

## Molecular Breeding of a Biotin-Hyperproducing *Serratia marcescens* Strain

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We previously reported that an acidomycin-resistant mutant of *Serratia marcescens* Sr41, SB304, and a mutant that was derived from SB304 and was resistant to a higher concentration of acidomycin, SB412, produced 5 and 20 mg of D-biotin, respectively, per liter of a medium containing sucrose and urea (N. Sakurai, Y. Imai, M. Masuda, S. Komatsubara, and T. Tosa, *Appl. Environ. Microbiol.* 59:2857-2863, 1993). In order to increase the productivity of D-biotin, the biotin (*bio*) operons were cloned from strains SB412, SB304, and 8000 (wild-type strain), and pLGM412, pLGM304, and pLGW101, respectively, were obtained through subcloning. These plasmids harbored 7.2-kb DNA fragments coding for the *bioABFCD* genes on a low-copy-number vector and were introduced into SB304, SB412, and 8000. Among the resulting recombinant strains, SB412(pLGM304) exhibited the highest D-biotin production (200 mg/liter) in the production medium. The plasmid was stably maintained in cells. Unexpectedly, SB412(pLGM412) grew very slowly, and the D-biotin productivity of this recombinant strain was not evaluated because pLGM412 was unstable.

In microorganisms, D-biotin is synthesized from pimeloyl coenzyme A (pimeloyl-CoA) in four enzymatic steps. The precursors of pimeloyl-CoA are still unclear, although some microorganisms have been reported to be capable of converting pimelic acid to pimeloyl-CoA (15). In *Escherichia coli*, the biosynthetic pathway from pimeloyl-CoA to D-biotin involves 7-keto-8-aminopelargonic acid (KAPA), 7,8-diaminopelargonic acid (DAPA), and dethiobiotin. The five biotin-biosynthetic enzymes are encoded by the biotin (*bio*) operon and are subject to strict feedback repression by biotin (Fig. 1) (1, 10). However, no one knows what mechanism is involved in the introduction of the sulfur atom into dethiobiotin and what kind of sulfur compounds participate in this reaction, despite many studies (9, 13, 26).

D-Biotin is a commercially important vitamin which is used mainly as an animal feed additive. It is known that D-biotin functions as the carrier of carbon dioxide in enzymatic carboxylation and transcarboxylation reactions (16). At present, this vitamin is manufactured industrially by using a complex chemical synthesis process (12).

Several research groups have been competing to develop fermentative methods for D-biotin production, and a lot of effort has been directed toward the construction of D-biotin-producing strains (3, 11, 14, 19, 27, 30, 36). Recombinant strains of *E. coli* and *Bacillus sphaericus* have been constructed by cloning the genes involved in the synthesis of D-biotin (3, 11, 14, 25, 27). We have estimated that the production of D-biotin is unlikely to be high enough for application to industrial fermentation when these recombinant strains are used; that is, fermentation methods require much higher levels of production to be cost competitive with the commercial chemical synthetic methods.

We previously reported that *Serratia marcescens* mutant SB412, which is very resistant to the biotin analog acidomy-

cin, produced 20 mg of D-biotin per liter (28). This strain was found to be highly constitutive for biotin-biosynthetic enzymes. In our laboratory, various L-amino-acid-producing strains of *S. marcescens* were improved by cloning related genes, and some of these strains have been used commercially (32-35). We are therefore acquainted with the genetic characteristics of *S. marcescens* and know how to apply gene cloning techniques to strain improvement.

With the background described above, we intended to apply gene cloning techniques to the construction of a D-biotin-hyperproducing strain of *S. marcescens*. In this paper we describe cloning of the wild-type and mutant *bio* operons, how we determined the mutations involved in D-biotin overproduction, and the gene dosage effect of the *bio* operon on D-biotin production by *S. marcescens*.

### MATERIALS AND METHODS

**Strains, plasmids, and media.** The derivatives of *E. coli* K-12 and *S. marcescens* Sr41 and the plasmids used are listed in Table 1. Nutrient medium containing 0.5% glucose, 1.0% peptone, 0.3% meat extract, 1.0% yeast extract, and 0.5% NaCl was used for routine colony isolation and for the growth study. The minimal medium of Davis and Mingioli (7) was modified by omitting sodium citrate and increasing the glucose concentration to 0.5%. For the complementation test in which *E. coli*  $\chi$ 1776 was used, we used the minimal medium described above with 0.1% casein hydrolysate (vitamin free), 0.01%  $\alpha,\epsilon$ -diaminopimelic acid, and 0.004% thymidine added. Antibiotics were added at the following concentrations for *E. coli* K-12: kanamycin, 100  $\mu$ g/ml; ampicillin, 100  $\mu$ g/ml; tetracycline, 10  $\mu$ g/ml; and streptomycin, 50  $\mu$ g/ml. For *S. marcescens* antibiotics were added at the following concentrations: kanamycin, 200  $\mu$ g/ml; and ampicillin, 500  $\mu$ g/ml. Medium F2, which was used for D-biotin production, contained 15% sucrose, 1.5% urea, 0.1% corn steep liquor, 0.1%  $K_2HPO_4$ , 0.2%  $MgSO_4 \cdot 7H_2O$ , 0.01%  $FeSO_4 \cdot 7H_2O$ , and 1.5%  $CaCO_3$ .

