Some Problems Involved in Seldomycin Fermentation

Mikio SHIMIZU, Itaru TAKAHASHI* and Takashi NARA
Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahicho, Machidashi, Tokyo 194
*Pharmaceutical Research Laboratory, Fuji Plant, Kyowa Hakko Kogyo Co., Ltd.,
Nagaiizumi-cho, Suntoh-gun, Shizuoka 411
Received November 21, 1977

Improvement of seldomycin factor 5 (SLD-5) fermentation with Streptomyces hofunensis nov. sp. (MK88, ATCC21970) was attempted both by alteration of cultural conditions and by mutation. SLD-5 production by strain MK88 was dependent on the presence of Bacto-Peptone in the fermentation medium. Mutant strains which were resistant to α-amino-β-hydroxyvaleric acid (a threonine analogue) were able to produce SLD-5 on media in which the Bacto-Peptone was replaced by a less expensive nitrogen source. Development of a chemically defined medium for SLD-5 production showed that vitamin B12 stimulated SLD-5 synthesis, while low concentrations of CuSO4·5H2O inhibited SLD-5 production greatly without any inhibitory effect on cell growth. Two actinophages active on MK88 were isolated from soil samples. Mutants resistant to one of the phages which is specific for MK88 became asporogenous but did not show any appreciable change in SLD-5 production.

Streptomyces hofunensis nov. sp. (MK88, ATCC21970) was isolated from soil samples in Hofu, Yamaguchi, Japan and produces several aminoglycoside antibiotics as reported previously.1~4 Seldomycin factor 5(SLD-5) (Fig. 1) is a novel aminoglycoside antibiotic active against both Gram-positive and -negative bacteria. SLD-5 production by strain MK88 was initially dependent on the presence of Bacto-Peptone in the fermentation medium. Mutants which would allow the use of a less expensive nitrogen source were sought. This paper describes the results obtained with these mutants.

MATERIALS AND METHODS

Organisms and phages. Streptomyces hofunensis nov. sp. (MK88, ATCC21970)1 was the parent culture for this study. Strains used for testing the host range of phages were stock cultures in our laboratory. Phage Ø88-a and Ø88-b were isolated from soil samples as described later. Streptomyces coelicolor strains A3 (2)-12, A3 (2)-1098 and A3 (2)-A332, and phage VP11 were generously provided by Dr. D. A. Hopwood.

Media. An agar slant medium for spore formation consisted of glucose 4 g, yeast extract (Difco) 4 g, malt extract (Kyokuto) 10 g, and agar (Ina Shokuhin) 20 g in a liter of deionized water (pH 7.2 before autoclaving). A seed medium contained soluble starch (Stabilose, produced by Matsuya Kagaku Co.) 20 g, glucose 20 g, Bacto-Peptone (Difco) 10 g, yeast extract (Difco) 5 g and CaCO3 1 g in a liter of deionized water (pH 6.0 before autoclaving). Fermentation experiments were studied in a standard medium of the following composition: soluble starch, 30 g; glucose (autoclaved separately), 20 g; Bacto-Peptone, 20 g; yeast extract, 4 g; CaCO3, 2 g; MgSO4·7H2O, 0.5 g; calcium phytate (Tokyo Kasei Co.), 0.5 g; d, L-methionine, 0.5 g; CaCl2·2H2O, 2 g; deionized water, 1,000 ml (pH 6.5 before autoclaving). A synthetic medium for fermentation contained soluble starch 15 g, glucose

![Fig. 1. Structure of Seldomycin Factor 5.](image-url)
15 g (autoclaved separately), sodium L-glutamate 2.5 g, 
MgSO₄·7H₂O 0.5 g, ZnSO₄·7H₂O 0.05 g, FeCl₃·6H₂O 
0.05 g, CaCl₂·2H₂O 2 g, vitamin B₁₂ (sterilized with 
Millipore filter type HA) 0.001 g, KH₂PO₄ 0.05 g, 
K₂HPO₄ 0.05 g in a liter of deionized water (pH 7.0 
before autoclaving). A medium for bioassay was 
nutrient agar broth (Nissui Co.) containing 0.1 M 
Tris-HCl buffer (pH 7.5). The minimal medium 
described by Hopwood⁶ was used for selection of mu 
tants resistant to amino acid analogues.

