0273-2289/99/81/0145/$12.00

Further Studies on the Bioconversion of Penicillin G into Deacetoxycephalosporin G by Resting Cells of *Streptomyces clavuligerus* NP-1

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Received April 27, 1999; Revised August 5, 1999; Accepted August 12, 1999

Abstract

Resting cells of *Streptomyces clavuligerus* NP-1, which possess deacetoxycephalosporin C synthase activity, have been shown previously to perform oxidative ring expansion of penicillin G in the presence of iron, ascorbic acid, and α-ketoglutaric acid to form deacetoxycephalosporin G. Further studies on this bioconversion indicated that use of MOPS or HEPES buffer at pH 6.5 more than doubled the extent of the reaction observed with the previously used Tris-HCl at pH 7.4. Levels of bioconversion as high as 16.5% were achieved at low penicillin G concentrations. Previously, conversion yields were <1%.

Index Entries: *Streptomyces clavuligerus*; β-lactam antibiotics; bioconversion; penicillin G; deacetoxycephalosporin G; resting cells.

Introduction

The production of deacetoxycephalosporanic acid (DAOC) from penicillin G is accomplished by a multistep chemical route with high cost and

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negative environmental impact. The resultant DAOC can be easily converted to 7-aminodeacetoxycephalosporanic acid (7-ADCA) by penicillin acylase. Since 7-ADCA is an important feedstock for production of semisynthetic cephalosporins, it is important to find a biological route to generate this commercially important intermediate.

Studies on the enzymatic ring expansion of penicillin N into deacetoxycephalosporin C have been carried out *(1–6)*. The enzyme of the biosynthetic pathway that carries out this conversion is deacetoxycephalosporin C synthase ("expandase") *(1)*. Expandase catalyzes the oxidative expansion of the five-membered thiazolidine ring of penicillin N into the six-membered cephem deacetoxycephalosporin C *(1,7)*. Penicillin N is not a good candidate for industrial conversion into deacetoxycephalosporins owing to its D-α-aminoadipic acid side chain, which cannot be removed easily from the resultant cephalosporin by penicillin acylase. Of particular interest is the use of other penicillins as substrates for the expandase reaction, e.g., the inexpensive natural hydrophobic penicillins produced by *Penicillium chrysogenum.* Ring expansion of penicillin G, e.g., would yield a cephalosporin (i.e., deacetoxycephalosporin G [DAOG]) whose side chain would be easily removable by penicillin acylase to yield 7-ADCA. Some unsuccessful attempts were made to convert penicillin G with cell-free extracts of *Streptomyces clavuligerus (5,8)*, partially purified expandase from the same microorganism *(9)*, or partially purified expandase from *Cephalosporium acremonium (6,10)*. A breakthrough was reported by Cho et al. *(11)*, who established conditions for the bioconversion of penicillin G into DAOG using resting cells and cell-free extracts of *S. clavuligerus* NP-1. In the present work, we report on improvements in the bioconversion and show that higher yields than previously observed with resting cells of *S. clavuligerus* NP-1 are obtained with lower concentrations of the substrate penicillin G.

Materials and Methods

Materials

Penicillin G, ascorbic acid, and α-ketoglutaric acid were from Sigma (St. Louis, MO). DAOG was from Antibioticos, S.A. (Leon, Spain) and Bacto-Penase from Difco (Detroit, MI).

Culture Conditions

S. clavuligerus mutant NP-1, which produces only traces of cephalosporins *(12)*, was used for these studies. A seed culture was made by inoculating a spore suspension (40 µL) into 40 mL of MST medium (90 m*M* MOPS buffer, 1% starch, and 3% trypticase soy broth without dextrose [BBL, Cockeysville, MD], pH 7.0) in 250-mL baffled flasks and incubating for 2 d at 30°C at 250 rpm. One milliliter of the seed culture was used as inoculum for the main culture, which contained 80 mL of MST medium in 500-mL unbaffled flasks. The flasks were incubated for 24 h at 30°C at 250 rpm.

Preparation of Resting Cells

Cells were harvested by centrifugation at 14,000*g* for 15 min at 4°C and washed twice with cold, deionized water. They were resuspended in 10 mL of water.

Ring Expansion Reaction

The standard reaction mixture (10 mL) contained 0.05 *M* Tris-HCl, pH 7.4; 8.0 m*M* KCl; 8.0 m*M* $MgSO_4 \cdot 7H_2O$; 4.0 m*M* ascorbic acid; 1.8 m*M* $FeSO_4 \cdot 7H_2O$; 1.28 m*M* α-ketoglutaric acid; 20 mg/mL of penicillin G; and 4.0 mL of cell suspension *(11)*. The order of addition of the components was as previously described *(13)*. The reaction started when penicillin G was added to the reaction mixture, which was incubated at 30°C at 220 rpm.