**D-Biotin production.** Cells were cultured in a shaking flask

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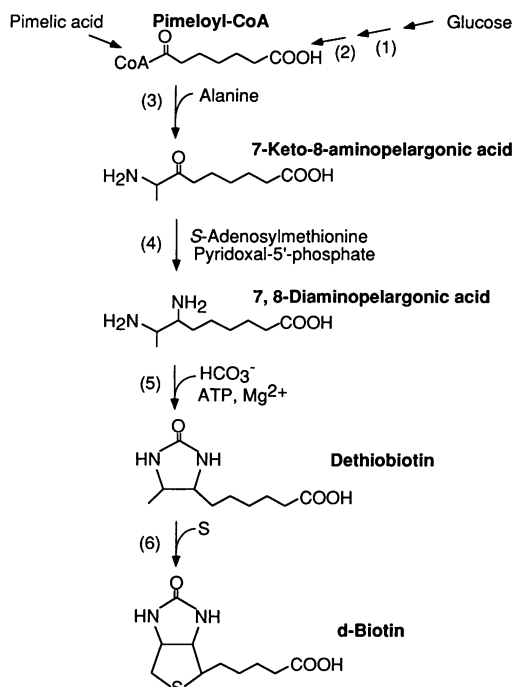


FIG. 1. Pathway of biotin biosynthesis in bacteria. (1) *bioH*, block before pimeloyl-CoA; (2) *bioC*, block before pimeloyl-CoA; (3) *bioF*, KAPA synthetase; (4) *bioA*, DAPA aminotransferase; (5) *bioD*, dethiobiotin synthetase; (6) *bioB*, biotin synthetase.

containing medium F2 under conditions described previously (28). Growth was estimated as described previously (28).

**Growth study.** To measure the growth rate, cells grown on a nutrient agar slant overnight were washed twice with cold saline and inoculated into a medium to give  $5 \times 10^7$  cells per ml, and the resulting culture was grown at 30°C in a shaking flask containing 100 ml of minimal medium. Portions were removed at 1-h intervals to measure the turbidity at the exponential growth phase. Growth was evaluated by measuring  $A_{660}$  with a Hitachi model U-1000 electric photometer.

**Cloning of the *bio* operons.** The chromosomal DNA of *S. marcescens* was prepared as described previously for *E. coli* DNA (22) and then was completely digested with endonucleases *EcoRI* and *HindIII*; this was followed by ligation with *EcoRI*- and *HindIII*-digested pBR322 DNA. The ligated DNA was transformed into *E. coli*  $\chi$ 1776, a *bio* operon deletion mutant, and then ampicillin-resistant (Ap<sup>r</sup>), tetracycline-sensitive (Tc<sup>s</sup>), and Bio<sup>+</sup> transformants were selected on minimal agar plates in the presence of 1  $\mu$ g of KAPA per ml. Transformants having the corresponding phenotypes were purified by single-colony isolation for further studies.

**Genetic procedures and recombinant DNA techniques.** Standard procedures were used for plasmid preparation, restriction enzyme digestion, ligation, and agarose gel electrophoresis (22). *E. coli* and *S. marcescens* were transformed by using the methods of Maniatis et al. (22) and Takagi and Kisumi (35), respectively. Plasmids were introduced into *S. marcescens* strains as follows. First, plasmid DNA extracted from *E. coli* strains was introduced into *S. marcescens* TT392, which is deficient in DNA restriction but proficient in DNA modification; this was followed by recov-

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source or reference
<i>E. coli</i> strains		
$\chi$ 1776	F <sup>-</sup> <i>tonA53 dapD8 minA1 glnV44</i> $\Delta$ ( <i>gal-bio-uvrB</i> )40 $\lambda^-$ <i>minB2 rfb-2 gyrA25 thyA142 osm-2 metC65 osm-1 (tte-1) <math>\Delta</math>(bioH-<i>asd</i>)29 cycB2 cycA1 hsdR2</i>	6
R879	<i>bioA24 IN(rrmD-rrmE)1</i>	8
R875	<i>bioB17 IN(rrmD-rrmE)1</i>	8
R872	<i>bioF103 IN(rrmD-rrmE)1</i>	8
R876	<i>bioC18 chlA18 IN(rrmD-rrmE)1</i>	8
R877	<i>bioD19 IN(rrmD-rrmE)1</i>	8
3104S <sup>b</sup>	Same as R879 except Sm <sup>r</sup>	This study
3105S	Same as R875 except Sm <sup>r</sup>	This study
3106S	Same as R872 except Sm <sup>r</sup>	This study
3107S	Same as R876 except Sm <sup>r</sup>	This study
3108S	Same as R877 except Sm <sup>r</sup>	This study
3104ST <sup>c</sup>	Same as 3104S except <i>srlC300::Tn10 recA56</i>	This study
3105ST	Same as 3105S except <i>srlC300::Tn10 recA56</i>	This study
3106ST	Same as 3106S except <i>srlC300::Tn10 recA56</i>	This study
3107ST	Same as 3107S except <i>srlC300::Tn10 recA56</i>	This study
3108ST	Same as 3108S except <i>srlC300::Tn10 recA56</i>	This study
JC10240	HfrC (PO45) <i>srlC300::Tn10 recA56 thr-300 ilv-318 rpsE300</i>	20
MG1063	F <sup>+</sup> ::Tn1000 <i>recA56</i>	23
<i>S. marcescens</i> strains		
8000	Wild type	24
SB304	Resistant to 0.5 mg of acidomycin per ml	28
SB412	Resistant to 2 mg of acidomycin per ml	28
TT392	Nuc <sup>-</sup> r <sup>-</sup> Ap <sup>s</sup> Km <sup>s</sup>	35
Plasmids		
pBR322	Ap <sup>r</sup> Tc <sup>r</sup>	2
pLG339	Km <sup>r</sup> Tc <sup>r</sup>	31
pBW101	pBR322:: <i>wbio</i> Ap <sup>r</sup>	This study
pBM304	pBR322:: <i>mbio</i> <sub>304</sub> Ap <sup>r</sup>	This study
pBM412	pBR322:: <i>mbio</i> <sub>412</sub> Ap <sup>r</sup>	This study
pLGW101	pLG339:: <i>wbio</i> Km <sup>r</sup>	This study
pLGM304	pLG339:: <i>mbio</i> <sub>304</sub> Km <sup>r</sup>	This study
pLGM412	pLG339:: <i>mbio</i> <sub>412</sub> Km <sup>r</sup>	This study

