screened for high-frequency transfer of OTC resistance. Although such strains were found at quite a high frequency (about 1 in 1000), it was subsequently discovered that a second plasmid SRP2 had actually been primed. This possessed much weaker sex-factor activity than SRP1. Neither plasmid has proved amenable to physical isolation or analysis, and with the advent of recombinant DNA techniques it was decided to clone the OTC genes onto specifically developed cloning vectors.

**Plasmid-cloning vectors**

An initial search for small multicopy plasmids in various *S. rimosus* isolates was soon overtaken by the development of a series of broad host range plasmids at the John Innes Institute (Kieser et al., 1982). The plasmid chosen was pJl303, which carried the *S. azureus* gene for thiostrepton resistance. When this plasmid was transformed into *S. rimosus* and thiostrepton resistance colonies selected, a low efficiency of transformation was observed. Plasmid DNA from the transformants was examined by agarose-gel electrophoresis, and found to be smaller in size than the original plasmid. This was interpreted as the result of restriction in vivo. A 6.2 kb plasmid pPZ12 was selected for use as a cloning vector for *S. rimosus*. pPZ12 DNA extracted from *S. rimosus* was able to transform *S. rimosus* at normal frequencies (~10⁶/µg) without detectable modifications. The following unique sites are available for cloning: PstI, ClaI, Kpnl and SacI.

When totally PstI-digested *S. rimosus* DNA was ligated into pPZ12 and transformed into an OTC-sensitive *S. rimosus* variant an OTC-resistant clone was isolated which contained an 8.7 kb insert. Southern-blotting analysis showed that this fragment was present in PstI-cut *S. rimosus* chromosomal DNA, but not present in DNA from the OTC-sensitive variant. This must, therefore, have suffered a deletion from the chromosome. Presumably at least some OTC biosynthetic genes must also have been lost or inactivated during the formation of such a variant; the continued production of OTC would have been suicidal.

In order to extend the cloned region, the 8.7 kb fragment was ³²P-labelled by nick translation and used to probe genomic banks of *S. rimosus* in bacteriophage lambda or in Escherichia coli.

**Chromosome walking**

The advantages of plaque hybridization in screening large numbers of clones lead us to clone *S. rimosus* DNA into the lambda vector L47.1. Size-selected (10-20 kb) Sau3A-cut *S. rimosus* DNA was ligated into the *Bam*H1-generated arms and packaged in vitro. Plaques which hybridized with the probe were examined in more detail and three overlapping clones representing a total of 30-40 kb from the OTC region were isolated. The inserts were subcloned into a *Streptomyces* plasmid as follows. We observed that the enzyme HindIII cuts *S. rimosus* DNA very infrequently, and that only one HindIII site exists in the cloned region. In the vector, however, there are HindIII sites just outside the *Bam* cloning sites. Treatment of the lambda clone DNA with HindIII therefore yielded a fragment representing the entire insert with small portions of lambda DNA at the ends. These fragments were ligated into a suitable plasmid vector, pPZ49. This was constructed by subcloning the erythromycin resistance gene from *S. erythraeus* into pPZ12. Cloning into the unique HindIII site causes insertional inactivation of the erythromycin resistance. Finally the plasmids were transformed into a restriction-deficient strain of *S. albus*. This system provides a rapid method of cloning, screening by hybridization and subcloning.

Alternative methods for cloning *S. rimosus* DNA using bifunctional *E. coli* plasmids have also been developed (Chambers & Hunter, 1984). To facilitate the transformation of DNA into *S. rimosus* from other species of *Streptomyces* or from *E. coli* a simple procedure for the isolation of restriction-deficient mutants has been worked out (Hunter & Friend, 1984). A total of 60 kb of *S. rimosus* DNA from the OTC-resistance region now has been cloned using these methods.

**Discussion**

Rapid and efficient methods for cloning *S. rimosus* DNA have been developed and applied to one of the OTC biosynthetic regions. The way is now open to study the expression of the OTC biosynthetic genes and the regulation of OTC biosynthesis. As our understanding of gene expression increases we will for the first time be able to devise deliberate strategies for the construction of strains with increased antibiotic production rates.


