Synthesis of Cephalosporin C from Sulfate by Mutants of Cephalosporium acremonium

H. F. NISS and C. H. NASH III
Antibiotic Manufacturing and Development Division, Eli Lilly and Company, Indianapolis, Indiana 46206

Received for publication 10 April 1973

The innate ability of Cephalosporium acremonium to use methionine preferentially over sulfate for synthesis of cephalosporin C can be influenced through mutation. Mutants of C. acremonium with altered capacity to utilize sulfate for synthesis of antibiotic were isolated and partially characterized with respect to the uptake of sulfate and the regulation of arylsulfatase.

Cephalosporin C, a sulfur-containing peptide antibiotic, is synthesized by Cephalosporium acremonium (14). Synthesis of this antibiotic occurs efficiently in the presence of methionine and less efficiently in the presence of sulfate (3, 5, 16). It has been reported that the sulfur for this antibiotic is derived almost exclusively from methionine in a complex medium (3).

Evidence is presented here to suggest that strict genetic regulation of sulfate assimilation may be responsible for the inferiority of sulfate in cephalosporin C synthesis. This report describes the isolation and partial characterization of specific mutants of C. acremonium with either enhanced or decreased potential to utilize sulfate for synthesis of cephalosporin C.

MATERIALS AND METHODS

Strains and media. A superior antibiotic-producing strain of C. acremonium, M8650-3 (6), was used as a parental strain for the sulfate-deregulated mutants. Cultures were maintained, and inocula were prepared as previously reported (3). In most experiments, cells were grown in a complex medium containing either sulfate or methionine as the primary sulfur source. The sulfate-medium was composed of soybean meal, 7.0%; cerelose, 0.5%; lard oil, 5.0%; calcium sulfate, 0.5%; and calcium carbonate, 0.2%. Methionine-medium contained 0.5% dl-methionine in place of calcium sulfate, and the content of calcium carbonate in this medium was adjusted to 0.6%. Cells also were grown in a synthetic medium (6) and were used to study the uptake of sulfates. Cultures were grown either in 500-ml Erlemeyer flasks containing 60 ml of medium or in 30-liter laboratory fermenters containing 15 liters of medium. Fermentations were established from 10% inocula. Flasks were incubated at 25 C on a rotary shaker at 250 rpm. Stirred vessels were agitated at 450 rpm and aerated at 1 volume of air per volume of medium at 25 C.

Isolation of mutants. Mutant M8650-sp-1 was isolated after ultraviolet mutagenesis for its ability to utilize sulfate as a sulfate source for antibiotic synthesis. Conidia (10⁸) were irradiated with ultraviolet light to elicit a 98% kill. Posttreatment survivors were spread onto sulfate-containing medium and incubated at 25 C for 7 days. Isolates were subsequently screened for antibiotic synthesis on sulfate. A second mutant, M8650-chr, was isolated on selective medium containing 500 μg of potassium chrome per ml, a toxicant known to impair sulfate uptake (10). The frequency for chromate resistance was about 3 × 10⁻⁶.

Assay for antibiotic. Penicillin N, present in culture filtrates of C. acremonium, was inactivated by treatment with penicillinase (Riker type A, 50 μg/ml for 1 h at 25 C). The titer of cephalosporin C then was determined chemically by the hydroxamate assay (2). Antibiotic titers are expressed in arbitrary units.

Assay for arylsulfatase. The level of arylsulfatase activity in cells was determined according to the procedure of Dennen and Carver (6).

Uptake of ³⁵S-sulfate. Mycelia harvested from synthetic medium were washed twice with phosphate buffer (0.05 M, pH 6.5) and then were suspended in phosphate buffer to a cell density of 1 mg (dry weight) per ml. Pellets were disrupted with a Teflon tissue homogenizer (TRI-R Instruments, Jamaica, N.Y.) to obtain a uniform cell suspension. The uptake assay was initiated by adding Na₂³⁵SO₄ at a final concentration of 1 μCi/ml to cell suspensions (6 ml). Suspensions were incubated at 25 C in a gyratory water bath shaker (New Brunswick model G76). Samples (1 ml) were removed, filtered onto membrane filters (Millipore Corp.), and washed successively with three portions of cold phosphate buffer containing 5 μg of sodium sulfate per ml. Filters were placed in vials containing 15 ml of Liquifluor scintillation fluid (New England Nuclear Corp., Chicago, Ill.). Radioactivity was determined in a Packard Tricarb Scintillation Counter (60% counting efficiency). Controls were prepared by treating mycelia with labeled sulfate and by washing them immediately with cold sulfate-containing buffer. Protein was determined by the method of Lowry et al. (9).
RESULTS

