1 minimal medium in the absence (A) and presence (B) of 500 mM NaCl. Symbols: ○, no addition; ■, 1 mM DHP; ●, 1 mM AZC; ▲, 1 mM TAC.

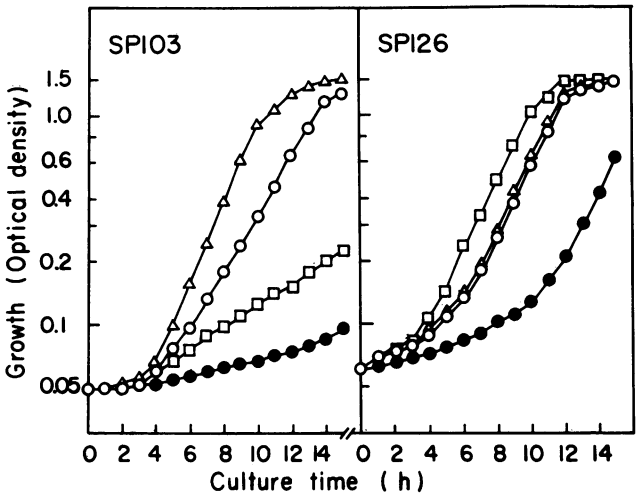

FIG. 4. Reversal of AZC-mediated growth inhibition of strains SP103 and SP126 by proline and glutamate during high osmotic stress. Late-log-phase cells were inoculated into M1 medium supplemented with 500 mM NaCl. Symbols: ○, no addition; ●, 1 mM AZC; △, 1 mM AZC plus 1 mM L-proline; □, 1 mM AZC plus 1 mM L-glutamate · sodium.

productivities. Representative AZC-resistant mutant SP187 and DHP-resistant mutant SP190 produced ca. 40 mg of proline per ml of medium containing 15% sucrose. Because both strains consumed sucrose exhaustively at 96 h, addition of higher concentrations of sucrose might result in an increase in proline productivity. As expected, strain SP187 produced 56 mg of L-proline per ml of the medium supplemented with 20% sucrose (Table 2).

Analyses of mutation in strain SP187. For a determination of the mutation of strain SP187, a transductional cross was performed between strain SP139 (*putA*$^-$ *proB*$^-$/*A*$^-$) as the recipient and strain SP187 as the donor, and Pro$^+$ transductants were selected. All of 30 Pro$^+$ transductants, represented by strain SP555, produced 4 to 5 mg of proline per ml (Table 3). This productivity was equivalent to that of strain SP105 (*putA*$^-$ *dpr-1*). Therefore, the mutation of the *proBA* region in strain SP187 may be the same as that in strains SP105 and SP126 (Sugiura et al., in press), i.e., strain SP187 must have acquired a new mutation (*acr-2*) located elsewhere than in the *proBA* genes. Moreover, hyperproducing

TABLE 2. Proline productivities by proline analog-resistant mutants obtained from strain SP126 under high osmotic conditions

Strain	Sucrose[a] (%)	Growth (mg [dry cell wt] per ml)			Residual sucrose (mg/ml)		Proline produced (mg/ml)		
		72 h	96 h	120 h	96 h	120 h	72 h	96 h	120 h
SP187	15	29	29	28	18	2	35	43	44
	18	29	30	29	49	23	33	52	54
	20	29	30	31	70	48	34	54	56
	22	28	29	29	96	70	29	48	53
SP190	15	27	26	NT[b]	5	NT	34	40	NT
	20	27	29	NT	65	NT	31	51	NT
SP126	15	28	29	NT	35	18	13	20	21

[a] Sucrose was added to F1 medium modified by increasing urea concentration to 2.5%.
[b] NT, Not tested.

TABLE 3. Proline productivity of transductant harboring *proB/A* region of strain SP187

Strain (genotype)	Proline productivity (mg/ml)[a]
SP555 (SP139 proB$^+$/A$^+$ from SP187)	4.5
SP187 (*putA*$^-$ *dpr-1 tcr-1 acr-2*)	43.2
SP105 (*putA*$^-$ *dpr-1*)	4.6
SP139 (*putA*$^-$ *proB*$^-$/A$^-$)[b]	0

[a] L-Proline productivity was determined by cultivation in F1 medium for 72 to 96 h.
[b] L-Proline was added at 0.5 mg/ml to the medium.

strains (over 50 mg/ml) could not be obtained all at once as AZC-NaCl-resistant mutants by transductional cross between strain SP187 as the donor and strain SP105 as the recipient, although such hyperproducing mutants could be obtained by transductional cross between strain SP187 as the donor and strain SP126 as the recipient (data not shown). These results suggest that the *acr-2* mutation might be different and distant from the *trc-1* mutation on the chromosome. Consequently, strain SP187 might carry four mutations, i.e., *putA*, *dpr-1*, *tcr-1*, and *acr-2*.