**Optimization of streptomycete strains producing polyether and macrolide antibiotics**

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Polyether and macrolide antibiotics represent numerous groups of compounds formed by condensation of acetate, propionate and, in some cases, butyrate units. However, their biogenesis has not yet been completely solved and theoretical discrepancies exist already at the level of the formation of basic building units. This holds primarily for the formation of the butyrate building unit. Aglycones of some 16-membered macrolides (e.g. magnamycin, tylosin, turimycin, platenolides, leuko-mycins, spiramycins) contain this unit. It was assumed that in case of magnamycin this unit is formed from succinic acid (Achenbach & Grisebach, 1964). The views of Woodward et al. (1965) were similar. Grisebach & Weber-Schilling (1968) later withdrew the assumption about the
incorporation of succinic acid. On the basis of incorporation experiments with 14C-labelled substrates Srivivasan & Srinivasan (1967) assumed that this unit is formed from acetate.

Studies in which the biosynthesis of 16-membered macrolides was investigated by means of 13C-labelled precursors and nuclear magnetic resonance led Omura et al. (1977) to the conclusion that the significant randomization of isotopes in the aglycone moiety can be, at least partially, explained by oxidation of butyric acid to succinic acid which is further isomerized to methymalonic acid (at the level of corresponding enzymes). After decarboxylation methymalonic acid yields the propionate unit. Carboxylation of butyric acid gives rise to ethymalonic acid which is isomerized, oxidized and decarboxylated yielding again methymalonic acid, but, in this case, with an altered isotopic labelling (Omura & Nakagawa, 1981). Ethymalonyl-CoA can only hardly be considered as a direct precursor of the butyrate unit as its absolute configuration is not known; in addition, the existence of both butyryl-CoA carbonylase and ethymalonyl-CoA epimerase has not yet been demonstrated (Hutchinson et al., 1981). Gersch et al. (1977) studying the biosynthesis of tumricin faced apparently different positions. Position 4' of the sugar mycarose of this antibiotic can be acylated by isovaleryl, propionyl, acetyl and iso- and n-butyryl. They degraded tumricin to mycarose and demonstrated that tumricin, produced not by this enzyme, might be a precursor of the butyrate unit of the lactone moiety. Reuter & Huttner (1976) reported that both theoretical considerations and experimental data give evidence against the direct synthesis of n-butyric acid from valine.

A similar situation with the biogenesis of the butyrate unit was observed when studying oligoketides. With the polyether type (Dorman et al., 1976). Within the basic building units of narasin they came to the conclusion that enzymes of the producer, i.e. Streptomyces aureofaciens, are capable of the interconversion of propionate and butyrate. The mechanism of these interconversions is not clear. It cannot be explained by simple schemes of α- and β-oxidation of basic building units. These authors were the first to demonstrate the conversion of propionate to butyrate.

Monensins A and B (differing only in a single butyrate and propionate unit) appear to be a highly valuable model which makes it possible to solve the origin of the butyrate unit. We found that Streptomyces cinnamonensis culture in a synthetic medium with L-valine produces predominantly monensine A, whereas in the polyether type (Vanek et al., 1982). When using radioactive substrates it is not necessary to degrade chemically the compounds under investigation but the differences in specific radioactivity between monensin A and monensin B can be directly related with the butyric acid unit. We found that 1-L-[1-13C]Valine might be a precursor of the butyrate unit of the lactone moiety. Reuter & Huttner (1976) reported that both theoretical considerations and experimental data give evidence against the direct synthesis of n-butyric acid from valine.

The successful incorporation of isobutyrate into monensin A demonstrates a new metabolic pathway involving its isomerization to n-butyrate. Both isobutyrate and n-butyrate can be converted to propionate by S. cinnamonensis with the carboxyl label retained. Such an effect has already been observed in lysocellin, tylosin, and probably also in lasalocid A. Butyrate synthase participates in the biosynthesis of monensin A. This finding explains a higher than theoretical radioactivity found by Day et al. (1973) in [1-13C]acetate-derived monensin A.

Our results show that in spite of simple building units, the biosynthesis of monensins is complex due mainly to multiple relationships among them.

Similar results were also reached by Omura et al. (1983) studying the formation of the butyrate unit in Streptomyces fradiae, a tylosin producer, and in Streptovacterium kitanensis producing leukomycin A. Both results indicate that the amino acids participating in the biosynthesis of these fatty acids are mainly valine and isoleucine, while leucine does so to a much lesser extent (Table 1).

