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Improvements in the Formation of Cephalosporins from Penicillin G and Other Penicillins by Bioconversion

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Abstract:

Chemical ring expansion of penicillin G plus enzymatic removal of the phenylacetyl side chain is currently being used in industry to obtain 7-aminodeacetoxycephalosporanic acid (7-ADCA) that is used for the manufacture of semisynthetic cephalosporins. A biological route requiring only two enzymatic steps (ring expansion and deacylation) might replace this chemical process, thereby reducing costs and environmental problems. Deacetoxycephalosporin C synthase, an iron-dependent and α-ketoglutarate-requiring dioxygenase, catalyses the expansion of the five-membered thiazolidine ring of the penicillin nucleus to form the six-membered dihydrothiazine ring of the cephalosporin nucleus. To improve the substrate specificity of this enzyme on hydrophobic penicillins (e.g., penicillin G, ampicillin, amoxicillin), different strategies have been recently developed. Biotransformation of penicillin G to deacetoxycephalosporin G by resting cells of *Streptomyces clavuligerus* has been improved by growing the cells in ethanol, eliminating agitation, adding water-immiscible solvents and catalase to the reaction, and using a hybrid strain obtained by directed evolution.

Introduction

Deacetoxycephalosporin C synthase (DAOCS, "expandase") is an iron- and α-ketoglutarate-dependent oxygenase that catalyzes the ring expansion of penicillin N to deacetoxycephalosporin C (DAOC) in all cephalosporin-producing microorganisms[1−4] (Figure 1). In bacteria, the subsequent hydroxylation of DAOC to deacetylcephalosporin C (DAC) is catalyzed by a closely related enzyme, deacetylcephalosporin C synthase (DACS), whereas in the fungus *Acremonium chrysogenum* (previously *Cephalosporium acremonium*), the activities of expandase and hydroxylase reside in a single functional protein.[2]

Ring expansion activity on penicillin N is known to be markedly increased by ascorbic acid, Fe^{2+},[5,6] α-ketoglutarate,[5,7] and oxygen.[7,8] The enzyme shows an absolute requirement for α-ketoglutarate,[5,6,9] and this cosubstrate cannot be replaced by other chemically related compounds such as α-ketoadipate, pyruvate, oxaloacetate, succinate, or α-ketobutyrate.[10,11] The properties of the expandase are very similar to those of other α-ketoglutarate-dependent dioxygenases which incorporate one atom of oxygen into α-ketoglutarate to form succinate and the other atom into the product molecule. However, expandase differs in that no oxygen atom appears in its ring expansion product, DAOC.

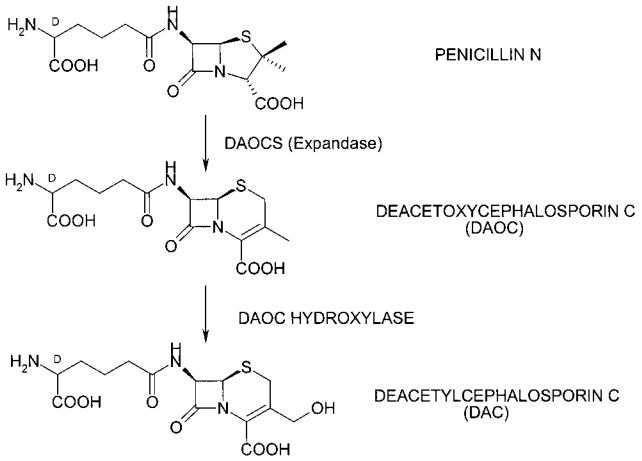

Figure 1. **Biosynthetic pathway from penicillin N to deacetoxycephalosporin C and deacetylcephalosporin C.**

Several research groups have reported on the narrow substrate specificity and lack of detectable activity of expandase on inexpensive and available penicillins such as penicillin V and G.[1,10,12,13] Chemical ring expansion of penicillin G plus enzymatic removal of the phenylacetyl side chain is currently being used in industry to obtain 7-aminodeacetoxycephalosporanic acid (7-ADCA) that is used for the manufacture of semisynthetic cephalosporins. However, this chemical process requires several steps and is expensive

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and polluting.[14] A biological route requiring only two enzymatic steps (ring expansion and deacylation) might replace the chemical process, thereby reducing costs and environmental problems.