Cultural condition and determination of SLD-5. A 
few loopfuls of well-sporulated slant culture (15 days 
old, 30°C) were inoculated in a 70-ml large test tube 
containing 10 ml of seed medium (1st seed). The cul 
ture shaken on a reciprocal shaker (220 rpm) at 30°C 
for 3 days was transferred to the second seed medium 
with 10% (v/v) of inoculum. The second seed culture 
shaken at 30°C for one day was transferred to a 300-ml 
Erlenmeyer flask containing 30 ml of the fermentation 
medium with 10% of inoculum. The fermentation was 
carried out at 30°C for 5–8 days on a rotary shaker 
(220 rpm). The beer was diluted with 0.1M Tris-
HCl (pH 7.5) and the potency of SLD-5 was estimated 
by a disc agar dilution method with Bacillus subtilis no. 
10703 as the test organism using SLD-5 sulfate (Lot 
no. T49301, 61.9% base) as the reference standard. 
The organism is at least forty times more sensitive to 
SLD-5 than to the other components produced by the 
strain MK88.

Isolation of mutants of MK88. Spores from slants 
were suspended in the solution used for mutagen treat 
ment. The spore suspension was blended in a Micro 
nizer (Nihon Seiki Co.) in order to separate each spore, 
filtered through layers of sterile cotton, and treated 
at a killing rate of approximately 99.9% with ultraviolet 
irradiation,⁷ N-methyl-N'-nitro-N-nitrosoguanidine 
(Wako Jun-yaku Co.),⁸ γ-ray irradiation (γ-Rays 
Irradiator, Yoshizawa Lead Co.), 8-methoxypsoralen (K 
& K Laboratories) and near-ultraviolet irradiation,⁹¹⁰ 
or ethylmethanesulfonate (Nakarai Kagaku Co.).¹¹ 
The treated spores were grown to allow segregation of 
the mutants and permit the phenotypic expression of the 
induced mutation. The grown cells were blended in a 
Micronizer and spread on a selective medium. Each 
clone which grew on a selective medium was cultured 
and assayed for SLD-5 potency as mentioned above.

Isolation of actinophages. About one gram of a 
soil sample and spores (a final concentration of 10⁹ 
cells/ml) of MK88 were added to 10 ml of the seed 
medium containing 4 mm CaCl₂·2H₂O in a 70-ml large 
test tube. The broth was shake-cultured at 30°C for 
3 days and centrifuged at 3000×g for 30 min. The 
supernatant was filtered through a Millipore filter (type 
HA) and the filtrate was tested for the presence of 
phages by the general technique of double layer 

method.¹¹ Phages VP5, VP11 and HA2 were used to 
test the procedure.

RESULTS AND DISCUSSION

Effect of nitrogen sources and other com 
ponents on SLD-5 production

Strain MK88 and several early mutants were 
tested for SLD-5 production with sixteen differ 
ent nitrogen sources. Good yields of SLD-5 
were only obtained with the Bacto-Peptone. 
Since an economical replacement for Bacto-
Peptone is required for industrial fermentation, 
muts were sought which would give good 
yields of SLD-5 with a less expensive nitrogen 
source. Strain 12-7, a mutant (Fig. 2) which 
is resistant to the threonine analogue α-
amino-β-hydroxyvaleric acid (Sigma Chemical 
Inc.), was found to give fair yields of SLD-5 
when Kyokuto-Peptone was used in the fermen 
tation medium instead of Bacto-Peptone (Table 
I). Strain 12–7 also gave fair yields of SLD-5 

