MICROBIOLOGICAL RING EXPANSION OF PENICILLIN N

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A mutant of *Cephalosporium acremonium* producing high amounts of penicillin N was isolated. This antibiotic was purified and characterized. It was possible to convert this penicillin N to deacetoxycephalosporin C enzymatically. The reaction could be carried out with enzyme systems prepared from *C. acremonium* mutants producing either no β-lactam antibiotics or excreting only penicillin N. It was surprising that a high level of transformation capacity was just found in a cephem negative mutant which overproduces penicillin N. For that reason the inability of the latter mutant to produce cephem compounds cannot be explained by a functional block of the ring expanding enzyme complex. The enzyme preparations used to carry out this reaction were made by ether-treatment or sonication of *C. acremonium* cells, or by submitting them to osmotic shock. The ring expanding enzyme system is strongly dependent on ATP and behaves as a 2-oxoglutarate dependent dioxygenase.

Recently several authors have demonstrated the enzymatic conversion of penicillin N to deacetoxycephalosporin C using cell-free extracts of *C. acremonium*[1,2]. Even with optimal reaction conditions[1] the conversion rate was very low, and the investigators had difficulty in reproducing their results. Many other earlier experiments were made using impure penicillin N of undefined quality.

Reaction rate and reproducibility could be increased using the methods described below. We were able to demonstrate that an enzyme similar to a 2-oxoglutarate dependent dioxygenase carries out the conversion. We used newly isolated mutants of *C. acremonium* with the purpose of measuring enzymatic conversion of penicillin N to deacetoxycephalosporin C. Natural penicillin N was isolated from a mutant producing ten times more than the parent. Penicillin N was highly purified and characterized by NMR spectroscopy and two different HPLC methods, and by gaschromatography of the hydrolyzate. We report here that the ring expansion can be measured in permeabilized[3,4] and sonicated cells. The *C. acremonium* cells used were osmotically fragile and utilized sulfate for antibiotic formation.

Materials and Methods

Microorganisms

A superior cephalosporin C producing strain designated 18.2.15 derived from *C. acremonium* 8650-S^{-5}[5], was used as the parental strain from which a cephem-negative, penicillin N overproducing mutant M269 and a β-lactam negative mutant M92 were obtained for use in this study. A β-lactam negative mutant (N-2) which accumulates the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine was a gift from Dr. Y. NAKAO, Takeda Chem. Ind. Ltd., Japan[6].

Culture Media and Conditions

The culture conditions were the same as described previously[3] except for the liquid seed medium (preculture) and the fermentation culture. The seed medium was: 10 g corn steep powder, 10 g sac-

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charose, 4.5 g ammonium acetate, 0.5 g calcium sulfate, 0.5 g magnesium sulfate in 1 liter of deionized water. The pH after sterilization (20 minutes, 120°C) was 7.3. The fermentation medium (A20) was: 11 g ammonium sulfate, 55 g corn steep powder, 24 g peanut meal, 13 g starch, 6 g magnesium sulfate, 30 g glucose in 1 liter of deionized water. The pH of the medium after sterilization (20 minutes, 120°C) was 6.3. The fermentation medium of mutant N-2 has been described by SHIRAFUJI *et al.*[9]. The strains were grown on complete medium agar slants for 7 days at 25°C and inoculated into 40 ml of the seed culture in a 200 ml flask. The flask was incubated at 25°C for 3 days on a rotary shaker (250 rpm). One ml of the seed culture was inoculated into 17 ml of the fermentation medium in a 50 ml flask and incubated at 25°C for 5 days under the same conditions as the seed culture.

Selection of β-Lactam Negative Mutants

The β-lactam negative mutants were selected independently as penicillin N or cephalosporin C negative colonies from the survivors of the conidia (10^3 per agar plate) of the parent strain 18.2.15 after exposure to UV irradiation (10~15 seconds) under a 10 W lamp at different distances yielding 10, 5 and 1% survivors. The survivors were placed on mycophil agar plates. After 8 days of incubation, the productivity of the picked colonies was tested by overlaying on agar plates containing *Alcaligenes faecalis*[6]. Colonies which gave no inhibition zone when incubated overnight at 37°C were isolated as cephalosporin C or penicillin N negative mutants. Absence of cephalosporin production or the production of penicillin N was confirmed by cultivation of the mutants in the fermentation medium (A20) at 25°C for 5 days. The culture filtrate was tested on *A. faecalis* and *Sarcina lutea*[6].