Expandase Proteins and Genes. The expandase/hydroxylase of *A. chrysogenum* is a monomer with a molecular mass of 41 000 Da and an isoelectric point of 6.3.[2] Cloning of the *cefEF* gene encoding this bifunctional protein in *Escherichia coli* was achieved using probes based on amino acid sequences of fragments of the purified protein. The cluster in *A. chrysogenum* containing the *cefEF* and *cefG* genes is located on chromosome II.[15] The genes are oppositely oriented and expressed from divergent promoters located within a 938 bp intergenic region.[16] When isopenicillin N, penicillin G, penicillin V, ampicillin, and 6-aminopenicillanic acid (6-APA) were tested as substrate analogues, no ring expansion was observed.[2,10,17]

The *Streptomyces clavuligerus* expandase is a monomer of 34 600 Da with two isoelectric points of 6.1 and 5.3.[3] By using a 22-residue amino-terminal sequence of the purified protein, the *cefE* gene coding for expandase was cloned and expressed in *E. coli*.[18] Comparison of this gene and the *cefEF* gene of *A. chrysogenum* showed 67% similarity, whereas at the protein level there was 57% similarity. The bacterial protein is 21 amino acids shorter than the fungal enzyme. As was previously observed by several authors working with cell-free extracts,[9,13] purified expandase was unable to expand isopenicillin N, penicillin G, penicillin V, ampicillin, or 6-APA.[3,17]

Cloning and expression of the *Nocardia lactamdurans cefE* gene in *S. lividans* revealed an enzyme of 34 532 Da and an isoelectric point of 4.9.[19] The gene has high similarity to the genes of *S. clavuligerus* (74.7%) and *A. chrysogenum* (69.2%). At the protein level, there is also high similarity to the expandase of *S. clavuligerus* (70.4%) and the expandase/hydroxylase of *A. chrysogenum* (59.5%). This expandase also showed a high specificity for the nature of the side chain, and only penicillin N was expanded.

The gene cluster involved in cephabacin F (a 7-formyl amino cephalosporin) biosynthesis in *Lysobacter lactamgenus* has also been characterized.[20] The 35 557 Da protein encoded by the *cefE* gene shows 54 and 57% similarity to the expandases of *S. clavuligerus* and *A. chrysogenum*, respectively.

Broadening the Substrate Specificity of Expandase. *Resting Cells.* Although previous attempts by several research groups to expand the thiazolidine ring of penicillin G and other penicillins had failed,[1,3,10,13] success was achieved with resting cells of *S. clavuligerus*.[4,21] The cofactor requirements for penicillin G expansion to deacetoxycephalosporin G (DAOG) were examined with resting cells of *S. clavuligerus* NP1 [a mutant that produces only trace levels of cephalosporins[22]]. When Fe^{2+}, α-ketoglutarate, or ascorbic acid was absent, the amount of product obtained was about 30% of that in the control. On the other hand, ATP, Mg^{2+}, K^{+}, and DTT did not play a significant role in the reaction with resting cells. The omission of DTT actually increased production by 50%. When the α-ketoglutarate concentration was raised from the previously used concentration of 0.64 mM[13] to 1.28 mM, activity of resting cells was doubled. When the concentration of Fe^{2+} was raised 45-fold, the ring expansion activity was markedly increased.