Bioassay

Product formation was estimated by the paper disk–agar diffusion bioassay as previously described *(11)*, using DAOG as standard. *Escherichia coli* strain Ess, a β-lactam supersensitive mutant, was used as assay microorganism.

Results

Effect of Different Buffers in Reaction Media

In previous studies *(11)*, the buffer used for bioconversion was 0.05 *M* Tris-HCl at pH 7.4. Because of the pH lability of the substrate, we have not ventured far from neutrality. However, we found (Fig. 1) that 0.05 *M* MOPS buffer at pH 6.5 or 0.05 *M* HEPES buffer at pH 6.5 supported more than a doubling of activity when compared to Tris-HCl at pH 7.4. The remaining experiments were done using 0.05 *M* MOPS, pH 6.5, as the buffer.

Effect of Preincubation of Resting Cells

It can be seen in Fig. 1 that product formation was rapid for 1 h and then either stopped or proceeded for a few more hours at a low rate. We had observed this phenomenon previously *(14)* using cell-free extracts, and it was attributed to inactivation of expandase in the presence of Fe^{+2} plus either ascorbate or α-ketoglutarate during the reaction.

We carried out further studies on this phenomenon. To determine whether a component(s) present in the reaction mixture inhibited or inactivated the enzyme, resting cells were preincubated for 3 h in the presence of individual components or various combinations. After the preincubation, the rest of the components were added to start the reaction. When preincubation was done with buffer alone, product formation was slower and the concentration of product made was about 50% lower than in the control. Preincubation in the presence of Fe^{+2}, ascorbic acid, or α-ketoglu-

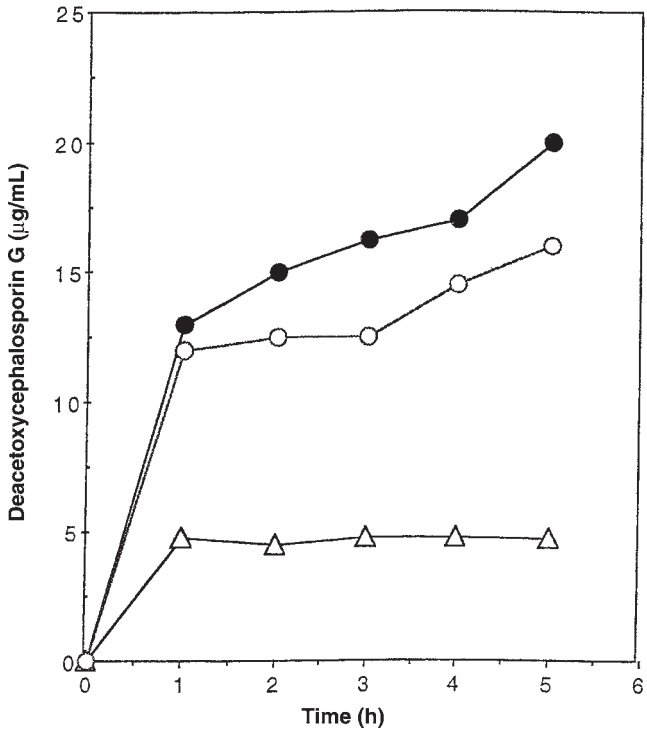

Fig. 1. Effect of different buffers on the ring expansion of penicillin G by resting cells. ●, 0.05 *M* MOPS, pH 6.5; ○, 0.05 *M* HEPES, pH 6.5; △, 0.05 *M* Tris-HCl, pH 7.4.

taric acid led to similar values. On the other hand, when resting cells were preincubated in the presence of Fe^{+2} plus ascorbic acid in the presence or absence of α-ketoglutaric acid, the activity was virtually eliminated (Fig. 2). These results are in agreement with our previous observations with cell-free extracts. Inactivation was not merely owing to the presence of oxidized Fe^{+2} because when the bioconversion reaction was carried out with Fe^{+3} (ferric sulfate) instead of Fe^{+2}, there was no inhibition (Fig. 2).

