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 δ-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 δ-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 δ-biotin production (200 mg/liter) in the production medium. The plasmid was stably maintained in cells. Unexpectedly, SB412(pLGM412) grew very slowly, and the δ-biotin productivity of this recombinant strain was not evaluated because pLGM412 was unstable.

In microorganisms, δ-biotin is synthesized from 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 δ-biotin involves 7-keto-8-aminopenicillanic acid (KAPA), 7,8-diaminopenicillanic 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).

δ-Biotin is a commercially important vitamin which is used mainly as an animal feed additive. It is known that δ-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 δ-biotin production, and a lot of effort has been directed toward the construction of δ-biotin-producing strains (3, 11, 14, 19, 27, 30, 36). Recombinant strains of E. coli and Bacillus subtilis have been constructed by cloning the genes involved in the synthesis of δ-biotin (3, 11, 14, 25, 27). We have estimated that the production of δ-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 acidomycin, produced 20 mg of δ-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 δ-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 δ-biotin overproduction, and the gene dosage effect of the bio operon on δ-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 χ1776 was used, we used the minimal medium described above with 0.1% casein hydrolysate (vitamin free), 0.01% d,dl-diaminopimelic acid, and 0.004% thymidine added. Antibiotics were added at the following concentrations for E. coli K-12: kanamycin, 100 μg/ml; ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; and streptomycin, 20 μg/ml. For S. marcescens antibiotics were added at the following concentrations: kanamycin, 200 μg/ml; and ampicillin, 500 μg/ml. Medium F2, which was used for δ-biotin production, contained 15% sucrose, 1.5% urea, 0.1% corn steep liquor, 0.1% K₂HPO₄, 0.2% MgSO₄·7H₂O, 0.01% FeSO₄·7H₂O, 1.5% CaCO₃.

δ-Biotin production. Cells were cultured in a shaking flask...
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 x 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 A660 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* χ1776, a bio operon deletion mutant, and then ampicillin-resistant (Ap'), tetracycline-sensitive (Tc'), and Bio' transformants were selected on minimal agar plates in the presence of 1 µg 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 recovery 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 pLG304.** 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, pLG304, and pLG412, respectively (Fig. 2). Plasmids in which each of the bio genes was inactivated were derived from pLG304 as follows. To inactivate the bioA gene,
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 SalI 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 pLGM304dBFC, 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- strains (21). First, the streptomycin resistance (Sm') marker was introduced into E. coli Bio- strains to distinguish spontaneous tetracycline-resistant (Tc') and kanamycin-resistant (Km') 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' colonies that arose spontaneously were purified and were designated 3104S, 3105S, 3106S, 3107S, and 3108S. Next, the recA56 mutation was introduced into these Sm' Bio- strains to avoid recA-mediated recombination in Tn1000 transpositions and to stabilize the Tn1000-inserted plasmids in these strains. Tc' recombinants were selected on LB medium plates containing streptomycin and tetracycline after mating with Hfr strain JC10240 (ser+::Tn10 near recA56). The Tc' 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'). 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', Tc', and Km' were selected and tested for each of the Bio- phenotypes. The plasmids were extracted from the transconjugants having the relevant Bio- 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 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. α-ε-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, χ1776 was the host, and EcoRI and HindIII were the restriction enzymes (Fig. 2). E. coli χ1776 is deficient in the bioABFCD genes and the bioH gene because of deletion. Following the introduction of S. marcescens SB412 DNA in pBR322, Bio+ transformants of χ1776 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::mBio412), pBM304 (pBR322::mBio304), and pBW101 (pBR322::mBio). 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::mBio), pLGM304 (pLG339::mBio304), and pLGM412 (pLG339::mBio412) (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 Saka-guchi 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 pLG304 to test d-biotin production. Strain 8000(pLG304) 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)
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 (y8) insertions, and insertion sites were mapped by restriction enzyme cleavage (Fig. 3A). We obtained 26 insertions, and among these 26 insertions, there were 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+ BioB− BioF+ BioC− BioD+ phenotype, and 5 derivatives (insertions 22 to 26) had the BioA− BioB+ BioF− BioC+ BioD− 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 operon organization is suggested.