<sup>a</sup> Abbreviations: Nuc<sup>-</sup>, no production of extracellular nuclease; Ap<sup>s</sup>, enhanced sensitivity to ampicillin; Km<sup>s</sup>, enhanced sensitivity to kanamycin; r<sup>-</sup>, defect in host restriction enzyme; *wbio*, wild-type biotin operon; *mbio*<sub>304</sub>, biotin operon allele of SB304; *mbio*<sub>412</sub>, biotin operon allele of SB412.

<sup>b</sup> The suffix S indicates Sm<sup>r</sup>.

<sup>c</sup> The suffix T indicates *recA56*.

ery of the plasmid DNA and introduction of it into *S. marcescens* strains, which are not deficient in DNA restriction.

**Construction of derivatives from primary plasmids and pLGM304.** To construct plasmids containing pLG339, a low-copy-number vector, 7.2-kb *EcoRI-HindIII* fragments carrying the *bio* operons from pBW101, pBM304, and pBM412 were ligated to pLG339 DNA digested completely by *EcoRI* and partially by *HindIII*, yielding pLGW101, pLGM304, and pLGM412, respectively (Fig. 2). Plasmids in which each of the *bio* genes was inactivated were derived from pLGM304 as follows. To inactivate the *bioA* gene,

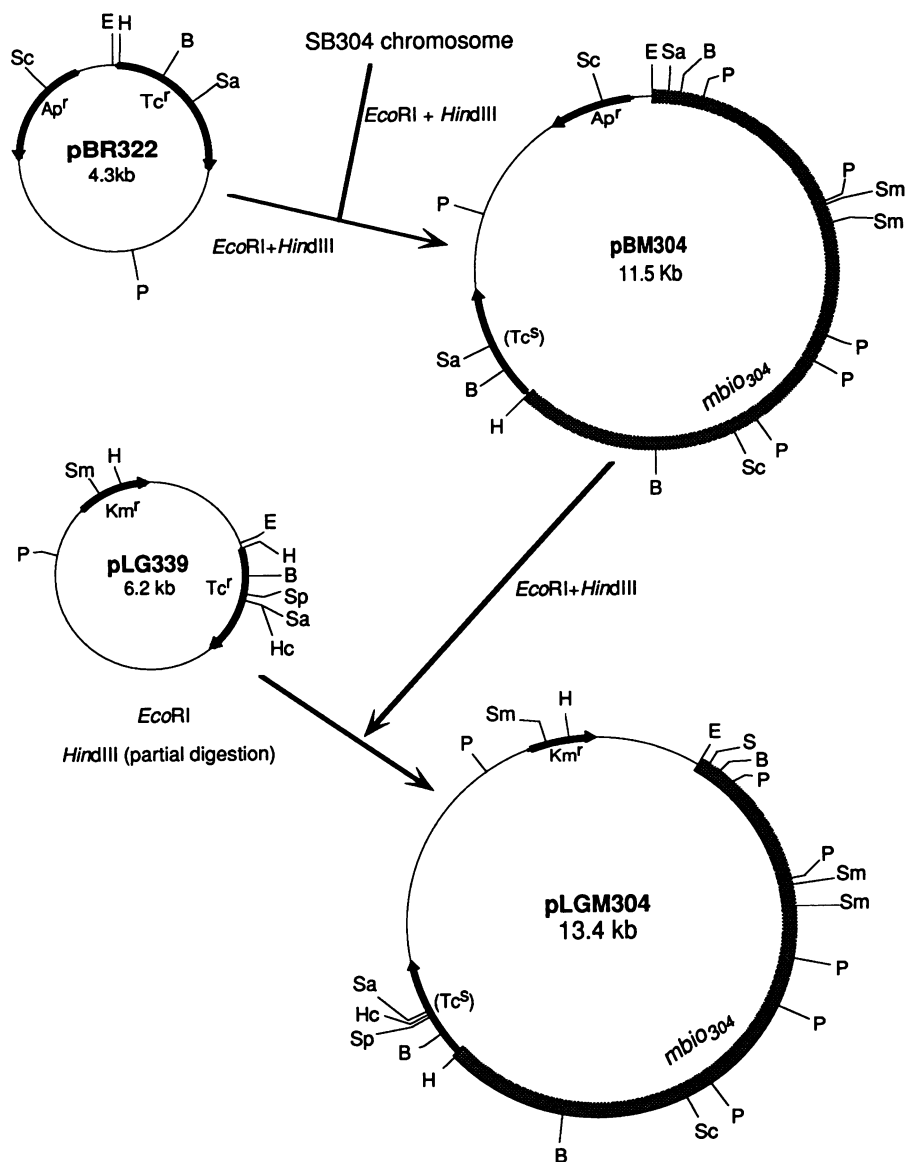


FIG. 2. Cloning and subcloning of the *bio* operon from SB304. The *mbio<sub>304</sub>* fragment was obtained by shotgun cloning and was subcloned onto pLG339, yielding pLGM304 as described in Materials and Methods. The *bio* operons were also cloned from wild-type strain 8000 and SB412. pLGM101 (pLG339::*wbio*) and pLGM412 (pLG339::*mbio<sub>412</sub>*) were constructed by using the method used for construction of pLGM304. Abbreviations: E, *EcoRI*; H, *HindIII*; B, *BamHI*; P, *PvuII*; Sa, *SacI*; Sc, *SacI*; Sm, *SmaI*; Sp, *SphI*; Hc, *HincII*.

