SULFATE UTILIZATION BY PENICILLIN-PRODUCING MUTANTS OF
PENICILLIUM CHRYSOGENUM

PHILIP L. TARDEW AND MARVIN J. JOHNSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

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A great deal of work has been directed toward increasing the yield of penicillin in fermentations with Penicillium chrysogenum. The spectacular increase in yield which has been achieved has resulted, in the main, from two approaches. In the first approach, mutant strains of P. chrysogenum produced by spontaneous variation or by the action of mutagenic agents such as nitrogen mustard or ultraviolet light were selected for enhanced penicillin production. The development of the "Wisconsin family" of high yielding mutant strains of P. chrysogenum strain NRRL-1551-B25 has been described by Backus and Stauffer (1955). Further enhancement of the yield of penicillin has resulted from a more complete definition of the environmental conditions (pH, temperature, constituents of the medium) necessary for the excretion of penicillin by the mold (Johnson, 1952).

However, changes in the sulfur metabolism which accompany an increase in penicillin synthesis brought about either by mutation of the mold or by changes in the conditions of the fermentation, have not been investigated. It is the purpose of this paper to report differences in the utilization of sulfate and the excretion of sulfur compounds by various strains of P. chrysogenum when grown on a chemically defined medium with inorganic sulfate as the sole source of sulfur, with and without precursor addition, and to relate these differences with the capacity of the strain to synthesize penicillin. The strains of P. chrysogenum which were used in these experiments are listed in table 1.

MATERIALS AND METHODS

Fermentation techniques and media. All fermentations were conducted in 500-ml Erlenmeyer flasks on a rotary type shaker (about 250 rpm) at 25 C. A 10 per cent inoculum of a 40 to 48 hr vegetative culture was used in all experiments. Each flask contained 100 ml after inoculation. Methods for handling and sampling cultures have been described previously (Jarvis and Johnson, 1947).

The inoculum medium used in all experiments contained the following ingredients in g per L: glucose, 40.0; ammonium lactate, 21.0; calcium carbonate, 13.0; KH₂PO₄, 3.0; Na₂SO₄, 0.740; magnesium acetate, 0.25; ZnCl₂, 0.02; MnCl₂·4H₂O, 0.02; FeCl₃·6H₂O, 0.02; CuCl₂·2H₂O, 0.005; CaCl₂·2H₂O, 0.05. The glucose and the calcium carbonate were sterilized separately in distilled water and added to the salts before inoculation.

The fermentation medium contained the following ingredients in g per L: lactose, 40.0; glucose, 10.0; ammonium lactate, 8.0; ammonium acetate, 3.5 KH₂PO₄, 6.0; magnesium acetate, 0.25; ZnCl₂, 0.02; FeCl₃·6H₂O, 0.02; MnCl₂·4H₂O, 0.02. Sulfur was added as desired (usually 200 μg per ml) as sodium sulfate. The sulfate was labeled with carrier-free S³⁵O₄²⁻, 0.10 to 0.50 μc being added to each flask before sterilization. The sugars were sterilized separately in distilled water and were added to the other ingredients immediately before inoculation. The pH of the mixture of salts was adjusted to 6.5 before sterilization.

For the low-yielding strains the fermentation medium was modified, since the above medium resulted in a pH plateau which was too high for optimal penicillin production. In the case of these strains no sodium sulfate was added to the medium, 0.825 g per L of ammonium sulfate (equivalent to 200 μg of sulfur per ml) were added and the quantity of ammonium lactate was reduced to 6.67 g per L to keep the total amount of nitrogen in the medium constant.

Analytical procedures. Mycelial nitrogen and penicillin were determined by the methods described in a previous paper (Jarvis and Johnson,
TABLE 1

Properties of Penicillium chrysogenum strains

<table>
<thead>
<tr>
<th></th>
<th>Wild Strains*</th>
<th>Mutant Strains†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS61 (a)</td>
<td>PS65 (b)</td>
</tr>
<tr>
<td>Maximum penicillin yield (units per ml)</td>
<td>6-7</td>
<td>6-8</td>
</tr>
<tr>
<td>Peak mycelial nitrogen (amoles per ml)</td>
<td>95-110</td>
<td>98-110</td>
</tr>
<tr>
<td>pH plateau ‡</td>
<td>7.65-</td>
<td>7.4-</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>7.9</td>
</tr>
</tbody>
</table>

The letters in parentheses after each strain refer to figure 1, where the letters along the abscissa of the figure refer to the strains as listed above.