The mutant M92 displayed a complete lack of β-lactam antibiotic production whereas M269 turned out to be a cephem negative mutant synthesizing ten times more penicillin N than the parent 18.2.15 which also produces cephalosporin C.

Preparation of Enzyme Systems

Three systems were used, namely, ether-treated cells[8], sonicated cells[7], and osmotically shocked cells. The washing medium was 70 mM phosphate buffer pH 7.0. Osmotically shocked cells correspond to control (intact) cells as described earlier[3]. These mutants seem to be very sensitive to changes in osmotic pressure (transfer from a production medium to phosphate buffer). In the course of the preparation procedure the original culture volume was concentrated twofold.

Ring Expansion Reaction

In a first assay the conditions of SAWADA *et al.*[1] were applied. 0.5 ml ether-treated or 0.5 ml sonicated cells were added per ml reaction mixture. Our optimized reaction conditions were as follows: We prepared 2.5 ml of a stock solution containing 4 mM 2-oxoglutaric acid, 0.32 mM $FeSO_4 \cdot 7H_2O$, 1.6 mM ascorbic acid, 6 mM ATP, 70 mM phosphate buffer pH 7.0, 1.6 mM penicillin N, 2 mM $MgSO_4 \cdot 7H_2O$ and 2 mM KCl. This solution was incubated together with 2.5 ml ether-treated, sonicated or osmotically shocked cells in 50 ml shake flasks at 25°C and 250 rpm for 3 hours.

Action of β-Lactamases on the Reaction Product Mixture

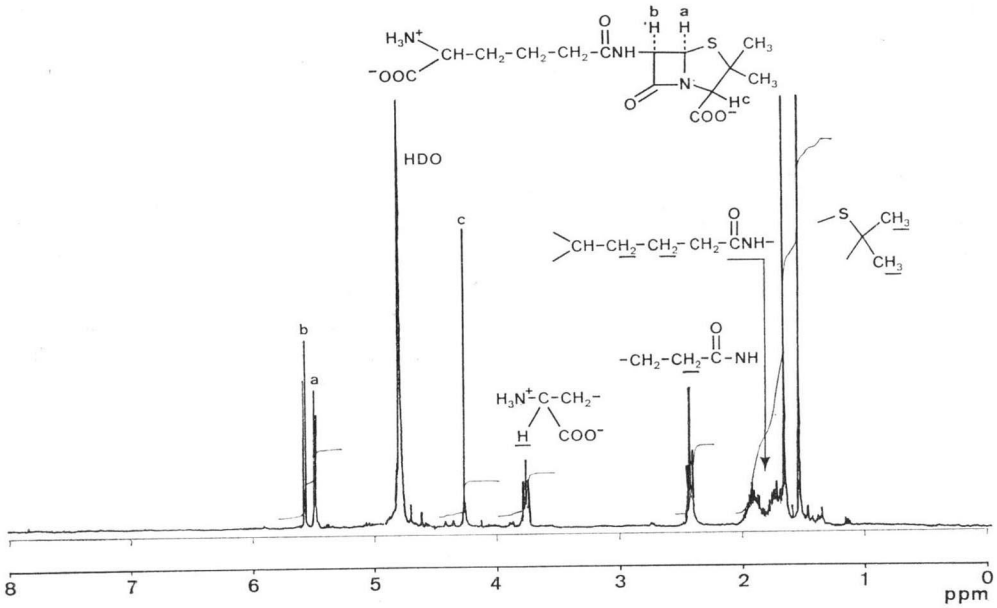
The product deacetoxycephalosporin C can be destroyed by β-lactamase P-99 from *Enterobacter cloacae*[8] and the substrate penicillin N by β-lactamase I from *Bacillus cereus* (288 EU/ml, 2912 EU/mg protein, 1 EU destroys 1 μmole penicillin G per minute). Generally we incubated 2 μl β-lactamase I with 10 μl of the filtrate for 15 minutes at 25°C before analysis. Both β-lactamases were a gift from J. KONECNY (Ciba-Geigy Ltd., Basel).