Effect of Substrate Concentration on Conversion Yield

Early studies done with cell-free extracts or resting cells revealed a very low yield: 0.71% *(11)*. To evaluate further the dependence of product formation and bioconversion yields on substrate concentration, different concentrations of penicillin G were tested. As Fig. 3 depicts, the concentration product formed was much higher when higher concentrations of penicillin G were used, but the yield, based on substrate added, was dramatically increased when lower substrate concentrations were used (Fig. 4). At 8 mg/mL of penicillin G, bioconversion was about 0.4%; at 2 mg/mL (the standard concentration), the yield was 1.0%; and at 0.063 mg/mL, the yield reached 9.0%. In a subsequent experiment in which substrate concentration was lowered to 0.015 mg/mL, the yield was 16.5% (data not shown).

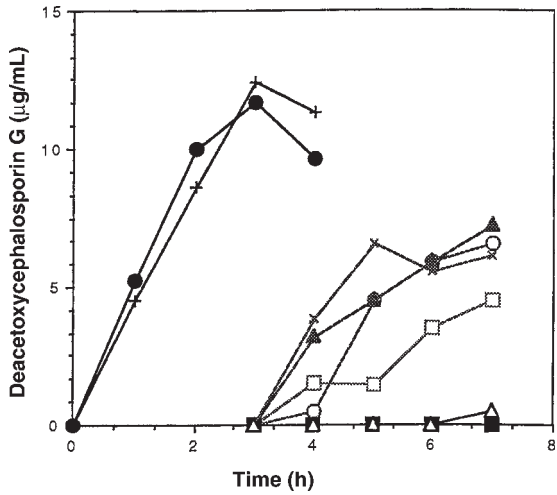

Fig. 2. Effect of preincubation of resting cells in the presence of different components of the expandase reaction mixture. ●, control, no preincubation; +, control with Fe^{+3} replacing Fe^{+2}. Preincubation for 3 h with ○, buffer; □, Fe^{+2}; ▲, ascorbic acid; **x**, α-ketoglutaric acid; ■, ascorbic acid + Fe^{+2}; △, ascorbic acid + Fe^{+2} + α-ketoglutaric acid.

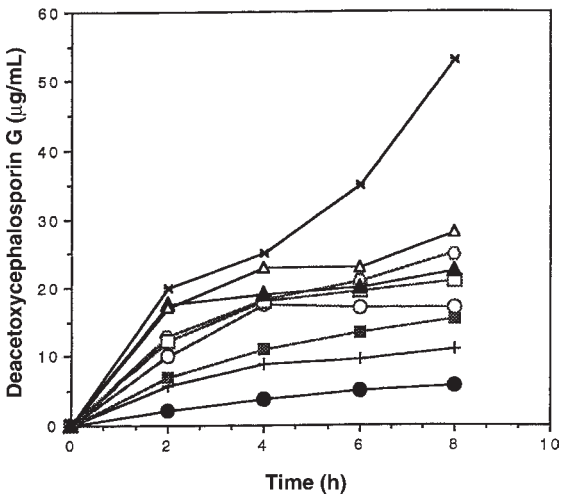

Fig. 3. Effect of substrate concentration on product formation. ●, 0.063 mg/mL; +, 0.125 mg/mL; ■, 0.25 mg/mL; ○, 0.5 mg/mL; ▲, 1 mg/mL; □, 2 mg/mL; ⬡, 4 mg/mL; △, 6 mg/mL; **x**, 8 mg/mL.

Combined Effect of Biomass and Substrate Concentrations

To determine whether increasing concentrations of biomass improve the bioconversion, three different cell concentrations were evaluated, ranging from 1.2 to 6.0 g (wet wt/10 mL of reaction mixture), in the presence of two different levels of penicillin G (0.063 and 2 mg/mL). At both concentrations of penicillin G, the percentage of bioconversion was markedly reduced by increasing biomass concentration (Fig. 5).

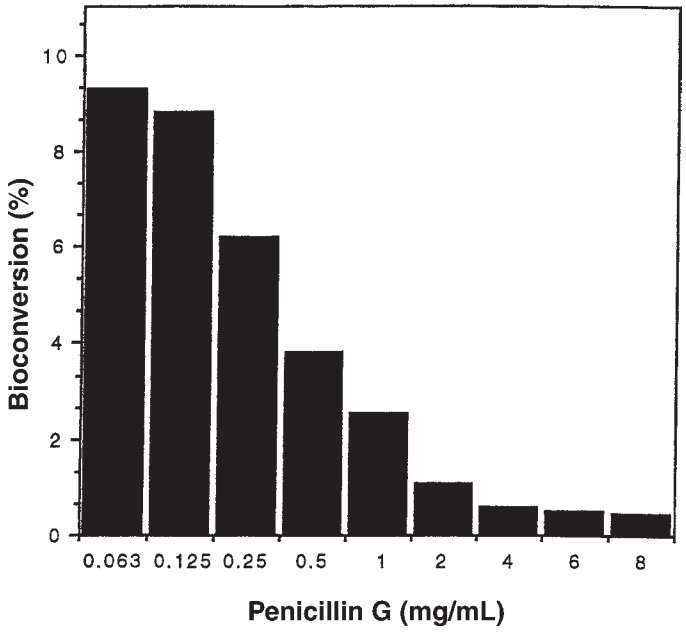

Fig. 4. Yields of bioconversion (based on concentration of substrate added) at different concentrations of penicillin G.