pLGM304 DNA was digested completely by *BamHI* and then partially by *HindIII*, and then the cohesive ends were repaired with the Klenow fragment to create blunt ends and the DNA was recircularized by ligation. The resulting plasmid, pLGM304dA, lacked the 1.5-kb *HindIII*-*BamHI* fragment carrying part of the *bioA* gene. To inactivate the *bioB* gene, pLGM304 DNA was digested by *SacI*, and the cohesive ends were treated with mung bean nuclease to generate pLGM304dB, whose *bioB* gene was inactivated because four nucleotides were deleted. To inactivate the *bioF* gene, the 0.35-kb *StuI* fragment was removed from pLGM304 DNA, and this was followed by selection of pLGM304dF. The *bioC* gene was inactivated by inserting *XhoI* linkers into the *PmaCI* site located in the *bioC* gene, and pLGM304dC was selected. To inactivate the *bioD* gene, pLGM304 DNA was

cleaved with *SaI* and then digested with *Bal31*; this was followed by selection of pLGM304dD, in which 1.5 kb of nucleotides was deleted. To construct pLGM304dP, pLGM304 was digested with *ScaI* after partial digestion with *SacI* to remove the 0.75-kb DNA fragment containing the putative promoter region of the *bio* operon. The unpaired ends of the residual DNA of pLGM304 were filled with the Klenow fragment before blunt-end ligation, forming pLGM304dP. To construct pLGM304dBFCD, pLGM304 was cleaved with *SacI* and *EcoRI*, the digested ends were filled in with the Klenow fragment, and the plasmid was recircularized to produce pLGM304dBFCD.

**Analysis of the *bio* operon by *Tn1000* insertion.** To locate the biotin-biosynthetic genes on pLGM304, derivatives with *Tn1000* insertions were obtained; this was followed by

performing complementation tests for Tn1000 insertions with various *E. coli* Bio<sup>-</sup> strains (21). First, the streptomycin resistance (Sm<sup>r</sup>) marker was introduced into *E. coli* Bio<sup>-</sup> strains to distinguish spontaneous tetracycline-resistant (Tc<sup>r</sup>) and kanamycin-resistant (Km<sup>r</sup>) mutants from the desired exconjugants in the subsequent conjugation experiments. Exponentially growing R879, R875, R872, R876, and R877 cells in Luria-Bertani (LB) medium were spread on LB agar plates containing streptomycin, and the preparations were incubated for 24 h at 37°C without mutagen treatment. The Sm<sup>r</sup> colonies that arose spontaneously were purified and were designated 3104S, 3105S, 3106S, 3107S, and 3108S. Next, the *recA56* mutation was introduced into these Sm<sup>r</sup> Bio<sup>-</sup> strains to avoid *recA*-mediated recombination in Tn1000 transpositions and to stabilize the Tn1000-inserted plasmids in these strains. Tc<sup>r</sup> recombinants were selected on LB medium plates containing streptomycin and tetracycline after mating with Hfr strain JC10240 (*srl::Tn10* near *recA56*). The Tc<sup>r</sup> exconjugants that arose from the brief mating were screened for the closely linked *recA56* allele by measuring their sensitivity to UV irradiation, resulting in 3104ST, 3105ST, 3106ST, 3107ST, and 3108ST.

A mating-out assay was used for Tn1000 transposition into pLGM304 as follows. MG1063 containing the Tn1000-bearing conjugative F replicon was transformed with target plasmid pLGM304 (Km<sup>r</sup>). MG1063(pLGM304) was then mated with recipient 3104ST, 3105ST, 3106ST, 3107ST, or 3108ST. Single colonies of the donor strain and the recipients were grown overnight at 37°C in LB medium, diluted 0.1 ml to 3 ml, and grown for 1 h. Equal volumes (1.5 ml each) of the donor and recipient strains were mixed and spread on LB medium plates containing streptomycin, tetracycline, and kanamycin after 30 min of incubation at 37°C. Transconjugants that were Sm<sup>r</sup>, Tc<sup>r</sup>, and Km<sup>r</sup> were selected and tested for each of the Bio<sup>-</sup> phenotypes. The plasmids were extracted from the transconjugants having the relevant Bio<sup>-</sup> phenotypes and were used for further restriction analysis.

**Enzyme assays of DAPA aminotransferase and dethiobiotin synthetase.** Cells were cultured in a minimal medium at 30°C with shaking, and cell extracts were prepared from exponentially growing cells by the method described previously (28). The activities of DAPA aminotransferase and dethiobiotin synthetase were determined as described previously (28). Protein concentrations were determined by using a Bio-Rad protein assay kit and bovine serum albumin as the standard protein.