<table>
<thead>
<tr>
<th>Nitrogen (2%)</th>
<th>SLD-5 yield (mcg: base/ml) at 6 day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0ᵃ</td>
</tr>
<tr>
<td>Bacto-Peptone</td>
<td>145</td>
</tr>
<tr>
<td>Kyokuto-Peptone</td>
<td>50</td>
</tr>
<tr>
<td>Kyokuto-Neopeptone</td>
<td>54</td>
</tr>
<tr>
<td>Kyokuto-Meat Extract</td>
<td>16</td>
</tr>
<tr>
<td>Daigo-Eiyo Polypeptone</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Bacto-Neopeptone</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Bacto-Tryptone</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Bacto-Trypticase</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

ᵃ) pH before autoclaving.

with the inexpensive peptones, spray dried lard 
water (SDLW) (Inland Molasses Inc.) and liquid peptone WP–100 (Inolex Inc.) (Table II).
TABLE II. COMPARISON OF SLD-5 YIELDS BY MUTANTS WITH VARIOUS NITROGEN SOURCES

<table>
<thead>
<tr>
<th>Strain</th>
<th>Marker</th>
<th>Maximal yield of SLD-5 (mcg · base/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacto-Peptone</td>
</tr>
<tr>
<td>MK88</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>U7</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>U7–1</td>
<td>phage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90</td>
</tr>
<tr>
<td>16G</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>12–7</td>
<td>αAHV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>245</td>
</tr>
<tr>
<td>E</td>
<td>αAHV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>420</td>
</tr>
<tr>
<td>E7–17</td>
<td>αAHV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>350</td>
</tr>
</tbody>
</table>

<sup>a</sup> HKM, Hokuyo-Kosen meal (fish paste); SDLW, spray dried lard water, WP-100: liquid peptone.
<sup>b</sup> phage<sup>88-a</sup>, resistance to phage 88-a; αAHV<sup>b</sup>, resistance to α-amino-β-hydroxyvaleric acid.

Further mutants were tested with Kyokuto-Peptone as the nitrogen source. Strain E (Fig. 2) was found to give good yields of SLD-5 with Bacto-Peptone, Kyokuto-Peptone, SDLW and WP-100 (Table II). Studies with strain E showed that the addition of sodium L-glutamate and sodium L-aspartate improved SLD-5 yield. Soluble starch was the best carbon source<sup>1</sup>) and further addition of glucose greatly improved the production. Figure 3 shows a production of SLD-5 was mostly proportional to cell growth with a decrease of SLD-5 potency at the later stage of fermentation. A decrease in potency has often been reported in fermentations of various antibiotics.

**Strain improvement**

After treatment with a mutagen, randomly selected colonies and colonies resistant to analogues of amino acids, to antibiotics or to phage φ88-a were screened for improved yields of SLD-5. The development of phage-resistant strains is sometimes important for industrial fermentation in order to prevent contamination by phage infection.<sup>12,13</sup>) Therefore, a similar trial was done with a strain U7. All colonies resistant to phage φ88-a turned out to be asporogenous and to show no appreciable changes in SLD-5 production (strains U7 and U7–1 in Table II). In contrast, it was reported that in Streptomyces mediterranei,<sup>13</sup>) phage-resistant strains became asporogenous, but resulted in higher yields of rifamycin. Mutants resistant to 28 different amino acid analogues were tested. Strain 12–7, a mutant resistant to α-amino-β-hydroxyvaleric acid, proved to be less stringent regarding the type of peptone required for SLD-5 production as described above. This result may suggest that primary metabolism can affect production of secondary metabolites. Since methionine sometimes seemed to stimulate SLD-5 yield and SLD-5 contains methyl group in the molecule, mutants resistant to analogues of methionine were ex-
pected to show some effects on SLD-5. However, appreciable changes were not found in SLD-5 yields in the mutants. Some other approaches\(^\text{14}\) for strain improvement were tried with no appreciable success. Recently, plasmid-determined antibiotic production has been reported.\(^\text{15}\) However, treatment of strain MK88 with ethidium bromide or acridine orange did not give nonproducers of SLD-5.