Mutants of *C. acremonium* altered for sulfate utilization have been isolated, and these strains have been evaluated for competence to synthesize cephalosporin C from sulfate and from methionine. The parental strain, M8650-3, synthesized 15 to 40% more antibiotic in methionine-medium than in sulfate-medium. In contrast, mutant M8650-sp-1 synthesized comparable amounts of antibiotic from either methionine or sulfate (Table 1). Equivalency for cephalosporin C synthesis by this mutant when grown in either of two media is even more apparent when the kinetics of antibiotic synthesis are compared (Fig. 1). The rate of cephalosporin C formation by the mutant grown in either methionine or sulfate-medium was approximately that of the parental control in methionine-medium. These data indicate that the mutation resident in M8650-sp-1 enables sulfate to be as effective as methionine in providing sulfur for cephalosporin C.

Mutant M8650-sp-1 was atypical when compared with other strains of the M8650 series (6). Strains previously assigned to this series exhibit increased potential for synthesis of antibiotic in methionine-medium as well as in sulfate-medium (Table 2). Mutant M8650-sp-1 was superior to its parental culture only in sulfate-medium (Table 1).

The nature of the mutation present in M8650-sp-1 was partially characterized. The mutant exhibits increased permeability for sulfate (Fig. 2). The rate of sulfate uptake by M8650-sp-1 exceeded that of its parent by about 50%. Mutant M8650-sp-1 thus is altered for sulfur metabolism at the level of sulfate permeation.

Strains of the M8650 series possess increased arylsulfatase activity in the presence of methionine (6). Mutant M8650-sp-1 and its parent were evaluated for arylsulfatase activity in the presence of graded concentrations of methionine and sulfate (Fig. 3). Arylsulfatase activity in the parental strain rose to 22 units at a high concentration of methionine and remained negligible in the presence of increased sulfate. Enzyme activity in the mutant was not significantly increased in the presence of methionine. Only 5% of the maximal enzyme activity observed with the parental strain was exhibited by the mutant, even at high concentrations of

Table 1. Synthesis of cephalosporin C by *Cephalosporium acremonium* from methionine of sulfate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fermenter</th>
<th>Time (h)</th>
<th>Antibiotic titer</th>
<th>Methionine medium</th>
<th>Sulfate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>M8650-3</td>
<td>Flask</td>
<td>116</td>
<td>537</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vessel</td>
<td>95</td>
<td>662</td>
<td>513</td>
<td></td>
</tr>
<tr>
<td>M8650-sp-1</td>
<td>Flask</td>
<td>116</td>
<td>524</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vessel</td>
<td>95</td>
<td>576</td>
<td>671</td>
<td></td>
</tr>
<tr>
<td>M8650-3</td>
<td>Flask</td>
<td>116</td>
<td>525</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 1. Kinetics of cephalosporin C synthesis from different sulfur sources by parental strain M8650-3 and mutant M8650-sp-1.

TABLE 2. Synthesis of cephalosporin C by the M8650 series of *Cephalosporium acremonium*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methionine medium</td>
</tr>
<tr>
<td>M8650-1</td>
<td>140</td>
</tr>
<tr>
<td>M8650-2</td>
<td>270</td>
</tr>
<tr>
<td>M8650-3</td>
<td>537</td>
</tr>
</tbody>
</table>
methionine. Thus, another consequence of mutation present in M8650-sp-1 is insensitivity of this strain to derepression of arylsulfatase by methionine.

Among strains resistant to chromate, mutant M8650-chr was isolated. A mutation introduced into this strain did not alter its competence to synthesize antibiotic from methionine. The mutant was, however, severely impaired for synthesis of antibiotic from sulfate. This mutant formed less than 10% of the amount of antibiotic synthesized by its parent in sulfate-medium (Table 1). The mutant was partially blocked for uptake of sulfate (Fig. 2). After 90 s of incubation, mutant 8650-chr transported only 20% of the sulfate transported by its parent, strain M8650.3.