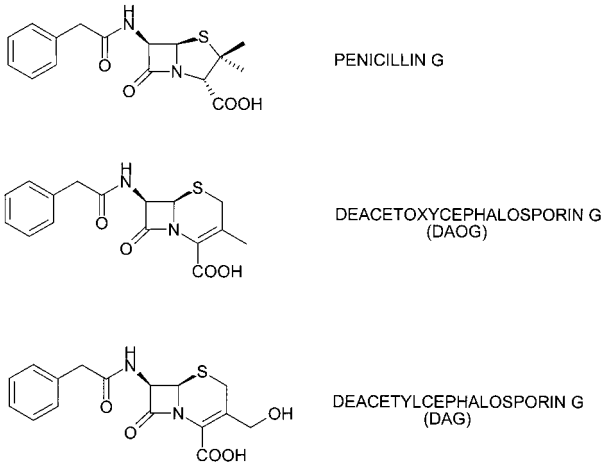

Figure 2. **Chemical structures of penicillin G, deacetoxycephalosporin G (DAOG), and deacetylcephalosporin G (DAG).**

Increasing cell mass concentration enhanced production of DAOG formed from penicillin G, the optimum concentration being 19 mg/mL (dry cell weight). Higher cell concentrations inhibited the reaction probably because oxygen supply became limiting under such conditions. Cho and coworkers[21] used 50 mM Tris-HCl [Tris(hydroxymethyl) aminomethane] at pH 7.4 as buffer for the reaction. It was later found that 50 mM MOPS [3-(*N*-morpholino) propanesulfonic acid] buffer or HEPES [*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)] buffer at pH 6.5 improved activity.[23] Increasing the concentration of penicillin G substrate (from 2 mg/mL) increased the concentration of DAOG produced but decreased the yield. Decreasing penicillin G concentration decreased the DAOG concentration but increased yield from <1% to as high as 16.5%.[23]

The products obtained during this biotransformation were identified by an HPLC method that allowed the separation of peaks corresponding to penicillin G, DAG and DAOG.[24] Figure 2 shows the structures of these compounds.

Cell-Free Extracts.[21,35] Cell-free extracts from *S. clavuligerus* NP1 were prepared by sonication. Using the conditions previously developed for resting cells as described

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above, ring expansion activity was observed on penicillin G and 14 other penicillins.[21] The studies done on cell-free extracts and resting cells revealed a similar but low bioconversion yield, about 1% when 2 mg/mL of penicillin G was used as substrate.

Recombinant Expandases. Expression of the *S. clavuligerus* NRRL 3585 *cefE* gene in *E. coli* was achieved for the first time using the λP_L promotor[18] although the protein produced was predominantly insoluble. Cloning under control of the T7 promoter using the pET expression system resulted in production of large amounts of soluble expandase.[25−27] Although induction at 37 °C led to low levels of the glutathione (GST)-expandase fusion protein (<5% of total soluble protein), when cultures were induced at 20 and 28 °C, high levels of recombinant protein were obtained (>20% of total soluble protein).[27] Recently, expression and secretion of this synthase has been achieved in a eukaryotic background using the *Pichia pastoris* expression system.[28]

The recombinant expandase obtained by Sim and Sim[27] was able to convert penicillin G not only under the conditions reported by Cho et al.[21] where the concentrations of the two most important cofactors, $FeSO_4$ and α-ketoglutarate were 1.8 and 1.28 mM, respectively, but also with the conditions used by Maeda et al.[13] using lower concentrations of both cofactors (0.04 and 0.64 mM, respectively). The rate of conversion was 2-fold higher using the conditions of Cho et al.[21] Ring expansion activity was also observed when ampicillin, amoxicillin, or penicillin V was used as substrate.[27,29] Studies on the effect of cofactors in the ring expansion reaction using penicillin G or ampicillin as substrate revealed that Fe^{2+} and α-ketoglutarate omission results in more than 50% reduction of activity. Conversion of penicillin G by recombinant expandase was enhanced by 11-fold when DTT was omitted.[27] The inhibitory nature of DTT had been noted earlier by Cho et al.[21] The significance of this effect is not known. On the other hand, omission of ascorbate had a greater negative effect on expandase activity in resting cells[21] than with the recombinant enzyme. When both DTT and ascorbate were omitted in the ring expansion reaction using the recombinant enzyme, the rate of conversion of penicillin G and ampicillin was enhanced by 21- and 35-fold, respectively.[27]

Further improvements in the conversion of hydrophobic penicillins have been obtained with a mutant enzyme in which the hydrophilic residue N304 was replaced by leucine (a noncharged, hydrophobic amino acid).[30] Using penicillin G as substrate, an improvement of 83%, relative to that of wild-type *S. clavuligerus* expandase activity, was obtained, whereas improvements in ampicillin and amoxicillin conversion were 75 and 180%, respectively.

