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Elucidation of conditions allowing conversion of penicillin G and other penicillins to deacetoxycephalosporins by resting cells and extracts of *Streptomyces clavuligerus* NP1

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ABSTRACT **Using resting cells and extracts of *Streptomyces clavuligerus* NP1, we have been able to convert penicillin G (benzylpenicillin) to deacetoxycephalosporin G. Conversion was achieved by increasing by 45× the concentration of $FeSO_4$ (1.8 mM) and doubling the concentration of α-ketoglutarate (1.28 mM) as compared with standard conditions used for the normal cell-free conversion of penicillin N to deacetoxycephalosporin C. ATP, $MgSO_4$, KCl, and DTT, important in cell-free expansion of penicillin N, did not play a significant role in the ring expansion of penicillin G by resting cells or cell-free extracts. When these conditions were used with 14 other penicillins, ring expansion was achieved in all cases.**

Biosynthesis of cephalosporins in *Cephalosporium acremonium* and *Streptomyces clavuligerus* proceeds through a biosynthetic pathway that includes expansion of the five-membered thiazolidine ring of the intermediate penicillin N into the six-membered dihydrothiazine ring of deacetoxycephalosporin C (DAOC) (for reviews, see refs. 1 and 2). This step is catalyzed by the α-ketoglutarate-dependent dioxygenase, DAOC synthase ("expandase") (3). In the fungus *C. acremonium,* the activity of expandase resides in a bifunctional enzyme (4) that catalyzes not only ring expansion but also the hydroxylation of the methyl group of DAOC to deacetylcephalosporin C whereas in the bacterium *S. clavuligerus* the two activities are associated with separate proteins (5, 6).

We and others (3–10) have found that the enzyme, in cell-free extracts as well as after purification, has a very narrow substrate specificity and no detectable activity on readily available and inexpensive penicillins (such as penicillin G and V produced by *Penicillium chrysogenum*). Indeed, chemical ring expansion plus an enzymatic removal of the phenylacetyl side chain is used in industry to convert penicillin G into 7-aminodeacetoxycephalosporanic acid, an important intermediate for the manufacture of semisynthetic cephalosporins. However, this process requires several steps and is expensive and polluting. A simple biological route might replace the chemical process, requiring only two steps, i.e., ring expansion and enzymatic deacylation, thereby reducing costs and environmental problems. We now have identified the conditions that allow conversion of penicillin G to deacetoxycephalosporin G (DAOG; phenylacetyl-7-aminodeacetoxycephalosporanic acid). In this report, we describe the conversion of a number of penicillins, including penicillin G, into cephalosporins by using resting cells and cell-free extracts of the cephalosporin-deficient mutant *S. clavuligerus* NP1 (11).

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MATERIALS AND METHODS

Microorganism. *S. clavuligerus* NP1, a mutant producing only trace levels of cephalosporins (11), was used in this work. The absence of significant levels of cephalosporins in this strain facilitated detection of cephalosporins produced from added penicillins by ring expansion.

Media and Culture Conditions. Mycelia were obtained by using 250-ml baffled flasks containing 40 ml of MST medium based on Jensen *et al.* (12): 1% soluble starch (Sigma), 3% trypticase soy broth without dextrose (BBL), and 90 mM Mops buffer (pH adjusted to 7.0 before autoclaving). Each flask was inoculated with 40 μl of a spore suspension (prepared and stored at −80°C in 20% glycerol) and was incubated at 30°C, 250 rpm for 48 h.

Preparation of Cell-Free Extracts. Fermentation broths were centrifuged at 8,000 × *g* and 4°C for 10 min. Pellets were washed twice by using 50 mM Tris·HCl supplemented with 0.1 mM DTT. The cells were resuspended in the same buffer and were disrupted by four 25-sec sonication treatments, power setting 5 and duty cycle 50%, in an ice-water bath by using a Branson 350 sonifier. Cell debris was removed by centrifugation (14,000 × *g*, 30 min, 4°C). The resulting extracts containing 8–10 mg protein/ml were placed on ice and were used immediately. Protein concentrations were measured by using the Bio-Rad protein assay. BSA was used as standard.