Isolation and Characterization of Penicillin N

Fermentation broth of strain M269 was harvested after 120 hours. The culture filtrate (0.95 liter) was lyophilized and subjected to chromatographic purification on the macroreticular resin Diaion HP-20; 75.8 g were redissolved in 250 ml of deionized water and percolated through a resin bed of 1.5 liters. Elution was performed with water at a rate of 1.5 liters/hour. Fractions of 150 ml were collected and assayed conductometrically and by analytical HPLC. The peak fractions 8 and 9 were lyophilized (4.27 g) and further purified by preparative HPLC. A modular system was used, consisting of an Altex pump with a preparative head, a preparative column by DuPont (23 mm ID × 250 mm, RP-C_8, particle size 7 μ) and a Uvikon LCD 725 UV-monitor operated with preparative cells. Frac-

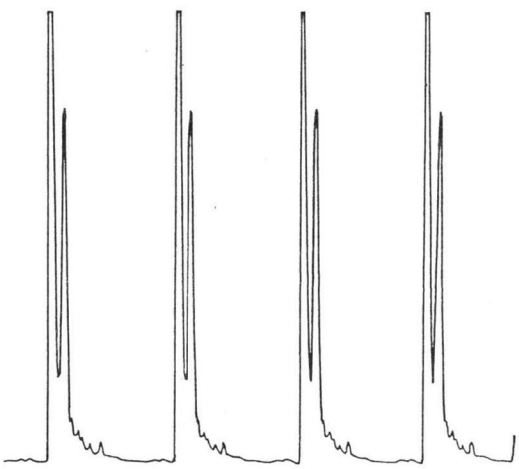
tions of eluent were collected according to their UV-absorption at 200 nm. For each separation step 1.0 ml of a 30% solution of the lyophilizate was injected. An isocratic separation mode was adopted, using 0.5% ammonium phosphate buffer adjusted to pH 6 as the mobile phase and a flow rate of 25 ml/minute. Under these conditions a back pressure of 78 bar was generated. The sensitivity range of the detector was set at 1.0. Separations were repeated every 30 minutes with good reproducibility (see Fig. 2). Under the above described conditions, penicillin N was eluted 7.8 minutes after injection. The collected peak fractions were pooled, lyophilized and subjected to the same procedure again. The residue obtained after the second passage was desalted by chromatography on a preparative HPLC column (16 mm ID × 500 mm from Knauer), filled with about 100 ml of Diaion HP-20 resin, with doubly distilled water as the eluent at a flow rate of 3 ml/minute. Two ml of a 20% solution were injected at a time. Eluant fractions showing absorption at 220 nm were collected and lyophilized.

Fig. 1. Proton resonance spectrum (360 MHz) of penicillin N (in D_2O, at 310°K, internal standard: HDO = 4.80 ppm).



The purity of the resultant white fluffy powder was checked by high resolution NMR-spectroscopy at 360 MHz. The assignment of the main signals is shown in Fig. 1. The minor peaks are due to traces of the penicilloyl degradation product, the amount of which increases slowly when the solution is left at room temperature and dramatically upon addition of penicillinase. The chirality of the α-aminoadipic acid side chain was examined by gaschromatography on Chirasil-val stationary phase after acid hydrolysis and derivatization. A sample of 5 mg of penicillin N was hydrolyzed in 0.5 ml of 6 N HCl under standard conditions (24 hours at 150°C in a closed glass tube). The hydrolyzate was dried, redissolved in 0.3 ml of 1.25 N HCl/2-propanol and kept at 105°C for 2 hours. The solution containing the 2-propylester was flushed with nitro-

Fig. 2. Separation of penicillin N by preparative HPLC.



gen gas. CH_2Cl_2 (0.3 ml) and 0.2 ml of trifluoroacetic acid were added to the dried residue. After 2 hours at room temperature the mixture was again completely dried in a stream of nitrogen. Samples of 1 μl of the fully derivatized reaction product dissolved in 0.5 ml of CH_2Cl_2 were used for gaschromatographic separation. The percentages of the D- and L-enantiomers were obtained by integration of the respective peak areas.

Analytical HPLC Methods

For analytical determination of penicillin N two different chromatographic methods were worked out:

a) Reversed-phase HPLC on a Whatman column (Partisil PXS 5/25 ODS) using elution conditions similar to those used for preparative separations.

b) Ion-exchange chromatography on a column (ID 3.2 mm×150 mm) filled with Lichrosorb 10 AN (Merck). The eluent consisted of 87.5% of 0.069 M phosphate buffer adjusted to pH 4.2 and 12.5% of methanol. The flow rate of 1.3 ml/minute generated a pressure of 100 bar. The absorption peaks were monitored at 200 nm using a DuPont variable wave-length detector (model 852001–902) with a sensitivity setting of 2.56. Under these conditions, the retention time for penicillin N was around 3.6 minutes. When the samples were left at room temperature for about 1 hour, an additional peak due to the penicilloic acid degradation product appeared after 2.3 minutes.