**Assay for D-biotin and biotin-related compounds.** D-Biotin was assayed with *Lactobacillus plantarum* ATCC 8014 (29). The total amount of D-biotin plus dethiobiotin (the amount of biotin-related compounds) was determined with *Candida tropicalis* IFO 0058 (28). The extent of the growth of this strain on dethiobiotin is very similar to the extent of growth on D-biotin when the same amount of each compound is added to the assay medium. The approximate amount of dethiobiotin was estimated from the total amount of D-biotin plus dethiobiotin and the amount of D-biotin determined separately.

**Chemicals.** D-Biotin was a product of Tanabe Seiyaku Co., Ltd. Dethiobiotin was prepared and supplied by the Production Technology Division of Tanabe Seiyaku Co., Ltd. Restriction endonucleases, T4 DNA ligase, mung bean nuclease, and the Klenow fragment were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan.  $\alpha,\epsilon$ -Diaminopimelic acid (a mixture of the LL, DD, and *meso* isomers) was obtained from Sigma Chemical Co., St. Louis, Mo. Other

chemicals were also obtained commercially and were not purified further.

## RESULTS

**Cloning of the *bio* operons from two D-biotin-producing strains and the wild-type strain.** We intended to increase D-biotin production by elevating the levels of biotin-biosynthetic enzymes. First, the *bio* operons were cloned from *S. marcescens* SB412, which has the highest D-biotin-producing activity known, and SB304, which is a parent strain of SB412 and has lower productivity, by performing a shotgun cloning experiment in which pBR322 was the vector,  $\chi$ 1776 was the host, and *Eco*RI and *Hind*III were the restriction enzymes (Fig. 2). *E. coli*  $\chi$ 1776 is deficient in the *bioABFCD* genes and the *bioH* gene because of deletion. Following the introduction of *S. marcescens* SB412 DNA in pBR322, Bio<sup>+</sup> transformants of  $\chi$ 1776 were selected on minimal agar plates containing KAPA, which complements the *bioH* deletion. The transforming DNA was tested for complementation of the *bioA*, *-B*, *-F*, *-C*, and *-D* mutants of *E. coli*, resulting in the identification of a 7.2-kb DNA fragment coding for the entire *bio* operon region. Next, a similar DNA fragment was cloned from strain 8000, the wild-type strain of *S. marcescens* Sr41, to compare the *bio* operons of mutant types and a wild type. We designated the resulting plasmids pBM412 (pBR322::*mbio*<sub>412</sub>), pBM304 (pBR322::*mbio*<sub>304</sub>), and pBW101 (pBR322::*wbio*).

**Subcloning of the *bio* operons into a low-copy-number plasmid.** Plasmid pBR322 is frequently used as a vector for gene cloning, but the introduction of pBW101, pBM304, and pBM412 into SB412 caused strong growth inhibition in addition to segregational instability of the plasmids. Apparently, such instability was not suitable for practical use. Hence we separately subcloned the 7.2-kb fragments containing the two mutant and wild-type *bio* operons into a low-copy-number plasmid, pLG339, which yielded pLGW101 (pLG339::*wbio*), pLGM304 (pLG339::*mbio*<sub>304</sub>), and pLGM412 (pLG339::*mbio*<sub>412</sub>) (Fig. 2). Plasmid vector pLG339 has a pSC101-derived replicon and has been reported to stably replicate at a level of five to six copies per chromosome in cells of *S. marcescens* (33).

**D-Biotin production by recombinant strains of *S. marcescens*.** To examine the gene dosage effect of the *bio* operon on the production of D-biotin, we constructed various recombinant strains carrying the pLG339 derivatives containing wild-type and mutant *bio* operons and tested them for D-biotin production by using medium F2 in Sakaguchi shaking flasks (Table 2). At first we expected that SB412 carrying pLGM412 might produce the most D-biotin. However, the growth of SB412 was strongly inhibited by the introduction of pLGM412, whereas the growth of wild-type strain 8000 was not inhibited. Thus, we could not evaluate the D-biotin production of SB412(pLGM412). Hence, we used recombinant strains carrying pLGM304 to test D-biotin production. Strain 8000(pLGM304) produced 3 mg of D-biotin per liter, whereas strain 8000, the host strain, produced less than 0.001 mg of D-biotin per liter. Strains SB304 (pLGM304) and SB412(pLGM304) produced 130 and 200 mg of D-biotin per liter, respectively. Although medium F2 contained no kanamycin, pLGM304 was almost stably maintained in the cells during the entire incubation.

**Activities of biotin-biosynthetic enzymes in a recombinant strain.** To examine the effect of gene dosage on the biotin-biosynthetic enzymes, the specific activities of DAPA aminotransferase (BioA) and dethiobiotin synthetase (BioD)

TABLE 2. D-Biotin production by recombinant strains<sup>a</sup>

Strain	Dry cell wt (g/liter)		Amt of D-biotin produced (mg/liter)		Amt of biotin-related compounds produced (mg/liter) <sup>b</sup>	
	120 h	144 h	120 h	144 h	120 h	144 h
8000(pLG339)	22	NT <sup>c</sup>	<0.001	NT	0.4	NT
8000(pLGW101)	22	NT	0.45	NT	2.3	NT
8000(pLGM304)	21	NT	0.89	NT	3.3	NT
8000(pLGM412)	21	NT	1.8	NT	4.8	NT
SB304(pLGM304)	20	12	130	130	170	180
SB412(pLG339)	16	16	40	41	45	46
SB412(pLGM101)	21	17	92	92	102	110
SB412(pLGM304)	22	13	180	200	320	300
SB412(pLGM304dA)	23	14	160	180	420	430
SB412(pLGM304dB)	21	16	60	60	410	490
SB412(pLGM304dC)	19	14	61	60	66	65
SB412(pLGM304dF)	23	14	170	180	330	410
SB412(pLGM304dD)	19	13	181	190	380	420

<sup>a</sup> Cells were grown in medium F2 in shaking flasks at 30°C.