The strain Streptomyces cinnamonensis C-100-5 produced roughly identical amounts of monensins A and B. In further studies we attempted to investigate the origin of the butyrate unit in strains producing predominantly monensin A.

We used amino acid analogues of the group of aspartic acid and derived from pyruvic acid, i.e. 2-aminobutyrate, DL-norvaline, L-norleucine, 2-amino-3-chlorobutyrate, and, in addition, DL-ethionine, an inhibitor of transmethylation reactions (Pospišil et al., 1984).

The usual procedures were applied. We verified the sensitivity of S. cinnamonensis to the antimetabolite in the presence of different N-buten source and tested the effect of amino acids on the increased inhibitory role of DL-norvaline and L-norleucine. After treatment with mutagens (u.v. light, NH2OH·HCl; survival 0.1–0.5%) the spore suspension was transferred to a liquid medium. The required concentrated

As expected, C-15 of monensin A is predominantly labelled by sodium[1-13C]butyrate; a lower level of enrichment was observed at C-1, -3, -5, -11, -17, -21, and -23, i.e. at carbons arising from C-1 of propionate. In monensin B only C-1 propionate sites (now also C-15) were labelled. Sodium[1-13C]butyrate produced the same incorporation pattern. Using double-labelled acetate, we found that five intact acetate units are incorporated into monensin B but seven into monensin A. The additional labelled carbons are those of butyrate origin.

Table 1. Fatty acids of S. cinnamonensis

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Normal acids (%)</th>
<th>Iso-acids (%)</th>
<th>Anteiso-acids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:0</td>
<td>0.21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7:0</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10:0</td>
<td>0.16</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>12:0</td>
<td>0.17</td>
<td>0.39</td>
<td>-</td>
</tr>
<tr>
<td>13:0</td>
<td>0.13</td>
<td>1.17</td>
<td>1.29</td>
</tr>
<tr>
<td>14:0</td>
<td>2.24</td>
<td>8.06</td>
<td>0.10</td>
</tr>
<tr>
<td>15:0</td>
<td>0.10</td>
<td>4.64</td>
<td>10.90</td>
</tr>
<tr>
<td>16:0</td>
<td>9.88</td>
<td>11.44</td>
<td>-</td>
</tr>
<tr>
<td>17:0</td>
<td>1.36</td>
<td>-</td>
<td>9.50</td>
</tr>
<tr>
<td>18:0</td>
<td>0.50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19:0</td>
<td>0.13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1984
solutions of the antimetabolites were added after a 42h incubation which was continued for a further 72h. The results of these experiments are summarized in Table 2. In the strains resistant to 2-amino-3-chlorobutyrate the production of monensin A varied from 85 to 90%.

When investigating the excretion of amino acids into the synthetic culture medium valine (0.41 μmol/ml) was detected in the strains resistant to 2-aminobutyrate and only traces of this amino acid were found in the 2-amino-3-chlorobutyrate-resistant strains. The strains also differed in the spectrum of other amino acids excreted into the culture medium, indicating that the regulation of the biosynthesis of the amino acids investigated in these strains is also different.

Therefore, in further experiments, we tried to prepare mutant strains of *S. cinnamonensis* resistant to 2-amino-3-chlorobutyrate and even to some other antimetabolites. These experiments were successful and stable mutant strains producing 95–97% of monensin A were obtained.


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Table 2. Mutant strains resistant to antimetabolites of amino acids

<table>
<thead>
<tr>
<th>Antimetabolite</th>
<th>(+) strains</th>
<th>(−) strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of</td>
<td>Mon*</td>
</tr>
<tr>
<td>2-Aminobutyrate</td>
<td>31</td>
<td>70–81</td>
</tr>
<tr>
<td>DL-Norvaline</td>
<td>14</td>
<td>70–80</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>8</td>
<td>70–80</td>
</tr>
<tr>
<td>DL-Ethionine</td>
<td>20</td>
<td>70–80</td>
</tr>
<tr>
<td>2-Amino-3-chlorobutyrate</td>
<td>9</td>
<td>85–90</td>
</tr>
</tbody>
</table>

*Mon* content of monensin A.