Mechanistic Studies on the Ring Expansion Reaction. On the basis of the elucidation of the crystal structure of the *S. clavuligerus* expandase,[31] a mechanism was proposed for the formation of the reactive ferryl species, a catalytic intermediate common to many non-heme oxygenases.[32] The apo-expandase, a crystallographic trimer, reacts first with iron, which is ligated by three protein residues (His183, His243, Asp185) and three solvent molecules, causing dissociation of the apoenzyme into monomers, which represent the catalytically active forms. Then, the enzyme-Fe^{2+} complex reacts with α-ketoglutarate, whose binding replaces two solvent molecules around the iron, and with dioxygen. The ferryl form of expandase, which reacts with penicillin N, is created by the splitting of dioxygen and oxidative decarboxylation of α-ketoglutarate to succinate. Studies employing site-directed mutagenesis were carried out to replace the residues His183, Asp185, and His243.[26] Substitution at these sites completely abolished ring expansion, confirming that these residues are essential for catalysis.

The utilization of alternative cosubstrates for the ring expansion reaction had been investigated earlier.[10,11] Expandase crystal structure revealed the presence of several arginine residues within the active site, with Arg258 being involved in cosubstrate binding.[25,31] Site-directed mutagenesis of this residue resulted in mutant enzyme R258Q whose activity was reduced in the presence of α-ketoglutarate but could be fully restored using aliphatic 2-oxoacids as alternative cosubstrates.[33] Wild-type expandase has, at most, only traces of activity with the 2-oxoacids tested (other than α-ketoglutarate and 2-oxoadipate). These results show that the side chain of Arg258 is a major determinant of the 2-oxoacid cosubstrate selectivity. The decrease in activity of the R258Q mutant observed with α-ketoglutarate seems to be due to the loss of a favorable ionic interaction between the 5-carboxyl group of α-ketoglutarate and the guanidine group of Arg258.

The crystal structure of expandase has revealed the formation of a trimeric unit in which the C terminus of one expandase molecule is inserted into its neighbor in a cyclical fashion.[25,31] Further studies suggested that residues located at the C terminus might be involved in orienting or binding or both of the penicillin substrates during the reaction. Construction of different mutants with truncated C termini showed that deletion of up to 11 residues does not affect binding of α-ketoglutarate but that there were significant differences in the way in which the enzyme catalyzes penicillin N oxidation as compared to penicillin G oxidation.[33] With penicillin N, deletion of five to six residues did not significantly affect activity, but when penicillin G was used as substrate, activity was very much reduced.

Expandase is inactivated by compounds such as *p*-hydroximercuribenzoate, DNTB [5,5′-dithio-bis(2-nitroben-

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zoic acid)], or *N*-ethylmaleimide,[3] suggesting that at least one sulfhydryl group may be important for structural integrity or catalysis or both. Lee et al.[34] mutated three cysteine residues (Cys100, Cys155, and Cys197) that were possible candidates for involvement in disulphide bond formation. Mutation at these positions (singly or in combination) to alanine residues led to mutant enzymes with activity on penicillin N. Only one mutant, C100A, showed an activity similar to that of the wild-type expandase when penicillin G was used as substrate.