<sup>b</sup> The biotin-related compounds were D-biotin and dethiobiotin.

<sup>c</sup> NT, not tested.

were measured by using cell extracts of SB412 and SB412(pLGM304). The levels of DAPA aminotransferase activity and dethiobiotin synthase activity were three- and sixfold higher (14 and 93 nmol/min/mg of protein, respectively) in SB412(pLGM304) than in host strain SB412 (5.6 and 15 nmol/min/mg of protein, respectively). In addition, the synthesis of these enzymes was not sensitive to biotin-mediated feedback repression.

**Structure of the *bio* operons of *S. marcescens*.** To locate the individual genes involved in the *bio* operon, we isolated independent derivatives of pLGM304 with Tn1000 ( $\gamma\delta$ ) insertions, and insertion sites were mapped by restriction enzyme cleavage (Fig. 3A). We obtained 26 insertions, and among these 26 insertions were insertions that had inactivated each of the five genes. A total of 17 of these independent derivatives (insertions 1 to 17) exhibited no polar effects although Tn1000 was inserted into the *bioA*, *bioB*, *bioF*, *bioC*, or *bioD* gene. Of the 26 independent derivatives, 9 exhibited incomplete polar effects; 4 derivatives (insertions 18 to 21) had the BioA<sup>+</sup> BioB<sup>-</sup> BioF<sup>+</sup> BioC<sup>-</sup> BioD<sup>+</sup> phenotype, and 5 derivatives (insertions 22 to 26) had the BioA<sup>+</sup> BioB<sup>-</sup> BioF<sup>-</sup> BioC<sup>-</sup> BioD<sup>+</sup> phenotype. These unusual polarities might have been due to an outward promoter on Tn1000 or pLG339 for downstream gene expression that resulted in nonpolar insertions. However, a complex operonal organization is suggested.

To localize the *bio* genes more precisely, eight plasmids which prevented the complementation of various Bio<sup>-</sup> strains of *E. coli* were constructed from pLGM304 (Fig. 3B). One of these plasmids, deletion plasmid pLGM304dP, which lacks the 0.75-kb *SacI-ScaI* fragment, did not complement all of the *bioA*, *-B*, *-F*, *-C*, and *-D* strains.

The results described above indicated that the *bioB*, *-F*, *-C*, and *-D* genes might be transcribed from a single promoter which lies within the 0.75-kb *SacI-ScaI* segment, confirming that the *bioB*, *-F*, *-C*, and *-D* genes of *S. marcescens* form an operonic structure.

On the other hand, transcription of *bioA* might be independent of transcription of the *bioBFCD* cistron since deletion of the putative *bioA* region did not influence the expres-

sion of the other genes, whereas all of the *bio* genes were repressed by removing the 0.75-kb *SacI-ScaI* fragment.

We deduced that the DNA fragment carrying these five genes was 5.8 kb long or longer. The approximate sizes of the *bioA*, *bioB*, *bioF*, *bioC*, and *bioD* genes seemed to be 1.3, 1.0, 1.1, 0.8, and 0.7 kb, respectively.

**Contribution of each of the *bio* genes to D-biotin overproduction.** To examine the contribution of the genes in the *bio* operon to D-biotin overproduction, we introduced the five types of deletion plasmids from pLGM304 into SB412 and tested for the production of D-biotin and biotin-related compounds (Table 2).

The strains carrying pLGM304dA (defective in the *bioA* gene), pLGM304dF (defective in the *bioF* gene), and pLGM304dD (defective in the *bioD* gene) produced the same amounts of D-biotin and biotin-related compounds as SB412(pLGM304). On the other hand, SB412(pLGM304dB), which was defective in the *bioB* gene of the plasmid, produced only 60 mg of D-biotin per liter, as did SB412, but produced more than 400 mg of biotin-related compounds per liter, as did SB412(pLGM304). SB412(pLGM304dC), from which the *bioC* gene was deleted, produced only small amounts of both D-biotin and biotin-related compounds, as did SB412.

The results described above indicated that overproduction of the *bioB* and *bioC* products played an important role in D-biotin overproduction but that amplification of the *bioA*, *bioF*, and *bioD* products might not be significant for D-biotin overproduction. In a bioautographic study *Saccharomyces cerevisiae* was used as an indicator organism; this organism can grow on D-biotin, dethiobiotin, KAPA, and DAPA. This study revealed that the recombinants described above accumulated no detectable amounts of biotin precursors other than dethiobiotin (data not shown).

**Colony sizes and growth rates of the strains carrying the *bio* operon recombinant plasmids.** As mentioned above, the recombinant strains carrying plasmid pLGM412 grew extremely poorly in both nutrient medium and medium F2. Hence, we examined the effects of various *bio* recombinant plasmids on colony size and compared the growth rates of the wild-type strain, D-biotin-producing mutants, and recombinant strains to determine the profile of growth inhibition.