Neither M8650-sp-1 nor M8650-chr is auxotrophic for reduced sulfur, because both strains grow normally in minimal medium containing sulfate.

**DISCUSSION**

Specific mutants of *C. acremonium* were obtained which were altered for sulfur metabolism and, as expected, were modified for their potential to synthesize cephalosporin C from sulfate.

Cysteine is recognized as a component of the antibiotics and is a pivotal intermediate for sulfur metabolism in fungi (3, 19, 20). The sulfur of cysteine can be derived either from sulfate, by the sulfate reduction pathway or from methionine, by reverse transsulfuration (4, 7, 18). Methionine has been recognized to be a more expedient source of sulfur for synthesis of cephalosporin C than sulfate (3, 5, 16). This preference for methionine is subject to mutation as has been demonstrated by the fermentation characteristic of M8650-sp-1. This mutant, relative to its parent, is able to take up more sulfate, forms cephalosporin C with equal competence from methionine or sulfate, and is insensitive to derepression of arylsulfatase by methionine.

Marzluf (10) has reported that in *Neurospora* sulfate transport is an energy-dependent, enzymatic process and that methionine can strongly impede transport of sulfate. Inhibition of sulfate transport by methionine has also been reported in *P. chrysogenum* (17, 22), and mutants of *Penicillium* have been obtained that appear to be deregulated for assimilation of sulfate (17, 18). Auxotrophic mutants of the...
cys-3 locus in Neurospora are recognized to be pleiotropic and to exhibit multiple effects from single mutation (11). The cys-3 locus of Neurospora exerts coordinate controls over the synthesis of sulfate permease as well as arylsulfatase. Mutant M8650-sp-1 of Cephalosporium may be analogous to cys-3 mutants of Neurospora. The Cephalosporium mutant apparently is facilitated for sulfate transport and repressed for arylsulfatase.

The M8650 series of Cephalosporium represent a succession of mutants producing progressively more cephalosporin C from methionine as the sole sulfur source. This series of mutants is increasingly derepressed for arylsulfatase and concomitantly exhibits increased potential for synthesis of antibiotic from methionine (6). The derepression of the enzyme presumably relates to the loss of the intermediate cysteine into excreted antibiotic and subsequent depletion of a sulfur compound regulating arylsulfatase. Mutations that drain cysteine from metabolism to accommodate increased antibiotic synthesis would also reduce the availability of sulfide, because sulfide can arise from catabolism of cysteine (13, 20). Sulfide is believed to be a corepressor of arylsulfatase in fungi (12). Depletion of endogenous sulfide would derepress arylsulfatase. Accumulation of sulfide in mutant M8650-sp-1 could account for the observed repression of arylsulfatase. Derepression of cysteine for antibiotic synthesis could derepress sulfate permease. Sulfide and cysteine exhibit key regulatory roles for sulfur metabolism in P. chrysogenum (1, 19) and in other fungi (12, 15, 21).

Potential for sulfate utilization by a chromate-resistant mutant of C. acremonium, although apparently restricted, did not impede growth. The restricted uptake of sulfate, however, reduced the efficiency of sulfate as a source of sulfur for antibiotic synthesis. The mutant appears to be analogous to chromate-resistant strains of Neurospora which grow normally in minimal medium but which are partially impaired for transport of sulfate (10).

An interesting auxotrophic mutant of C. acremonium has recently been described (15). The mutant is unable to form sulfide via the reduction of sulfate and is more efficient for uptake of methionine. The strain can synthesize significantly more cephalosporin C from methionine than can its sulfide-proficient parent. This auxotrophic mutation and the mutations discussed in the present paper indicate that specific regulatory controls in C. acremonium govern the flow of sulfur in primary metabolism.

In this paper we have demonstrated that the control of inorganic sulfur assimilation is subject to mutation and influences profoundly the biosynthesis of cephalosporin C.

ACKNOWLEDGMENTS

We are grateful to Richard Pieper for his excellent technical assistance and to our colleagues in the Antibiotic Manufacturing and Development Division for many fruitful discussions concerning this work. We are especially indebted to Paul A. Lemke for his help in preparing this manuscript and to D. Dennen for his support.

LITERATURE CITED