For further investigation of the mechanism of proline overproduction, intracellular concentrations of proline and glutamate were determined (Table 4). The proline and glutamate contents in nonproducing strains (strains 8000 and SP103) were below 1 and 7 to 8 mM, respectively. The proline contents in all producing strains were increased to between 13 and 18 mM. In contrast, the glutamate content in proline-producing strains SP105 and SP126 was decreased to 3 to 5 mM. This depletion, however, was restored in strain SP187. These results suggest that strain SP187 might have acquired a mutation which resulted in an increased glutamate content. There are two major pathways for glutamate biosynthesis in bacteria. One is GDH and the other is GOGAT. We determined both enzyme levels of proline-producing strains in M1 medium (Table 5). The GDH level of strain SP187 was high compared with those of other strains, although the GOGAT level was not changed. Because GDH functions only during growth in excess ammonia (18), the real role of GDH and GOGAT on proline production was not identified. Both enzyme levels of proline-producing strains were determined during cell growth in F1 medium (Table 5). The GDH level of strain SP187 was ca. 5 to 6 times higher than those of strains SP105 and SP126. GOGAT levels were almost equal in all strains, although the levels were lower than those in M1 medium. Consequently, the increase of GDH level in strain SP187 certainly contributed to the increase of glutamate content and proline productivity. For a determination of whether this mutation (*acr-2*) was located in *gdhA* (structural gene for GDH), a transductional cross was performed between strain SP187 as the donor and glutamate auxotrophic strain SGD-11 (*gdhA*$^-$ *gltB*$^-$) as the recipient. The GDH levels of 12 Glu$^+$ (*gdhA*$^+$ *gltB*$^-$) transductants, represented by strain SP457, were the same as that of wild-type strains (Table 4). This result indicated that the *acr-2* mutation, which enhanced the GDH level, was not located in the *gdhA* region.

TABLE 4. Intracellular contents of proline and glutamate in proline-producing strains

Strain	Intracellular concn (mM)	
	Proline	Glutamate
8000	<1	7.8
SP103	<1	8.7
SP105	13.0	2.5
SP126	18.0	4.8
SP187	16.7	8.2

DISCUSSION

Osmotic stress is one of the important environmental parameters for the growth of organisms. In microorganisms, internal osmolarity is controlled by the accumulation of inorganic cations (22), carbohydrates (17), and amino acids. For amino acids, proline and glutamate are effective osmobalancers in nonhalophilic bacteria (2, 16, 21). Csonka reported that proline-overproducing mutants of *Salmonella typhimurium* were resistant to high osmotic stress (5). At the beginning of our study, we expected that the sensitivity to proline analogs would decrease during a high osmotic stress because the intracellular proline content would probably increase under high osmotic conditions. However, contrary to expectation, the sensitivites to proline analogs were significantly enhanced during high osmotic stress. Csonka recently reported the third proline permease specified in high osmotic stress in *Salmonella typhimurium* (6). The changes in the intracellular content of metabolites and proline analogs during high osmotic stress are now under investigation and will be reported in a separate paper.

Growth inhibition by proline analogs under high osmotic stress was reversed by glutamate as well as by proline in a proline-producing strain, SP126. The real antagonist of proline analogs is proline itself, because the growth inhibition in wild-type strains was reversed completely only by proline (Sugiura et al., in press). Accordingly, the antagonism by glutamate in proline-producing strains may be responsible for the increase in proline formed from glutamate. The AZC-resistant mutant SP187 restored the depletion of glutamate observed in strain SP126. The GDH level in strain SP187 was high compared with those of parent strains when cells were grown in both minimal and fermentation medium. Accordingly, the increased GDH level would enhance the glutamate content and the glutamate might be converted to proline. Transductional analysis revealed that the *acr-2* mutation, which increased the GDH level, might be not located in the *gdhA* region and be distant from the other three mutations of strain SP126. The levels of other tricarboxylic acid-cycle enzymes, citrate synthase and

TABLE 5. GDH and GOGAT activities of proline-producing strains

Strain	Sp act[a]			
	M1 medium		F1 medium	
	GDH	GOGAT	GDH	GOGAT
8000	0.092	0.104	NT[b]	NT
SP103	0.086	0.126	NT	NT
SP105	0.072	0.120	0.044	0.043
SP126	0.060	0.194	0.056	0.041
SP187	0.272	0.086	0.312	0.065
SP457	0.088	<0.002	NT	NT
SGD11[c]	0.010	<0.002	NT	NT

[a] Expressed as micromoles of NADP per minute per milligram of protein.
[b] NT, Not tested.
[c] Cultured in medium containing 1 mM glutamate.

α-ketoglutarate dehydrogenase, were not changed in strain SP187 (data not shown). Despite the central position of glutamate in metabolism, the mechanisms controlling the production still await clarification (18). This mutation is, therefore, attractive for examination of the control mechanisms of GDH formation. Strain SP187 produced 56 mg of L-proline per ml of fermentation medium. This is the highest value of proline productivity so far reported.

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