Vector pLG339 had no effect on the colony sizes of wild-type strain 8000 and strain SB412, which formed smaller colonies than the wild-type strain. Recombinant plasmids pLGM304 and pLGM412 did not affect the growth of the wild-type strain but caused cells of SB412 to form smaller and much smaller colonies, respectively, although a number of unusually large colonies of SB412(pLGM412) were formed among the small colonies. Next, we examined the frequency of larger SB412(pLGM412) colonies. Cells from 26 independent small colonies were spread on nutrient agar plates containing kanamycin. After incubation for 3 days, we found 1,110 large colonies that were similar to the original large colonies in size among the 121,200 Km<sup>r</sup> colonies on the plates. Hence, the frequency of large-colony formation was as high as  $9.2 \times 10^{-3}$ . We found no marked differences in the frequency of large-colony formation among the cells from the 26 small colonies examined. On the other hand, no large colonies of SB412(pLGM304) were found.

Some of the strains described above were examined for growth rate in a minimal medium (Table 3). SB412 grew at one-half the rate of wild-type strain 8000. Plasmid pLGW101, carrying the wild-type *bio* operon, had no effect on the growth rates of 8000 and SB412. Plasmids pLGM304

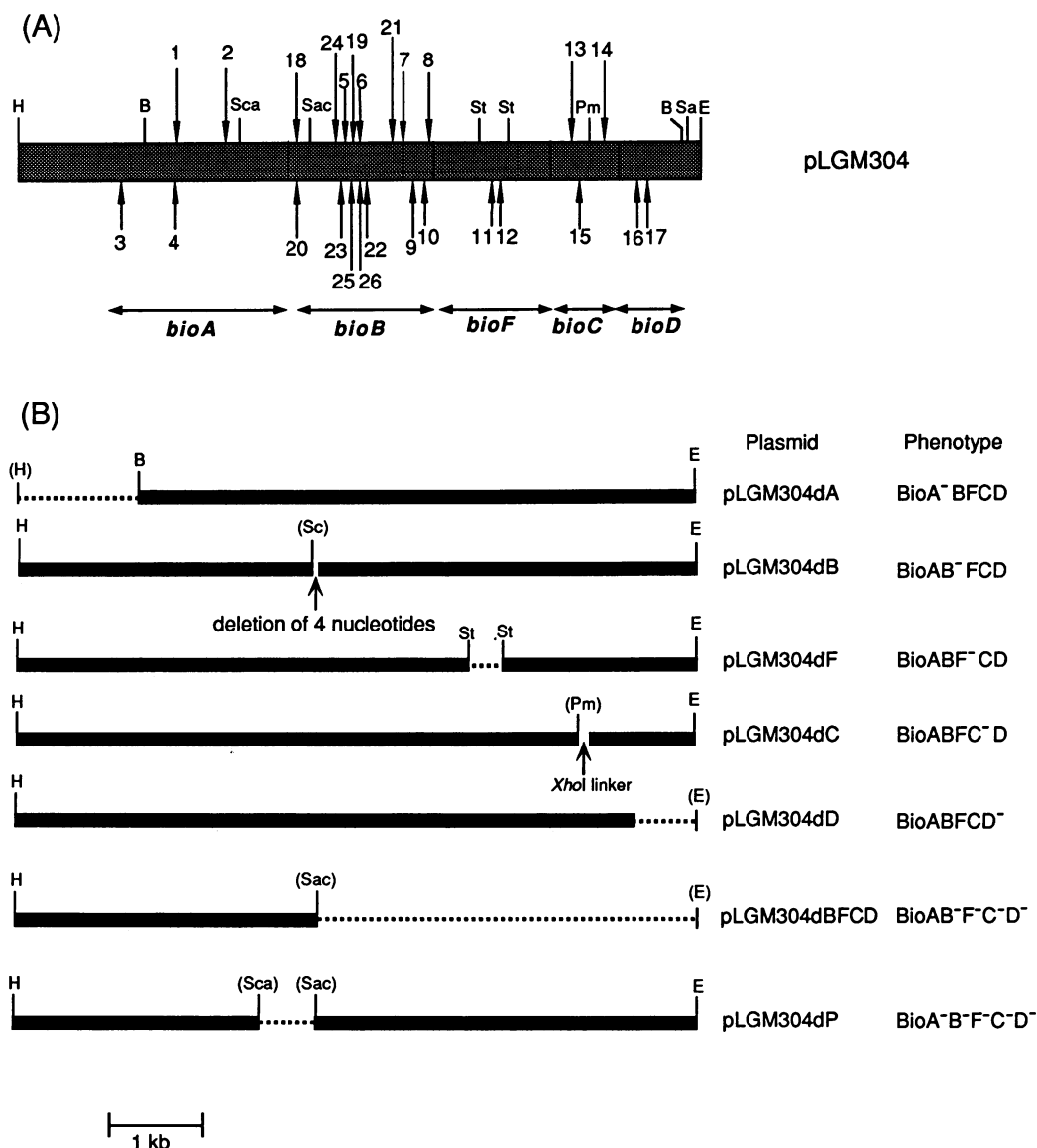


FIG. 3. Locations of the *bioA*, *-B*, *-F*, *-C*, and *-D* genes and derivatives of pLGM304. (A) Sites of Tn1000 insertions on pLGM304 are indicated by the vertical lines. Each number indicates the clone number of the plasmid. The insertions above the horizontal line are oriented with the  $\gamma$  end of the transposon to the left. The insertions below the horizontal line are in the opposite orientation. (B) Phenotypes of the derivatives of pLGM304 lacking each *bio* gene. pLGM304dA, pLGM304dB, pLGM304dF, pLGM304dC, pLGM304dD, pLGM304dBFCd, and pLGM304dP were constructed by inactivating *bioA*, *bioB*, *bioF*, *bioC*, *bioD*, *bioBFCd*, and the promoter region, respectively, as described in Materials and Methods. Abbreviations: E, *EcoRI*; H, *HindIII*; B, *BamHI*; Sa, *SalI*; Sac, *SacI*; Pm, *PmaCI*; St, *StuI*. (E), (H), (Sac), and (Pm) indicate *EcoRI*, *HindIII*, *SacI*, and *PmaCI* sites which were lost during construction of the derivatives of pLGM304.

and pLGM412 caused SB412 to grow slowly and very slowly, respectively, but had no effect on the growth rate of 8000. These observations were consistent with the profile of colony sizes described above and, in addition, indicated that more D-biotin production was accompanied by slower growth, presumably because of the metabolic disorder.