* Wild strains were isolated from natural sources. Strains PS61 and PS75 are randomly selected soil isolates. Strain 1951-B25 is a spontaneous variant from a wild culture selected for penicillin production. These cultures were obtained from the Northern Utilization Research Branch, U.S.D.A., Peoria, Illinois.

† Mutant strains have been selected after treatment with mutagenic agents, e.g., X-rays, ultraviolet light, and nitrogen mustard. Strain X1612 was obtained from strain NRRL1951-B25 by selection after irradiation with X-rays. All other mutant strains were derived from X1612. They are members of the "Wisconsin family" of mutants which has been described by Backus and Stauffer (1955). All mutant strains were obtained from Doctor M. P. Backus of the Botany Department, University of Wisconsin.

‡ The pH plateau is the pH range which obtained in the fermentation during the penicillin producing phase (usually 24 to 96 hr).

1947). The pH of each sample was determined, immediately after removal, by means of a glass electrode. The total sulfur and total organic sulfur present in the filtered broth at various times was determined by counting the radioactivity before and after precipitation of sulfate with barium chloride under acid conditions in the presence of added carrier sulfate. After removal of the precipitate of barium sulfate the broth was neutralized with sodium hydroxide solution. The filtered broth, or broth after sulfate precipitation, was diluted with a 1 per cent gelatin solution (pH 10 to 11 with sodium hydroxide) to give an estimated 1000 cpm per ml of diluted sample. One ml of this solution was pipetted on to a copper planchet and counted with a Geiger-Mueller thin-window counter after drying overnight. The specific activity of the sulfur in cpm per μg of sulfur was determined from the measured count rate of the sample taken at the time of inoculation, the sulfur content of this sample being known. The concentration (in μg) of sulfur present in any sample was calculated from the measured counts of the sample and the specific activity of the sulfur. At least 1000 counts were recorded in all radioactivity determinations. In all cases corrections were made for background, coincidence, and self-absorption.

RESULTS

Sulfate utilization by various strains. In table 1 the maximum yield of penicillin and of mycelial nitrogen obtained with the various strains of P. chrysogenum are listed. Table 1 also shows the pH plateau given by each strain during the penicillin-producing phase (usually 24 to 96 hr) on the chemically defined media used.

It can be seen from table 1 that the strains of P. chrysogenum which were used in this investigation differ mainly in the capacity to produce penicillin. Under the conditions of these experiments, mycelial growth as measured by the production of mycelial nitrogen does not vary greatly from strain to strain although, on an average, the low penicillin-yielding strains (PS61 to NRRL1951-B25 in table 1) may produce a little more mycelial nitrogen than the high penicillin-yielding strains (Q176 to 51-20F3 in table 1). Also, the pH plateau obtained with the low-yielding strains was, on the average, higher
than with the high-yielding strains. A further difference was observed between the two groups of strains in that the pH of the medium always rose at the end of the fermentation (100 to 130 hr) in the case of the low-yielding strains, whereas the pH invariably fell at a corresponding time in fermentations with the high-yielding strains. The effect of these differences on the uptake and excretion of sulfur is unknown, although experiments with the same strain suggest that pH values higher than 7.8 or 7.9 result in lowered uptake of sulfate. These results are consistent with the known reduction in the yield of penicillin at these pH values.

In figure 1 the various strains of *P. chrysogenum* are compared for the utilization of sulfate and the excretion of sulfur compounds during the penicillin-producing phase (usually from 24 to 96 or 120 hr). The quantity of sulfate utilized by the mold was the decrease in inorganic sulfate in the medium. Inorganic sulfate was determined as the radioactivity precipitated by barium chloride in acid solution after addition of carrier sulfate. “Total organic sulfur excreted” refers to soluble sulfur not precipitated by barium chloride. All figures are expressed as µg of sulfur per µg of mycelial nitrogen. In this way any differences which depend upon differences in the rate of growth are obviated. Each point is the average of three determinations made during the penicillin-producing phase of the fermentation (usually at 48, 72, and 96 hr).