Analysis of Product

The product deacetoxycephalosporin C was characterized by three different methods: Reversed-phase thin-layer chromatography[6], bioassay using test organisms *A. faecalis* and *S. lutea*[6] and HPLC on Lichrosorb 10 AN Merck (Fig. 3). The latter is more suitable than the reversed-phase method for the detection of very small concentrations of cephalosporin C components in culture filtrates. Almost all contaminating products are eluted right at the beginning of the chromatogram and they do not interfere with the cephalosporin peaks. Five μl of a sample were injected. The mobile phase used was the same as described above for analytical separations of penicillin N. The wave-length for detection was changed to 262 nm. A flow rate of 0.5 ml/minute was applied. Concentrations as low as 10 μmoles/liter of deacetoxycephalosporin C were detectable.

Results

Among 188 cephem negative mutants, four *C. acremonium* strains were isolated producing 13 ~ 26 mmoles of penicillin N per liter. One mutant (M269) excretes penicillin N as the only β-lactam compound and, in addition, the tripeptide α-aminoadipoylcysteinylvaline (characterization not shown here). Penicillin N was purified by preparative HPLC. A high degree of purity of the product obtained was demonstrated by high-field ^{1}H-NMR spectroscopy and analytical HPLC using two different methods. The D-configuration of the α-aminoadipoyl side chain was confirmed by gaschromatography on Chirasilval stationary phase after hydrolysis and derivatization: A fraction of 97.9% exhibited the D-configuration. The 2.1% of the L-analogue can be attributed to racemization under the conditions of acid hydrolysis. This indicates that no isopenicillin N is present in the filtrate. The NMR spectrum of penicillin N shows only traces of impurities (Fig. 1).

In the first experiments, the conversion of penicillin N to deacetoxycephalosporin C could be measured with low yields (30 μmoles/liter and 6 hours) in ether-treated and in sonicated *C. acremonium* cells using the conditions described by SAWADA *et al.*[1]. The detection of deacetoxycephalosporin C was carried out by HPLC on a strong anion exchange resin. An elution profile is shown in Fig. 3. The transformation was observed in the penicillin N overproducing, cephem negative mutant M269 as well as the β-lactam negative strain M92. Since mutant M269 was expected to be blocked in the ring expansion reaction which mediates the transformation of penicillin N to deacetoxycephalosporin C, it was

Fig. 3. Separation of cephalosporins using analytical HPLC on Lichrosorb AN (strong anion exchange) resin.

a) standard solution with 330 μM deacetoxycephalosporin C (DAO), 1.56 mM deacetylcephalosporin C (DA) and 3.61 mM cephalosporin C.
b) ether-treated mutant M269 converting penicillin N to DAO (0.11 mM).
c) ether-treated mutant M269, addition of β-lactamase P-99 to the reaction mixture or no penicillin N added as substrate.

At 2.57 or 2.60 minutes retention time ATP is eluted.

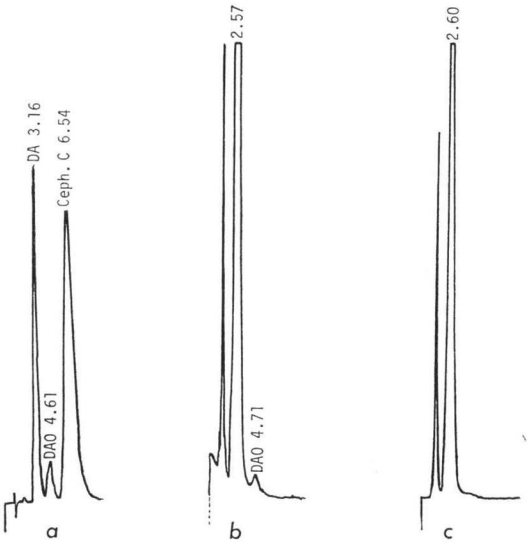


Fig. 4. Growth of penicillin N producing mutant M269.

Content of penicillin N (1 corresponds to 5.33mM) and of RNA (1 corresponds to 0.2 g/liter), determined as described by KÜENZI[12]. Rate of synthesis of penicillin N (RSP, 1 corresponds to 0.1 mmole/liter/hour).