Resting Cells. From a seed culture (in MST), 0.5 ml was transferred to new flasks containing 40 ml of the same medium. Cells were grown at 30°C, 250 rpm for 24 h. Mycelia from each flask were washed twice and, finally, were resuspended in 10 ml of distilled water. Four milliliters of this cell suspension were used in the reaction mixture.

Ring Expansion Reaction. The standard reaction mixture for expandase action was that described by Maeda *et al.* (10) except that penicillin G was used as substrate instead of penicillin N. Additions were made following the order established by Shen *et al.* (13). Reaction mixtures were incubated in test tubes (cell-free extract) or 250-ml baffled flasks (resting cells) at 220 rpm, 30°C. Reactions containing the protein extract were stopped at various times (see tables and figures) by mixing 0.5 ml of reaction mixture with 0.5 ml of methanol. In the case of resting cells, samples were centrifuged to remove cells, and supernatants were transferred to new tubes. Expandase activity was detected by paper disc-agar diffusion bioassay. Paper discs were saturated with 200 μl of the biosynthesis

Abbreviations: DAOG, deacetoxycephalosporin G; 6-APA, 6-aminopenicillanic acid.

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Table 1. Effect of cofactors on the ring expansion of penicillin G when using resting cells

Cofactor omitted	μg DAOG/ml
None	10.5
DTT (16 mM)	15.5
α-Ketoglutarate (1.28 mM)	3.7
$FeSO_4 \cdot 7H_2O$ (1.8 mM)	3.2
$MgSO_4 \cdot 7H_2O$ (8 mM)	10.1
KCl (8 mM)	11.5
Ascorbic acid (4 mM)	3.0
ATP (0.7 mM)	10.0

All reactions contained 50 mM Tris·HCl (pH 7.4) and 2 mg/ml of penicillin G. Dry cell weight was 13 mg/ml. A 1-ml sample from each reaction was taken after 2 h and was centrifuged (14,000 × *g*, 5 min). The supernatants were transferred to new tubes, and 200 μl was used in the bioassay.

reaction mixture (cell-free extracts) or supernatant (resting cells) as follows. Two discs were superimposed, and four 50-μl samples were applied. After each application, the discs were allowed to dry for 30 min under a laminar hood, and, finally, they were placed on Luria–Bertani medium (1% tryptone/0.5% NaCl/0.5% yeast extract/0.1% glucose) and 0.8% agar medium seeded with *Escherichia coli* Ess (a β-lactam supersensitive mutant; ref. 14), and the plates were incubated overnight at 37°C. The formation of DAOG was determined by adding 50,000 units per milliliter of penicillinase (Difco Bacto penase concentrate) to the assay plates. This is a narrow spectrum β-lactamase that attacks all penicillins but not cephalosporins. The diameters of zones of growth inhibition were measured and quantified with a calibration curve by using pure DAOG as standard.

Test Substrates. Preparation of all penicillins used in this work, except penicillin G and ampicillin (Sigma), were synthesized as described (10).

RESULTS

Cofactors Required for Expandase Reaction When Using Resting Cells and Penicillin G as Substrate. Although we previously had failed to expand the ring of penicillin G with cell-free extracts of *S. clavuligerus* (10), we were successful in the present work with resting cells (Table 1). Jensen *et al.* (5) had reported that ring expansion of penicillin N by using cell-free extracts of *S. clavuligerus* showed an absolute requirement for α-ketoglutarate whereas ATP and magnesium or potassium cations had lesser effects. Table 1 shows the cofactor requirement when ring expansion of penicillin G was carried out by using resting cells of *S. clavuligerus* NP1. When $FeSO_4$, α-ketoglutarate, or ascorbic acid was not present, the amount of product was ≈30% of that in the control. On the other hand, ATP, $MgSO_4$, KCl, or DTT did not play a significant role in the reaction with resting cells. The omission of DTT actually increased production by 50%. Similar results were obtained with cell-free extracts, although we usually retained $MgSO_4$ and KCl in the reaction mixture. In the cell-free system, DTT did not decrease production and was included to protect the enzyme.