It is evident from figure 1 that high-yielding mutant strains of *P. chrysogenum* show an enhanced sulfur metabolism in comparison to their low-yielding analogues. This enhancement of the sulfur metabolism manifests itself in a 2- to 3-fold increase in the quantity of sulfate taken up by the mold and of the quantity of sulfur excreted into the medium, and also in an increase in penicillin production from 6 to 40 times (table 1).

Strain X1612, which is intermediate between the high- and low-yielding strains in its capacity to excrete penicillin, seems to resemble the low-yielding strains in its capacity to utilize sulfate.

**Effect of R-group precursor (phenylacetate) on sulfate utilization.** The effect of the addition of certain compounds, notably those containing

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**TABLE 2**

*Effect of phenylacetate addition on growth and pH*

<table>
<thead>
<tr>
<th>Hr</th>
<th>Strain NRRL1951-B25</th>
<th>Strain 51-20F3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With precursor</td>
<td>Without precursor</td>
</tr>
<tr>
<td></td>
<td>Mycelial N mmoles/L</td>
<td>pH</td>
</tr>
<tr>
<td>48</td>
<td>63</td>
<td>7.9</td>
</tr>
<tr>
<td>72</td>
<td>78</td>
<td>7.4</td>
</tr>
<tr>
<td>96</td>
<td>88</td>
<td>7.5</td>
</tr>
<tr>
<td>120</td>
<td>74</td>
<td>8.1</td>
</tr>
</tbody>
</table>

The precursor used was phenylacetic acid, 0.08 g being added, as the potassium salt, to each flask at 40, 72, and 96 hr. All figures represent the average of duplicate flasks.
the phenacetyl group, in stimulating the production of penicillin by high-yielding mutant strains of \textit{P. chrysogenum} is well known (Behrens et al., 1948; Singh and Johnson, 1948). The addition of a compound which will function efficiently as an R-group precursor often results in the excretion of a single penicillin, e.g., benzylpenicillin when phenylacetic acid is added, rather than the mixture of penicillins which result when no R-group precursor is added.

In order to test the effect of R-group precursor addition on the utilization of sulfate and the excretion of sulfur compounds, two strains were selected, the high-yielding mutant strain 51-20F3 and its low-yielding ancestor, strain NRRL1951-B25. Flask fermentations were carried out with these strains, with and without phenylacetate addition. As may be seen from table 2, the addition of precursor had no effect on the amount of growth with either strain. With strain 51-20F3, however, the pH was higher during the later stages of the fermentation when precursor was added, probably because of utilization of phenylacetate ion. The sulfur distribution in these fermentations is shown in figures 2 and 3. As may be seen from figure 2, the precursor had no effect on the sulfate utilization with either strain. As was expected, more sulfate was used by the high-yielding strain. Figure 3 shows that the amount of organic sulfur secreted by the mold was also not affected by the addition of precursor. With strain NRRL1951-B25, the amount of penicillin sulfur excreted was also unaffected by precursor. With strain 51-20F3, however, the effect on penicillin sulfur is striking. Penicillin sulfur, in the absence of precursor, was less than 20 per cent of total excreted sulfur. In the presence of precursor, penicillin sulfur was from 60 to 90 per cent of the total excreted sulfur, the total excreted sulfur remaining unchanged. Thus, with the high-yielding strain the addition of precursor seems to cause a shift of sulfur from other excretory products to penicillin. In the low-yielding strain, no such effect was apparent.