**SLD-5 production in a chemically defined medium**

The best one of 18 carbon sources was glucose followed by rhamnose, xylose and soluble starch, and the best one of 40 nitrogen sources was sodium L-glutamate followed by sodium L-aspartate, NaNO\(_3\), ammonium citrate and ammonium tartrate. MgSO\(_4\)·7H\(_2\)O, ZnSO\(_4\)·7H\(_2\)O, inorganic phosphate, FeCl\(_3\)·6H\(_2\)O and especially vitamin B12 (Table III, Table III. **Stimulation of SLD-5 yield by vitamin B12 in strain E7-17 in a synthetic medium**

| SL}D-5 (mcg·base/ml) at 9 day\(^\text{a}\) | No addition | 0\(^\text{b}\) | Vitamin mixture | 7 | Vitamin mixture—VB\(_{12}\) | 0 | VB\(_{12}\) | 10\(^{-3}\) mcg/ml | 5 | 10\(^{-2}\) mcg/ml | 5 | 10\(^{-1}\) mcg/ml | 5 | 1 mcg/ml | 11 | 10 mcg/ml | 5 | NiCl\(_2\)·7H\(_2\)O | 25~200 mcg/ml | 0 | CoCl\(_2\)·7H\(_2\)O | 25~200 mcg/ml | 0 |

\(^\text{a}\) Seed culture was well-washed in saline and transferred to the synthetic medium with 10% (v/v) of inoculum.

\(^\text{b}\) A smaller size of inoculum (10% or less) from seed culture hardly showed a potency in the synthetic fermentation medium in contrast to a larger size of inoculum (30%) giving a potency of 100 mcg/ml in Fig. 4.

Fig. 4) were required for SLD-5 production. Thus, a synthetic medium for SLD-5 production was formulated as described in MATERIALS AND METHODS. The synthetic media used for streptomycin production\(^\text{16}\) were not effective on SLD-5 production. The production of SLD-5 was completely inhibited by CuSO\(_4\)·5H\(_2\)O at low concentrations where cell growth was not inhibited (Fig. 4). The effect of CuSO\(_4\) Fig. 4. **SLD-5 Production and Cell Growth by Strain E7-17 in a Synthetic Medium.**

Seed culture was well-washed in saline and transferred to the synthetic medium with 30% (v/v) of inoculum. ●—●, without addition; ○—○, plus 1 mcg/ml of vitamin B12; ●—○, plus 1 mcg/ml of vitamin B12 and 1 mcg/ml of CuSO\(_4\)·5H\(_2\)O