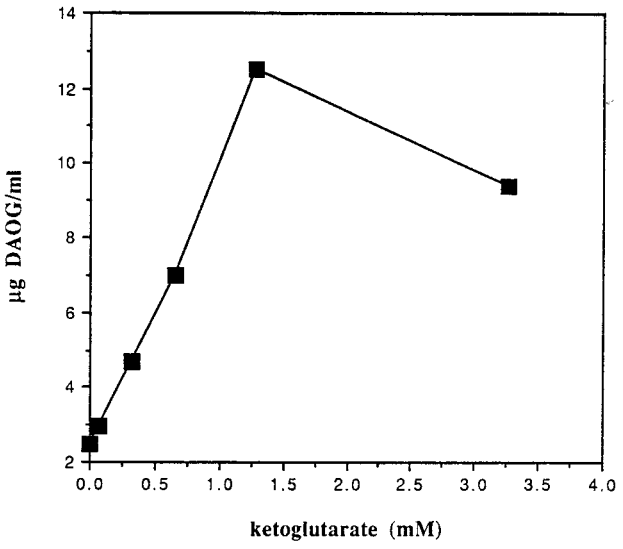

FIG. 1. Effect of α-ketoglutarate concentration on ring expansion of penicillin G by resting cells. The concentration of α-ketoglutarate previously used for unsuccessful cell-free ring expansion of penicillin G was 0.64 mM (10). Reaction contained 50 mM Tris·HCl (pH 7.4), 1.8 mM $FeSO_4$, 4 mM ascorbic acid, and 2 mg/ml penicillin G. Dry cell weight was 12 mg/ml. Samples were taken at 2 h and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

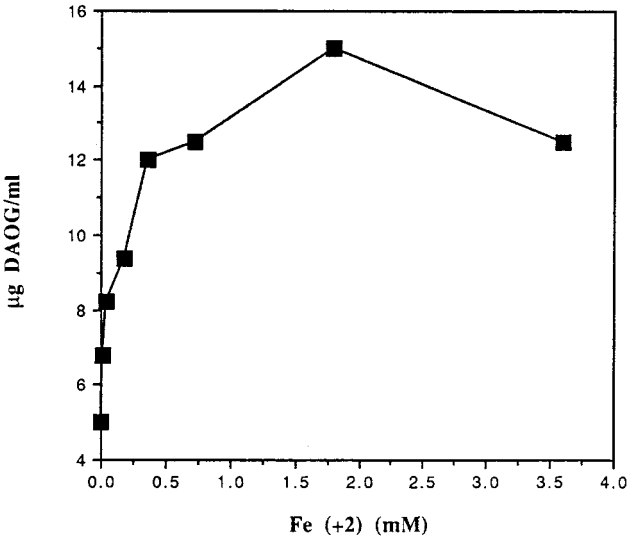

FIG. 2. Effect of $FeSO_4$ concentration on ring expansion of penicillin G by resting cells. The concentration of $FeSO_4$ previously used for unsuccessful cell-free ring expansion of penicillin G was 0.04 (10). Reaction contained 50 mM Tris·HCl (pH 7.4), 1.28 mM α-ketoglutarate, 4 mM ascorbic acid, and 2 mg/ml penicillin G. Dry cell weight was 10 mg/ml. Samples were taken at 2 h and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

Effect of α-Ketoglutarate Concentration When Using Resting Cells. α-Ketoglutarate is known to be necessary as a cosubstrate for enzymatic ring expansion of penicillin N (5, 7,

Table 2. Effect of ascorbate and lack of effect of ATP on the ring expansion of penicillin G when using resting cells

	Concentration, mM	μg DAOG/ml
Ascorbate	0	2.4
	0.8	4.0
	2	6.0
	4	6.4
	8	6.4
ATP	0	6.6
	0.14	6.4
	0.35	6.4
	0.7	6.6
	1.4	6.6
	3.5	4.7

The reactions contained 50 mM Tris·HCl (pH 7.4), 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, and 2 mg/ml penicillin G. Dry cell weight was 9.8 mg/ml. The concentrations previously used for unsuccessful cell-free ring expansion of penicillin G were 4 mM for ascorbate and 0.7 mM for ATP (10). Samples were taken after 2 h and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