#### DISCUSSION

Many research groups have been trying to develop fermentative methods for D-biotin production (3, 11, 14, 19, 27, 30, 36). A recombinant strain of *E. coli* has been reported to produce 105 mg of D-biotin per liter in a jar fermentor under

carefully controlled fed-batch culture conditions (14). This level of production is the highest level among the levels that have been reported. In this study we found that a recombinant strain of *S. marcescens* carrying the mutant *bio* operon produced D-biotin at levels as high as 200 mg/liter in a shaking flask under conventional culture conditions. Higher levels of D-biotin production can be expected when culture conditions are optimized and more sugar is added to the cultures. This D-biotin-producing strain is a promising candidate for industrial production of D-biotin.

Why does a recombinant strain of *S. marcescens* produce such high levels of D-biotin? Basically, *S. marcescens* grows faster, produces higher cell yields in a simple medium, and

TABLE 3. Effect of *bio* operon recombinant plasmids on growth

Strain	Specific growth rate in minimal medium (h <sup>-1</sup> ) <sup>a</sup>
8000(pLG339).....	0.67
8000(pLGM101).....	0.68
8000(pLGM304).....	0.66
8000(pLGM412).....	0.65
SB412(pLG339).....	0.35
SB412(pLGM101).....	0.33
SB412(pLGM304).....	0.22
SB412(pLGM412).....	<0.02 <sup>b</sup>

<sup>a</sup> Cells were cultured in minimal medium with shaking.  $A_{660}$  was measured at different times. The specific growth rate constant ( $k$ ) was defined as follows:  $k = \ln 2/\text{mass doubling time}$ .

<sup>b</sup> The specific growth rate constant for this strain was not determined correctly because of strong growth inhibition.

has higher sugar-assimilating activity than *E. coli*. These characteristics allow *S. marcescens* to produce abundant metabolites, including D-biotin, if metabolic regulatory mechanisms are genetically removed (17, 18). We previously reported that the biotin-biosynthetic enzymes in both SB304 and SB412 were free from biotin-mediated feedback repression (28). By performing a transductional analysis we detected a mutation in the *bio* operon locus of SB412 for D-biotin overproduction but could not determine whether SB304 acquired genetic alterations in the *bio* operon locus. This study revealed that SB304 also contains a mutation in the *bio* operon locus, although the contribution of this mutation to D-biotin overproduction was less important than the contribution of the mutation carried by SB412.

In *E. coli*, the five biotin-biosynthetic enzymes are encoded by the *bioA*, *-B*, *-F*, *-C*, and *-D* genes, all of which are clustered on a single operon (4). This operon is divergently transcribed leftward to the *bioA* gene and rightward to the *bioBFCD* genes (5). Both types of transcription are coordinately repressed by the biotin operon repressor (*birA*) protein combined with biotin (10). We demonstrated that the *bioA*, *-B*, *-F*, *-C*, and *-D* genes of *S. marcescens* are clustered in this order and that *bioB*, *-F*, *-C*, and *-D* are transcribed by a single promoter, indicating that these genes form an operon structure. This structure is very similar to the overall structure of the *E. coli* genes. In this study, we could not determine the transcriptional direction of *bioA*, but it might be similar to the transcriptional direction in the *E. coli* model.

As described above, we constructed recombinant plasmids pLGM304 and pLGM412 carrying the mutant *bio* operons from D-biotin-producing mutants SB304 and SB412, respectively, and this was followed by the construction of several recombinant strains. We expected that SB412 (pLGM412) might produce the highest levels of D-biotin since the *bio* operon of pLGM412 was cloned from SB412, which produced the most D-biotin among the acidomycin-resistant mutants which we isolated (28). Unexpectedly, we could not evaluate the actual production of D-biotin by this recombinant strain because of poor growth and the instability of the plasmid; that is, the introduction of pLGM412 impeded cell growth, and mutated plasmids arose that no longer overproduced D-biotin and became dominant in the culture, resulting in the low productivity.

The slow growth of SB412(pLGM412) described above might be explained as follows. It is possible that the metabolic disorder caused by biotin overproduction might result

in slow growth. A specific biotin intermediate prior to pimeloyl-CoA could be shared by other essential metabolites, the formation of which might decrease because of the competition for the common intermediate by the biotin-biosynthetic pathway. However, this possibility is unlikely because the total amounts of the biotin compounds were not decreased by inactivation of the *bioB* product, biotin synthetase.

Very recently, we have found that a recombinant plasmid carrying only the *bioB* gene of SB412 on pLG339, the vector which was used for pLGM412 construction, also inhibited the growth of SB412 (data not shown). This finding indicates that the poor growth of SB412(pLGM412) depended on excess production of the *bioB* product, which might be toxic to cells directly or indirectly. If we can elucidate the biochemical function of the *bioB* product, it may be possible to overcome the slow growth of this recombinant strain and to construct a strain which produces even more D-biotin.

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