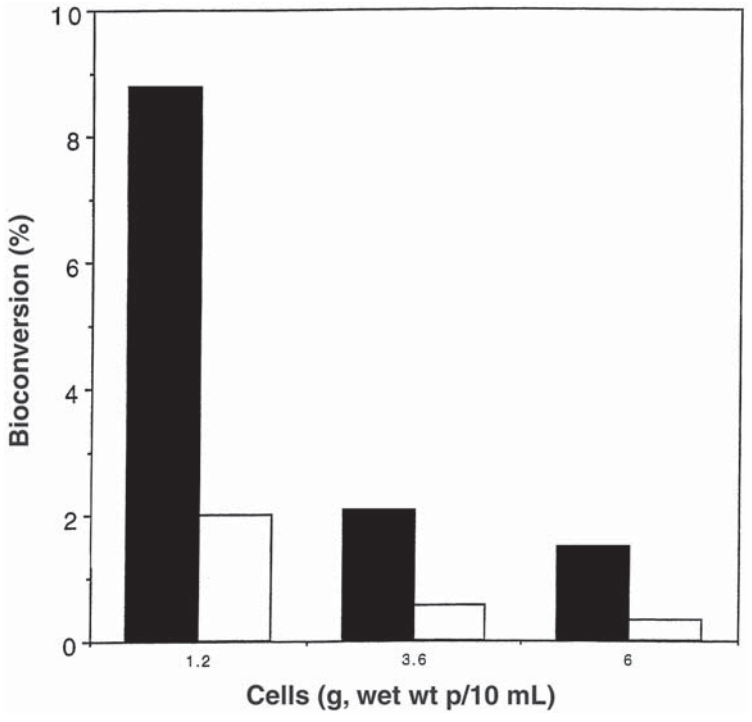

Fig. 5. Effect of different biomass levels on the yield of bioconversion at two levels of penicillin G: ■, 0.063 mg/mL; □, 2 mg/mL.

Discussion

Resting cells from the prokaryote *S. clavuligerus* have the capacity to utilize penicillin G as a substrate alternative to penicillin N to generate DAOG instead of deacetoxycephalosporin C. Oxidative ring expansion activity by resting cells works best in 0.05 *M* MOPS, pH 6.5, or HEPES, pH 6.5. The reaction was rapid only for 1 h and then proceeded at a very low rate. Preincubation of resting cells for 3 h in buffer alone or with individual reaction components decreased the subsequent reaction rate, whereas preincubation in the presence of combinations of Fe^{+2} and ascorbic acid with or without α-ketoglutaric acid virtually eliminated activity. We do not know the mechanism of inactivation, but similar results were reported by Adrio et al. *(14)* using cell-free extracts. The data with cell-free extracts indicated that inactivation was not due to production of hydrogen peroxide or superoxide radical.

Prior to our studies, the reaction yielded <1% conversion of added penicillin G. We observed conversion yields as high as 16.5% by reducing the concentration of penicillin G. On the other hand, increasing cell density decreased the bioconversion yield even at a low level of penicillin G (0.0625 mg/mL). This effect might be owing to diffusional limitations of oxygen because of the higher viscosity generated by the increased cell mass, oxygen being a substrate of the expandase reaction. This will be examined in future experiments.

The present work has furthered our knowledge concerning the use of resting cells of *S. clavuligerus* for converting inexpensive available substrates in the production of valuable β-lactam compounds. The bioconversion of penicillin G by resting cells provides a powerful and promising alternative to the chemical process for the industrial production of 7-ADCA. However, the yield must be increased.

Acknowledgments

We thank Antibióticos for financial assistance and DAOG and Ermanno Bernasconi and Francisco Salto for encouragement. We also acknowledge the Organization of American States Scholarship Department, Washington, DC, and the Instituto Tecnológico y de Estudios Superiores de Monterrey-Campus Monterrey, México, for financial support to M. A. Báez-Vásquez during his postdoctoral work at M.I.T.

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