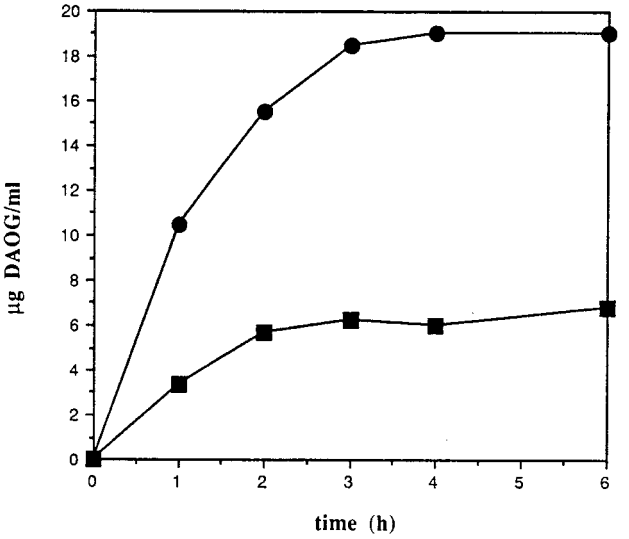

FIG. 3. Time course of the ring expansion of penicillin G by resting cells. The reaction contained 50 mM Tris·HCl (pH 7.4), 4 mM ascorbic acid, and 2 mg/ml penicillin G. Cell mass was 11.6 mg/ml in both cases. ■, old concentrations (0.04 mM $FeSO_4$, 0.64 mM α-ketoglutarate); ●, new concentrations (1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate). Samples were taken and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

15). Fig. 1 shows that a doubling of the activity of resting cells on penicillin G was observed when the concentration was raised from the standard concentration of 0.64 mM (10) to 1.28 mM.

Effect of $FeSO_4$ Concentration When Using Resting Cells. Fig. 2 shows that increasing concentrations of Fe^{2+} up to 1.8 mM increased ring expansion of penicillin G. The optimum concentration (1.8 mM) is 45-fold higher than that used for conversion of penicillin N by cell-free extracts (10).

Effect of Ascorbate and Lack of Effect of ATP When Using Resting Cells. Table 2 shows that the optimum ascorbate concentration for conversion of penicillin G by resting cells is 4–8 mM. Maeda *et al.* (10) used 5 mM ascorbate, and Lübbe *et al.* (16) used 4 mM ascorbate for cell-free conversion of

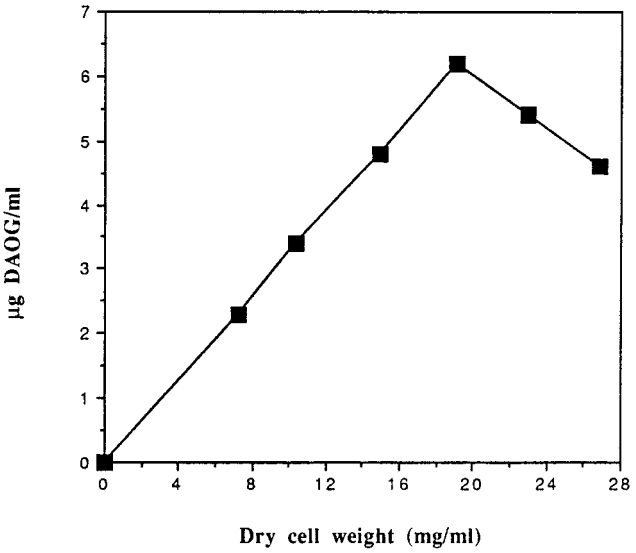

FIG. 4. Effect of cell mass on ring expansion of penicillin G. Reaction contained 50 mM Tris·HCl (pH 7.4), 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, 4 mM ascorbic acid, and 2 mg/ml penicillin G. Samples were taken at 2 h and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