Superimposition analysis of *S. clavuligerus* expandase and *Aspergillus nidulans* IPNS (isopenicillin N synthase) allowed Chin et al.[30] to identify several residues located in the substrate-binding pocket of *S. clavuligerus* expandase. Four of them (R74, R160, R266, and N304) were classified as the most hydrophilic. To modify the substrate specificity of expandase towards hydrophilic penicillins, these four positions were selected for single mutations to replace R (arginine) or N (asparagine) with L (leucine). Only mutant enzyme N304L was able to convert the three substrates tested (penicillin G, ampicillin, amoxicillin), and improvements in ring expansion activity from 5- to 40-fold were observed relative to that of wild-type expandase. The improved enzymatic activity showed by this mutant could be due to the orientation of the L residue at the C terminus guiding the entrance of substrate into the catalytic center of expandase, confirming the proposal made by Lee et al.[33] that modifications at this part of the enzyme might be involved in orienting, binding, or both of the penicillin substrates during the reaction.

Inactivation of Expandase During the Ring Expansion Reaction. The extent of expansion by cell-free extracts increased with increases in concentration of both protein and substrate.[21] However, in all cases, including that of purified recombinant expandase,[27] the concentration of product increased during the first 1−3 h and then either remained stable or decreased. To determine if the limiting factor after 2 h was the exhaustion of one or more components of the reaction mixture, different concentrations and combinations of reaction constituents were added to the reaction tubes at 2 h, but no additive was able to reactivate the system.[35] Lack of activity could not be attributed to enzyme instability during shaking. Preincubation of the cell-free extracts under different conditions of temperature and agitation for 2 h did not affect subsequent ring expansion activity.[35]

To determine whether one or more reaction mixture components might inactivate the enzyme, the cell-free extract was preincubated for 2 h in the presence of different reaction components. After this preincubation, the remaining reaction components as well as the substrate (penicillin G) were added. The amount of product obtained was markedly different, depending on which component was present during preincubation. When buffer alone was present, the subsequent reaction yielded up to 95% of the amount of product formed in the control reaction. When certain individual components ($FeSO_4$, ascorbate, α-ketoglutarate, $MgSO_4$, or KCl) were present during incubation, production remained between 64 and 85% of control. However, when preincubation was with Fe^{2+} plus ascorbic acid or Fe^{2+} plus α-ketoglutarate, no detectable product was obtained. The same phenomenon was also observed with resting cells.[23] As with extracts, inactivation was not merely due to the presence of oxidized Fe, because when the normal bioconversion reaction was carried out with Fe^{3+} (as ferric sulfate) instead of Fe^{2+}, there was no inhibition.

Inactivation during aerobic incubation with Fe^{2+} plus ascorbic acid or α-ketoglutarate or both might have been due to formation of hydrogen peroxide.[36−38] As for other α-ketoglutarate-dependent dioxygenases, expandase requires a reducing agent in addition to α-ketoglutarate and iron. This requirement could be fulfilled by several substances but, ascorbate is the most effective and is the reducing agent usually used with this type of enzyme.[39] However, ascorbate can have adverse effects, since incubation of some enzymes with ascorbate and oxygen leads to rapid loss of enzymatic activity due to production of hydrogen peroxide during autoxidation of this reducing agent.[39,40] Catalase is known to stimulate the activity of such enzymes;[41] however, when catalase was added to the preincubation mixture with cell-free extract, no protection was observed.[35] Nevertheless, when 15 mM hydrogen peroxide was added to a reaction mixture, activity was markedly reduced, and when catalase was also added, the inactivation due to peroxide was reversed, indicating that inactivation normally observed with Fe^{2+} plus ascorbate was not due, at least exclusively, to formation of hydrogen peroxide.

Oxygen-dependent inactivation of several key biosynthetic metabolic enzymes that involve mixed-function oxidation systems have been reported.[36] These systems catalyze synthesis of hydrogen peroxide and reduction of Fe^{3+} to Fe^{2+} followed by oxidation of enzyme-bound Fe^{2+} to generate oxygen radicals that attack a histidine (or other oxidizable amino acids) at the metal-binding site of the enzyme. Golan-Goldhirst et al.[42] reported that preincubation of different proteins in the presence of ascorbate and copper (or iron) led to their inactivation due to the autoxidation of ascorbic acid. In this reaction, four strong oxidant species were formed, and after preincubation, changes in the amino acid composition of all proteins were observed. There was a major loss of histidine [expandase has three essential residues, His183, His243, and Asp185 which ligate ferrous iron to give a catalytically active form[31]] and methionine residues.