\textbf{Distribution of sulfur in the broth and mycelium during the penicillin-producing phase.} The distribution of sulfur in the broth and mycelium of the low-yielding strain NRRL1951-B25 and the high-yielding strain 51-20F3 at 72 hr is shown in table 3. The differences between these two strains in the utilization of sulfate and excretion of sulfur compounds have already been discussed. However, from table 3 it can readily be seen

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2}
\caption{Sulfate uptake by two strains of \textit{Penicillium chrysogenum}. Analytical figures from the fermentations of table 2.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3}
\caption{Excretion of organic sulfur by two strains of \textit{Penicillium chrysogenum}. Analytical figures from the fermentations of table 2.}
\end{figure}
that the amount of sulfate in the mycelium of the high-yielding mutant strain is at least twice as great as in the case of the low-yielding strain. These results suggest that the high-yielding strain has an enhanced capacity to concentrate inorganic sulfate in its mycelium. Furthermore, the concentration of sulfate in the mycelium (on the basis of 5 per cent nitrogen in the dried mycelium and 80 per cent water in the wet mycelium) is higher than that in the broth. These results suggest that sulfate in the mycelium might exist as a complex rather than as free inorganic sulfate. The addition of phenylacetic acid as R-group precursor does not seem to affect the concentration of sulfate in the mycelium to any great extent.

The addition of R-group precursor seems to cause a reduction in the concentration of organic sulfur in the mycelium of the high-yielding mutant strain but does not seem to affect the concentration of organic sulfur in the case of the low-yielding strain. The quantity of bound sulfur (sulfur not extracted by 67 per cent aqueous ethanol at −15 C) seems to be substantially the same in all cases.

DISCUSSION

The results presented here suggest that, in the selection of higher penicillin-yielding mutants of *P. chrysogenum*, an increase in the ability of the mold to synthesize penicillin has resulted from an enhancement of its sulfur metabolism. The greater part (80 to 100 per cent) of the extra sulfate utilized by the high-yielding strains is subsequently excreted by the mold. Included among these excretory products are the penicillins. An important effect of mutation seems to involve the capacity of the mold to concentrate sulfate within the mycelium, since the concentration of sulfate inside the mycelium of the high-yielding strain 51-20F3 is at least four to five times the concentration of sulfate in the surrounding medium during the penicillin-producing phase. In contrast, with the low-yielding strain NRRL1951-B25, the concentration of sulfate in the mycelium seems to be no more than twice that in the surrounding medium during the penicillin-producing phase. The effect of R-group precursor (phenylacetate) on the high-yielding strain, namely the secretion of penicillin at the expense of other organic sulfur compounds, suggests that the capacity of the mold to synthesize sulfur-containing potential penicillin precursors is greater than its capacity to synthesize compounds that will function as R-group precursors. In the absence of such precursors, most of the organic sulfur is secreted as compounds other than penicillin, but in the presence of precursor, penicillin is the chief sulfur compound secreted.

ACKNOWLEDGMENT

We are indebted to Mrs. Margaret Larson for invaluable technical assistance.
SUMMARY

Quantitative studies were made of the utilization of sulfate by various strains of *Penicillium chrysogenum* grown on a chemically defined medium in which inorganic sulfate was the sole source of sulfur.

Differences in the utilization of sulfate and the excretion of sulfur compounds were observed between the high penicillin-yielding mutant strains of the “Wisconsin family” and a number of low penicillin-yielding strains isolated from natural sources. Included among the low-yielding strains was strain NRRL1951-B25 from which the mutants of the “Wisconsin family” were derived.

The differences observed may be summarized as follows:

1. The high-yielding mutants show enhanced (two to three times) utilization of inorganic sulfate.
2. The greater part of the increased sulfate utilized by the high-yielding strains appears among the sulfur-containing excretory products. Thus, the excretion of sulfur is much greater with the high-yielding strains than with the low-yielding strains. With the low-yielding strains, the penicillins account for less than 20 per cent of the excreted sulfur, even when precursor is added. With the high-yielding strains, however, precursor addition increases the proportion of penicillin in the excreted sulfur to 70 to 90 per cent.
3. The addition of phenylacetate causes no enhancement of the utilization of inorganic sulfate by the mold. The increased yield of penicillin obtained with the high-yielding strains is due rather to a change in the distribution of excreted sulfur. This change in the nature of the excreted sulfur, together with a reduction in the amount of organic sulfur which could be extracted from the mycelium with aqueous ethanol, seem to be the only effects of the addition of phenylacetate on the sulfur metabolism of the high-yielding mutant 51-20F3.
4. The addition of phenylacetate has little, if any, effect on the sulfur metabolism of the low-yielding strains.

REFERENCES