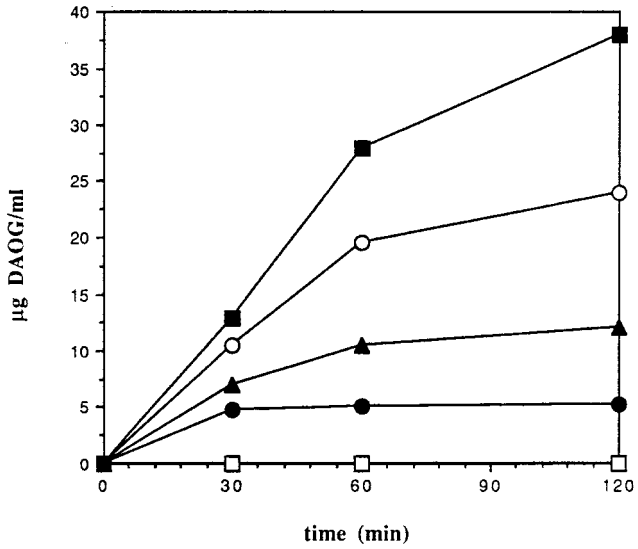

FIG. 5. Effect of protein concentration of cell-free extracts on ring expansion of penicillin G. The reaction contained 50 mM Tris·HCl (pH 7.4), 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, 8 mM KCl, 8 mM $MgSO_4$, 4 mM ascorbic acid, 14 mM DTT, and 2 mg/ml penicillin G. □, 0.2 mg/ml; ●, 1 mg/ml; ▲, 2 mg/ml; ○, 4 mg/ml; ■, 6 mg/ml. The concentration previously used for unsuccessful cell-free ring expansion of penicillin G was 1–2 mg/ml (10). Samples were taken and centrifuged (14,000 × *g*, 5 min); 200 μl of the supernatant was used in the bioassay.

penicillin N to DAOC. On the other hand, ATP did not improve the resting cell activity whereas cell-free extracts acting on penicillin N were stimulated by ATP (17).

Time Course of the Reaction When Using Resting Cells. Fig. 3 shows the time course of the penicillin G ring expansion reaction and the improvement in activity when carried out by using the increased concentration of $FeSO_4$ and α-ketoglutarate described above.

Effect of Cell Mass. As shown in Fig. 4, increasing the cell

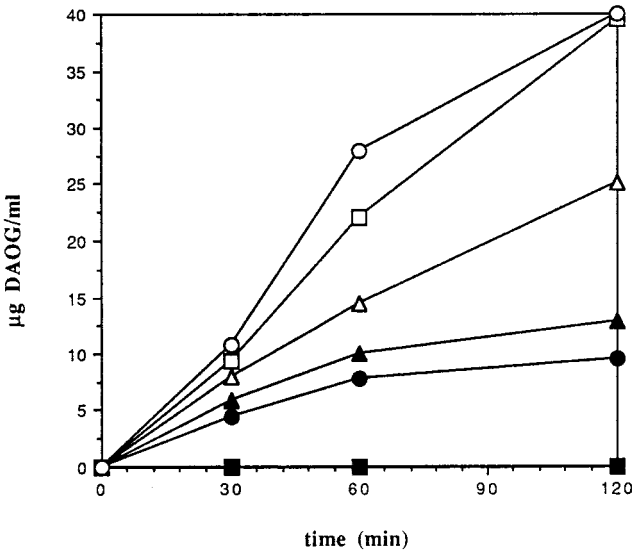

FIG. 6. Effect of penicillin G concentration on cell-free ring expansion of penicillin G. The reaction contained 50 mM Tris·HCl (pH 7.4), 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, 8 mM KCl, 8 mM $MgSO_4$, 4 mM ascorbic acid, and 14 mM DTT. ■, no penicillin G; ●, 0.5 mg/ml; ▲, 1 mg/ml; △, 2 mg/ml; □, 4 mg/ml; ○, 5 mg/ml. The concentration previously used for unsuccessful cell-free ring expansion of penicillin G was 0.35 mg/ml (10). Samples were taken and centrifuged (14,000 × *g*, 5 min); 200 μl of the supernatant was used in the bioassay.

Table 3. Effect of concentrations of cell-free protein, Fe^{2+}, α-ketoglutarate, and penicillin G on the ring expansion of penicillin G

Protein, mg/ml	$FeSO_4$, mM	α-Ketoglutarate, mM	Penicillin G, mg/ml	μg DAOG/ml
4	0.036	0.64	1	6.8
4	0.036	0.64	0.3	5.2
4	0.036	0.64	0.1	4.6
2	0.036	0.64	1	5.2
2	0.036	0.64	0.3	4.2
2	0.036	0.64	0.1	<4
1	0.036	0.64	1	4.2
1	0.036	0.64	0.3	<4
1	0.036	0.64	0.1	<4
4	1.8	1.28	1	11.2
4	1.8	1.28	0.3	6.8
4	1.8	1.28	0.1	5.6
2	1.8	1.28	1	6.8
2	1.8	1.28	0.3	4.6
2	1.8	1.28	0.1	4.2
1	1.8	1.28	1	4.2
1	1.8	1.28	0.3	<4
1	1.8	1.28	0.1	<4

The reactions also contained 50 mM Tris·HCl (pH 7.4), 4 mM ascorbic acid, 14 mM DTT, 8 mM $MgSO_4$, and 8 mM KCl. Samples were taken after 1 h and were centrifuged (14,000 × *g*, 5 min); 200 μl was used in the bioassay.

mass enhanced the formation of DAOG from penicillin G. The optimum concentration was 19 mg/ml. Higher cell concentrations inhibited the reaction probably because oxygen supply was limiting under such conditions.