To check if inactivation could be due to formation of reactive oxygen species such as superoxide, the effects of

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different radical scavengers such as mannitol and DMSO, as well as superoxide dismutase, were examined. However, none of these compounds was able to reactivate the enzyme after preincubation.[35] The addition of reducing agents such as DTT and β-mercaptoethanol failed to stimulate after preincubation with Fe^{2+} and ascorbate.

Further Improvements in the Bioconversion of Penicillin G to Deacetoxycephalosporin G. *Stimulatory Effect of Growth in Ethanol.* Biosynthesis of antibiotics rarely takes place during periods of rapid growth in rich media.[43] Rather their production occurs best under conditions of nutrient imbalance brought about by limitation of carbon, nitrogen, or phosphorus and at low growth rates. The discovery of heat shock-like proteins (GroEL-like proteins), potentially important in antibiotic export and in the assembly of multienzyme complexes for polyketide antibiotic synthesis in a variety of streptomycetes,[44] led to the first study on the relationship between non-nutritional stresses, such as heat shock or ethanol treatment and antibiotic biosynthesis.[45] In that study, jadomycin production was induced by heat shock or by ethanol.

The effect of growth in the presence of alcohols of *S. clavuligerus* NP1 was studied with regard to the ability of resting cells to biotransform penicillin G into cephalosporin-type antibiotics.[24] Cultures grown in MST medium [1% soluble starch, 3% trypticase soy broth without dextrose, 90 mM MOPS, pH 7.0[9]] or MT medium (MST-less starch) showed the typical mycelial masses of tangled hyphae. However, when MT medium was supplemented with ethanol, different morphologies were observed, depending on the alcohol concentration. In 1% ethanol, the hyphae were somewhat more dispersed, whereas in 2% ethanol, the hyphae were extensively fragmented and dispersed. In the presence of 1% ethanol or 1−2% methanol, growth extent was slightly less than in MT medium, but with 2% ethanol, growth was severely restricted; higher concentrations totally inhibited growth. Omission of starch (MT medium) led to a slight increase in specific production of DAOG as compared to production in MST medium. When MT medium was supplemented with 1% ethanol, there was a marked increase in specific production and a 6- to 7-fold increase when 2% ethanol was added.[24] Addition of alcohols at later times during growth (at 2, 6, and 12 h) did not have any stimulatory effect.

The mechanism of alcohol stimulation on biotransformation of penicillin G remains a mystery although it could be related to the known ability of ethanol to trigger a heat-shock (stress) response.[45−47] Alternatively, growth in alcohol might yield cells with increased membrane permeability. The morphological effects observed could reflect this latter mechanism.

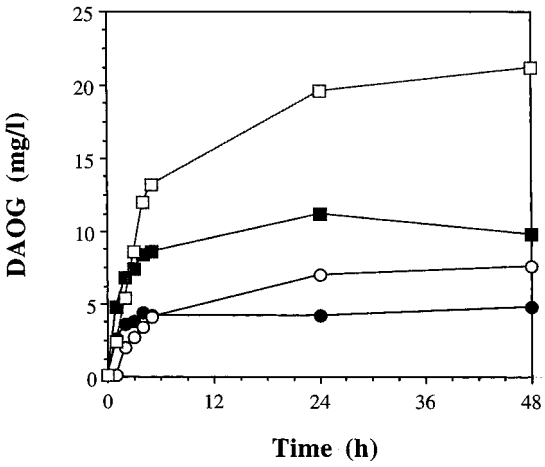

Figure 3. **Effect of growth substrate and agitation during reaction on DAOG formation. (●) starch-grown cells with agitation; (○) starch-grown cells without agitation; (■) ethanol-grown cells with agitation; (□) ethanol-grown cells without agitation.**