**Fig. 5. Thin-layer Chromatography of Products.** The beers of 8-day culture (8d), 8-day culture with CuSO\(_4\)·5H\(_2\)O (+Cu, 8d) and 15-day culture (15d) were charged on IRC50 (NH\(_4^+\)) column. The eluate with 0.5 N NH\(_4\)OH were subjected to thin-layer chromatography on silica gel sheets (Merck) in CH\(_3\)Cl-CH\(_2\)OH-NH\(_4\)OH-acetone (2:2:1:1) as described previously.\(^\text{3}\) Authentic samples were SLD-1, 2, 3, 4, 5, 3B and 3C.
TABLE IV. HOST RANGE OF PHAGE φ88-a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage sensitivitya)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>φ88-a</td>
</tr>
<tr>
<td><strong>Streptomyces hofunensis</strong></td>
<td>ATCC21970</td>
</tr>
<tr>
<td><strong>St. coelicolor A3(2)-12</strong></td>
<td>−</td>
</tr>
<tr>
<td><strong>A3(2)-1098</strong></td>
<td>−</td>
</tr>
<tr>
<td><strong>A3(2)-A332</strong></td>
<td>−</td>
</tr>
<tr>
<td><strong>St. ambofaciens</strong></td>
<td>ATCC15154</td>
</tr>
<tr>
<td><strong>St. aureofaciens</strong></td>
<td>ATCC10762</td>
</tr>
<tr>
<td><strong>St. caespitosus</strong></td>
<td>KY681</td>
</tr>
<tr>
<td><strong>St. erythreus</strong></td>
<td>HUT6144</td>
</tr>
<tr>
<td><strong>St. erythromycolus</strong></td>
<td>KY622</td>
</tr>
<tr>
<td><strong>St. fradiae</strong></td>
<td>ATCC10745</td>
</tr>
<tr>
<td><strong>St. griseus</strong></td>
<td>KY641</td>
</tr>
<tr>
<td><strong>St. kanamyceticus</strong></td>
<td>ATCC12853</td>
</tr>
<tr>
<td><strong>St. kasugaensis</strong></td>
<td>ATCC15714</td>
</tr>
<tr>
<td><strong>St. lavendulae</strong></td>
<td>ATCC8664</td>
</tr>
<tr>
<td><strong>St. prasinus</strong></td>
<td>NIHJ408</td>
</tr>
<tr>
<td><strong>St. rimosus</strong></td>
<td>ATCC14827</td>
</tr>
<tr>
<td><strong>St. tanashiensis</strong></td>
<td>ATCC15238</td>
</tr>
<tr>
<td><strong>Micromonospora chalcea</strong></td>
<td>ATCC12452</td>
</tr>
<tr>
<td><strong>M. olivosterospora</strong></td>
<td>ATCC21819</td>
</tr>
<tr>
<td><strong>M. sagamiensis</strong></td>
<td>ATCC21803</td>
</tr>
<tr>
<td><strong>Nocardia gardneri</strong></td>
<td>ATCC21519</td>
</tr>
<tr>
<td><strong>N. lutea</strong></td>
<td>ATCC21291</td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong></td>
<td>ATCC9634</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>Abbott no. 10707</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>KY4279</td>
</tr>
</tbody>
</table>

a) 10⁴ plaque-forming units of phages were spotted on soft agar plate containing spores or cells.

Fig. 6. Phage φ88-a.

was also shown in complete fermentation media.

Components produced in fermentation

Strain MK88 produces seldomycin factors
1, 2, 3, 5 and other components as described previously. At the later stage of fermentation, a decrease of SLD-5 yield was seen as shown in Fig. 3 and Fig. 4. This phenomenon has been reported in various antibiotic fermentations and inactivated forms of products have been shown in the culture broth. Thin-layer chromatography (Fig. 5) showed that the SLD-5 presented at 8 days was changed to other components (biologically inactive) at 15 days. Similar inactive components were detected in broth where SLD-5 production was inhibited by CuSO₄ (Fig. 5). Characterization of these inactive components is currently under investigation. It was found that cells or cell-free extracts of MK88 changed SLD-5 to inactive components in vitro. These components had higher Rf values than the inactive components produced in the later stage of fermentation. Therefore, the two types of inactive components seem to be different.

**Isolation of actinophage active on MK88**

At least two kinds of phages active on MK88 were isolated from soil samples collected from various parts of the field around our laboratory. One of eleven soil samples was found to contain phages (termed φ88-a) which make clear plaques and are stable. Four samples contained phages (termed φ88-b) which make turbid plaques and are unstable. Phage φ88-a has a silo-type head (ca. 43.3 x 93.3 nm) and a tail (ca. 13.3 x 169 nm) (Fig. 6) and shows a monovalent host range (Table IV). Many actinophages have been reported to have a wide host range as shown by phage VP11 (Table IV) and only a few have been reported specific for the host strains. It is interesting that mutants resistant to phage φ88-a were asporogenous as described above.

**Acknowledgements.** The authors wish to thank Drs. A. C. Sinclair, N. E. Wideburg and Miss M. Jackson (Abbott Laboratories, North Chicago) for their kind encouragement and for the critical comment on the manuscript. We are also thankful to Drs. D. A. Hopwood (John Innes Institute), H. Kawaguchi and K. Tomita (Bristol-Banyu Research Institute Ltd.) for kindly supplying strains and phages. We are grateful to Dr. I. Takano (JEOL Ltd.) for electronmicrography of phages.

**REFERENCES**