Effect of Protein and Substrate Concentrations When Using Cell-Free Extracts. Using the high concentrations of $FeSO_4$ and α-ketoglutarate established for resting cell conversion of penicillin G to DAOG, we found activity with cell-free extracts. Furthermore, high protein (Fig. 5) and high substrate concentrations (Fig. 6) enhanced product formation. Using the conditions reported by Maeda *et al.* (10), in which protein concentration in the cell-free extracts was 1–2 mg/ml, we observed only a low level of activity. The penicillin G concentration previously used by Maeda *et al.* (10) was 0.01–0.356 mg/ml.

Table 4. Substrate specificity of expandase when using cell-free extracts

Substrate	Inhibition zone, mm
No substrate	0
Adipyl-6-APA	20.0
Ampicillin	17.0
Butyryl-6-APA	17.5
Decanoyl-6-APA	7.0
Heptanoyl-6-APA	16.5
Hexanoyl-6-APA	19.5
Nonanoyl-6-APA	10.5
Octanoyl-6-APA	16.0
Penicillin F	21.0
Penicillin G	29.0
Penicillin V	7.5
Penicillin mX	30.5
Penicillin X	30.5
2-Thiophenylacetyl-6-APA	32.0
Valeryl-6-APA	15.0

Reactions were carried by using 4 mg/ml enzyme protein, 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, 8 mM KCl, 8 mM $MgSO_4$, 4 mM ascorbic acid, and 14 mM DTT. 6-APA, 6-aminopenicillanic acid. All substrates were used at 2 mg/ml, except for penicillin mX and octanoyl-6-APA (3 mg/ml). Samples were taken at 2 h and were centrifuged (14,000 × *g*, 5 min). Bioassay was done by using 250 μl of reaction mixture.

Effect of Varying the Concentrations of Cell-Free Protein, Penicillin G, $FeSO_4$, and α-Ketoglutarate. Table 3 shows the results obtained when different concentrations of these components were tested with cell-free extracts. It is clear that the highest concentrations of each component gave the best activities. Concentrations of α-ketoglutarate or $FeSO_4$ higher than 1.28 mM or 1.8 mM, respectively, did not improve the ring expansion (see Figs. 1 and 2).

Effect of Expandase on Other Penicillins. We examined the substrate specificity of the expandase in cell-free extracts by using the new conditions established in this work. Table 4 lists the 15 penicillins tested. Conversion was observed in all cases. The larger bioassay zones of inhibition were produced from penicillin G, X, mX and 2-thiophenylacetyl-6-APA whereas the smallest zones were obtained with decanoyl-6-APA and penicillin V.

DISCUSSION

Before this study, penicillin G was found to be inactive as a substrate for expandase present in cell-free extracts and in purified preparations, both under the conditions optimal for ring expansion of penicillin N (5, 6) and under various conditions tested by Maeda *et al.* (10). Maeda *et al.* (10) examined temperature, duration of reaction, buffers, and concentration of penicillin G without success. They were only able to observe ring expansion when penicillin N or D-carboxymethylcysteinyl-6-APA were used as substrate. Our success with resting cells acting on penicillin G allowed us to improve the ring expansion of penicillin G. By increasing the concentration of the substrate, cosubstrate (α-ketoglutarate), and one cofactor ($FeSO_4$) and by using high concentrations of cells, conversion of penicillin G to DAOG was improved markedly. Using such improved conditions, we were able to show activity on penicillin G and 14 other penicillins with cell-free extracts. Of interest, Baldwin *et al.* (18) found purified *C. acremonium* expandase to be inactive on penicillin G but pure expandase from recombinant *E. coli* containing the *S. clavuligerus* expandase gene to be active. However, the latter report is an abstract and no detailed publication has appeared yet.

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