Use of Immobilized Cells. The oxidative ring expansion of penicillin G by free and entrapped resting cells of *S. clavuligerus* NP1 was compared,[48] and immobilized cells were found to perform the expandase reaction more slowly and less extensively than free cells, probably due to strong diffusional limitations. Both types of cells virtually ceased production after 2 h of reaction. Increasing resting cell concentration yielded increased product formation although, again, the reaction markedly decreased in rate after 2 h. To examine multiple cycles of ring expansion, free or resting cells immobilized by entrapment in polyethyleneimine-barium alginate were allowed to carry out oxidative ring expansion for 2 h, followed by centrifugation in the cold for 5 min. The cells were washed with 50 mM MOPS (pH 6.5) and recentrifuged. The expandase reaction was again initiated. Two-hour cycles were carried out up to four times with assays for product formation done at the end of each cycle. The activity of free cells was reduced by about 60% from the first to the second cycle and was completely lost after the second cycle. On the other hand, immobilized cells showed only a small reduction of activity at each cycle and still had activity through four cycles.

Elimination of Agitation and Addition of Water-Immiscible Solvents. Since inactivation of expandase was thought to be an oxidative process,[34] the effect of eliminating shaking during the ring expansion reaction was examined. It was found that the bioconversion rate was lower, but more importantly, the conversion yield increased.[49] As shown in Figure 3, the positive effect was observed with cells grown either in MST medium or in MT2E (MT medium plus 2% ethanol).

A large number of different additives were tested for their effect on bioconversion by resting cells. Poly(ethylene glycol)s, alginate, Tween 80, DTT, and D,L-methionine showed moderate or slight stimulatory effects.[49] Water-immiscible solvents were tested because they often increase

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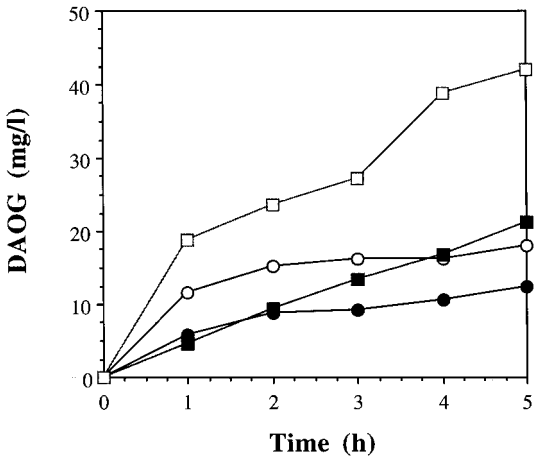

Figure 4. **Effect of decane on DAOG formation by ethanol-growing cells. (●) with agitation; (○) with agitation plus 32% (v/v) decane; (■) without agitation; (□) without agitation plus 32% (v/v) decane.**

enzyme activity and stability and prevent the hydrolysis of the substrate or product.[50,51] To check if they might decrease the inactivation of expandase by Fe^{2+} plus ascorbate or Fe^{2+} plus α-ketoglutarate, some of these solvents were added to the reaction. When compared to other solvents used for enzyme catalyzed reactions, alkanes are often best because of their high log *P* values (octanol−water partition coefficient) which are favorable for activity and stability of cells and enzymes.[52−54] Among them, *n*-decane has been reported to have minimal deleterious effects on microbial viability.[55] On the basis of this, the effect of adding *n*-decane to the reaction was examined.[49] As Figure 4 shows, addition of 32% (v/v) *n*-decane led to an increase in DAOG formation that was especially significant in those reactions carried out without agitation.

A series of other solvents were compared to decane at the 32% concentration. *n*-Butanol, DMSO, dioxane, isopropyl alcohol, and cyclohexanol totally inhibited the reaction.[56] Solvents that were as effective as *n*-decane were all alkanes, that is, hexane, heptane, octane, dodecane, and hexadecane.