To localize the bio genes more precisely, eight plasmids which prevented the complementation of various Bio− strains of E. coli were constructed from pLGM304 (Fig. 3B). One of these plasmids, deletion plasmid pLG304Dp, which lacks the 0.75-kb Sac1-Scal 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 Sac1-Scal 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 expression of the other genes, whereas all of the bio genes were repressed by removing the 0.75-kb Sac1-Scal 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(pLGM304d10), 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 pLG412 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 pLG304 and pLG412 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(pLG412) were formed among the small colonies. Next, we examined the frequency of larger SB412(pLG412) 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− plates on the plates. Hence, the frequency of large-colony formation was as high as 9.2 × 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 pLG304

Table 2. d-Biotin production by recombinant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dry cell wt (g/liter)</th>
<th>Amt of d-biotin produced (mg/liter)</th>
<th>Amt of biotin-related compounds produced (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120 h</td>
<td>144 h</td>
<td>120 h</td>
</tr>
<tr>
<td>8000(pLG339)</td>
<td>22</td>
<td>NT</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8000(pLGW101)</td>
<td>22</td>
<td>NT</td>
<td>0.45</td>
</tr>
<tr>
<td>8000(pLGM304)</td>
<td>21</td>
<td>NT</td>
<td>0.89</td>
</tr>
<tr>
<td>8000(pLGM412)</td>
<td>21</td>
<td>NT</td>
<td>1.8</td>
</tr>
<tr>
<td>SB304(pLGM304)</td>
<td>20</td>
<td>12</td>
<td>130</td>
</tr>
<tr>
<td>SB412(pLG339)</td>
<td>16</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>SB412(pLGM101)</td>
<td>21</td>
<td>17</td>
<td>92</td>
</tr>
<tr>
<td>SB412(pLGM304)</td>
<td>22</td>
<td>13</td>
<td>180</td>
</tr>
<tr>
<td>SB412(pLG304dA)</td>
<td>23</td>
<td>14</td>
<td>160</td>
</tr>
<tr>
<td>SB412(pLG304dB)</td>
<td>21</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>SB412(pLG304dC)</td>
<td>19</td>
<td>14</td>
<td>61</td>
</tr>
<tr>
<td>SB412(pLG304dD)</td>
<td>23</td>
<td>14</td>
<td>170</td>
</tr>
<tr>
<td>SB412(pLG304dD)</td>
<td>19</td>
<td>13</td>
<td>181</td>
</tr>
</tbody>
</table>

a Cells were grown in medium F2 in shaking flasks at 30°C.
b The biotin-related compounds were d-biotin and dethiobiotin.
c NT, not tested.
FIG. 3. Locations of the bioA, -B, -F, -C, and -D genes and derivatives of pLG304. (A) Sites of Tn1000 insertions on pLG304 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 pLG304 lacking each bio gene. pLG304dA, pLG304dB, pLG304dF, pLG304dC, pLG304dD, pLG304dBFCD, and pLG304dP 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, Sall; Sac, SacI; Pm, PmaCl; St, Stul. (E), (H), (Sac), and (Pm) indicate EcoRI, HindIII, SacI, and PmaCl sites which were lost during construction of the derivatives of pLG304.

and pLG412 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific growth rate in minimal medium (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8000(pLG339)</td>
<td>0.67</td>
</tr>
<tr>
<td>8000(pLGW101)</td>
<td>0.68</td>
</tr>
<tr>
<td>8000(pLGM304)</td>
<td>0.66</td>
</tr>
<tr>
<td>8000(pLGM412)</td>
<td>0.65</td>
</tr>
<tr>
<td>SB412(pLGM339)</td>
<td>0.35</td>
</tr>
<tr>
<td>SB412(pLGW101)</td>
<td>0.33</td>
</tr>
<tr>
<td>SB412(pLGM304)</td>
<td>0.22</td>
</tr>
<tr>
<td>SB412(pLGM412)</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* Cells were cultured in minimal medium with shaking. \( A_{600} \) was measured at different times. The specific growth rate constant \( k \) was defined as follows: 

\[ k = \frac{\text{mass doubling time}}{2} \]

* The specific growth rate constant for this strain was not determined correctly because of strong growth inhibition.

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