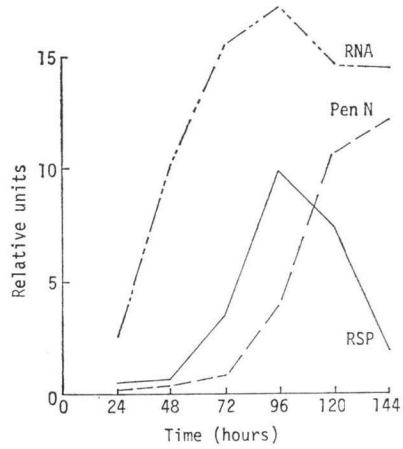
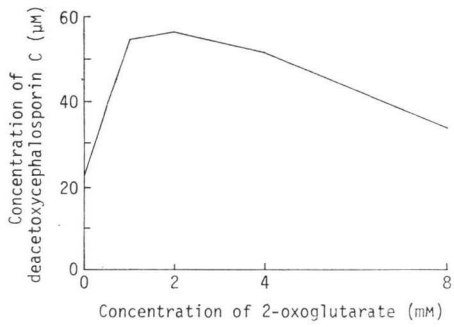


Fig. 5. Effect of 2-oxoglutarate concentration on ring expansion activity.

All other substrates were added in concentrations used by SAWADA *et al.*[1].



surprising to find that M269 was capable of maintaining a high level of transformation capacity. In contrast, no transformation activity could be found in the cephalosporin C producing parental strain 18.2.15 and the peptide mutant N-2. The culture had to be 4 days old for optimal transformation activity. No deacetoxycephalosporin C was detectable within the limits of detection in 3 or 5 days old cultures of M269. Similar results were obtained with the β-lactam negative mutant M92. *In vivo*, the rate of penicillin N synthesis in the mutant M269 reaches a maximum of 1 mmole/liter/hour at an age of 4 days (Fig. 4).

Using the ether-treated mutant M269 as an enzyme system under improved experimental conditions, the reaction rate could be drastically increased. The original incubation time of 6 hours turned out to be too long. No increase in the amount of deacetoxycephalosporin C was observed after 3 hours. The best substrate conditions were found to be quite similar to those described by SAWADA *et al.*[1]. The diverging optimal conditions for ATP and Fe^{2+} might arise from the different enzyme systems and strains used. In contrast to the observations of SAWADA *et al.*[1], 2-oxoglutaric acid promotes the reaction (Fig. 5). We used a higher penicillin N concentration (0.8 mmolar) to avoid substrate depletion which can

occur under our better reaction conditions. Initially we obtained 30 μmoles/liter deacetoxycephalosporin C after 6 hours of incubation. Under optimized reaction conditions the yield was increased to 0.37 mmoles/liter after 3 hours.

As in the first experiments the strains 18.2.15 and N-2 produced no detectable amounts (HPLC) of deacetoxycephalosporin C using optimal reaction conditions. As shown in Fig. 3c, deacetylcephalosporin C was not synthesized either. In contrast, the mutant M92 converted as much penicillin N to deacetoxycephalosporin C as mutant M269.

In a control experiment, the "intact" cells of the mutant M269 were compared with ether-treated cells. Ether-treatment was not necessary for the potent penicillin N producing, cephem negative strain M269, in contrast to the inferior cephalosporin C and penicillin N producing strain C462 which was described in an earlier publication[3]. M269 cells can be permeabilized by transfer from production medium to phosphate buffer. This osmotic shock treatment is widely used[4]. We could demonstrate the permeabilizing effect by measurement of the hexokinase/glucose-6-phosphate dehydrogenase system as described earlier[3].

To further characterize the ring expansion reaction we carried out several control experiments. In addition to HPLC, we run reversed-phase thin-layer chromatography plates (Table 1). Deacetoxycephalosporin C has the same Rf-value as the authentic product of the ring expansion. It can be destroyed by β-lactamase P-99 but not with β-lactamase I (penicillinase). The filtrate of the reaction mixture was tested on *A. faecalis* (Table 2). The deacetoxycephalosporin C concentrations determined with HPLC could be confirmed. The strain 18.2.15 inhibited *A. faecalis* too, but this was due to residual traces of cephalosporin C from the fermentation broth. By thin-layer chromatography using UV detection at 254 nm no deacetoxycephalosporin C or deacetylcephalosporin C could be determined (detection limit less than 10 mg/liter). Cephalosporin C was not synthesized *de novo* since no increase of the amount was detectable by HPLC. Moreover, the terminal step of the biosynthesis would require the presence of acetyl-coenzyme A[8].

Table 1. Reversed-phase thin-layer chromatography of products and β-lactam antibiotics in three solvent systems (Rf values).