When other conditions of the alkane-stimulated bioconversion were examined, it was found that the optimum conditions for this system included a pH range of 5.5−7.0 and a temperature of 28 °C, which was only slightly different from the conditions previously used, that is, pH 6.5 and 30 °C.[21,23]

Addition of Catalase. As mentioned above, oxidative inactivation of expandase during the ring expansion reaction by the cofactors Fe^{2+}, α-ketoglutarate, and ascorbate might possibly be due to the formation of hydrogen peroxide, superoxide anion, or both.[23,35] We decided to study the effect of enzymes capable of destroying these types of compounds. Although addition of catalase or scavengers did not prevent inactivation with cell-free extracts,[35] a positive effect was surprisingly observed with resting cells.[57] Addition of catalase to the bioconversion system, conducted in the absence of agitation and in the presence of 50% (v/v) hexane, was found to increase the bioconversion of penicillin G to DAOG. When other enzymes such as peroxidase or superoxide dismutase were added to the reaction, no enhancing effect was observed.

Hybrid Expandases. On the basis of the high degree of similarity at the nucleotide (74%) as well as at the amino acid (70%) sequence levels between the expandase genes of *S. clavuligerus* and *N. lactamdurans*,[19] it was of interest to determine whether homeologous recombination (recombination between partially homologous sequences) could take place at a significant frequency between these two genes when cloned in the same orientation on a plasmid. If so, hybrid expandases with potentially altered activity/specificity could be generated in vivo.[59] Studies were first conducted in a *recA*$^+$ *E. coli* strain. Seventeen colonies (27%) isolated after four rounds of propagation in such a strain revealed that two major types of DNA rearrangements had occurred in these plasmids. Sequencing of the crossover junctions in representative recombinant plasmids showed that recombination had taken place in fully conserved sequence stretches of 2−17 bp at five different positions within the first 550 bp of the chimeric *cefE* genes. All clones had undergone in-frame recombination events.

Recombination was also attempted in *Streptomyces lividans* 1326. A rapid screen to detect the putative clones harbouring a chimeric expandase gene was developed using as marker the *melC* gene from *Streptomyces glaucescens* which encodes the black pigment melanin.[59] Analysis of several white colonies ("melanin-negative") revealed the presence of plasmids of different sizes, ranging from 4 to 8 kb (the expected size was 6.3 kb). When the ring expansion abilities of several of these recombinants were determined, three white strains as well as controls (melanin-positive) showed activity producing growth inhibition zones in the agar bioassay and a peak corresponding to DAOG when analyzed by HPLC. Strain W25 produced approximately 20-fold more DAOG than *S. clavuligerus* NP1 and even more than B18 which contains the two intact cloned expandase genes.[59]

When different growth conditions were tested for this recombinant strain W25, it was found that the best conversion was obtained with cells grown in MT4E medium (MT medium plus 4% ethanol) for 1 day.[60] When bioconversion was carried out without shaking and in the presence of 50% (v/v) hexane, production was markedly increased as previously observed for *S. clavuligerus* NP1 cells. The hybrid strain performed better at 16−25 °C than at 28 °C. Furthermore, lowering the iron concentration to 0.45 mM and raising α-ketoglutarate concentration to 1.92 mM

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improved the activity of W25 cells. In a statistical study, the $FeSO_4$ concentration was found to be the most important factor, whereas ascorbate was the least. The 4-fold lower iron concentration preferred by the hybrid culture is probably the main reason for the improved bioconversion. This is based on the action of iron, not as cofactor in the biological oxidation reaction, but more importantly, as an inactivator of the expandase.[35] Catalase stimulated the bioconversion and could be replaced by the inexpensive additive, yeast extract, which contains a component known to destroy hydrogen peroxide.[58] In a number of experiments, DAOG concentrations higher than 200 mg/L have been obtained.

Closing Comments

Marked improvements have been made in the bioconversion of penicillin G to DAOG. These improvements have been accomplished by modifying the concentrations of reaction components, eliminating agitation, adding an alkane and catalase, and using the technique of directed evolution. The improvements have raised the conversion from less than 1% to about 10% of the charged penicillin G. Further improvements will be made by additional molecular genetic and biochemical modifications as well as engineering inputs. We feel strongly that down the road the bioprocess will become an economically relevant alternative to chemical ring expansion.

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