Material	Solvent system		
	I	II	III
Product	0.54	0.49	0.39
Penicillin N*	0.56	0.47	0.39
Deacetoxycephalosporin C	0.56	0.51	0.40
Deacetylcephalosporin C	0.76	0.73	0.60
Cephalosporin C	0.42	0.32	0.23

Solvent systems: I=water-dioxan (98 : 2); II=water, III=1 % aqueous sodium sulfate.
Detection: UV light at 254 nm.
* Not visible as the compounds were detected at 254 nm; penicillin N can be detected with ninhydrin spray reagent.

Discussion

To the best of our knowledge, no penicillin N overproducing mutants have been mentioned in the literature so far. We found several cephem negative mutants producing up to 50 mmoles penicillin N per liter. The titer of the cephem positive parent strains was about tenfold lower. The filtrate of the M269 culture contains exclusively penicillin N. Isopenicillin N, an assumed intermediate of the cephalosporin biosynthesis, was not detectable.

We could also measure the conversion of penicillin N to deacetoxycephalosporin C. We were able to increase the conversion rate from 15 μmoles/liter/hour[1] to 120 μmoles/liter/hour. Highly purified natural penicillin N was used as a substrate. The results were easily reproducible. Without the addi-

Table 2. Biological test of compounds.

Osmotically shocked ("intact") or ether-treated cells were incubated for 3 hours at 25°C with optimal conditions. The filtrates of the reaction mixture were concentrated 10-fold. The results refer only to the ether-treated and osmotically shocked cells. The sonicates were not tested.

Strain	Treatment	Inhibitory zones on *A. faecalis* (mm)
Penicillin N producing mutant M 269	ether-treated	17
	ether-treated+β-lactamase P-99*	0
	ether-treated+β-lactamase I*	15
	ether-treated, no addition of penicillin N	0
	"intact"	17
	"intact"+β-lactamase P-99*	0
	"intact"+β-lactamase I*	15
	"intact", no addition of penicillin N	0
Non-producing strains M 92	ether-treated	15
	ether-treated+β-lactamase P-99*	0
	ether-treated+β-lactamase I*	14
	ether-treated, no addition of penicillin N	0
Cephalosporin producing strain 18.2.15**	ether-treated	25
	ether-treated+β-lactamase P-99*	0
	ether-treated+β-lactamase I*	22
	ether-treated, no addition of penicillin N	25
Peptide producing mutant N-2	ether-treated	0
	"intact"	0
	Deacetoxycephalosporin C (in H_2O) 27 mM	28
	2.7 mM	19
	0.27 mM	0
	Penicillin N (in H_2O) 27 mM	0

* β-Lactamases added after 3 hours of incubation to the reaction mixture.

** Ether-treated cells of strain 18.2.15 are characterized by a release of cephalosporin C, which causes a high zero time value of biological activity. This activity is not increased by exogenously added penicillin N.

tion of penicillin N to the reaction mixture no deacetoxycephalosporin C was formed. The enzyme system carrying out the ring expansion behaved similar to a 2-oxoglutarate dependent dioxygenase which contrasts with the results of SAWADA *et al.*[1]. The ring expansion could be demonstrated in the penicillin N excreting strain M269 and the non-producing mutant M92. No reactions giving similar yields were detectable in the cephalosporin producing strain 18.2.15 and the peptide mutant N-2. However, SAWADA *et al.*[1] were able to detect much smaller amounts of deacetoxycephalosporin C using supersensitive test organisms. Our findings seem to support the hypothesis that the exogenously added penicillin N can only reach the ring expanding enzyme complex in the mutants M269 and M92. It can be assumed that the biosynthetic enzymes are not organized normally in those mutants. The overproduction of penicillin N can be attributed to a premature elimination of this metabolic intermediate from the biosynthetic complex. Several analogous cases have been described in the literature: In *E. coli*, such mutants were found for two systems. Some strains, unable to grow on non-fermentable carbon sources, display the release of their ATPase from the membrane complex[13,14]. Certain chlorate-resistant mutants are unable to form a proper membrane aggregate of nitrate reductase[15,16]. In *Neurospora crassa*,

some mutants, unable to use malate as a sole carbon source, have been reported to show redistribution of malate dehydrogenase within the cell[17]. The highly developed system of organellar membranes in fungi could make enzyme subcellular mislocation a relatively common mutational event. The ring expansion enzyme system behaves as a 2-oxoglutarate dependent dioxygenase.

Recent publications have shown that oxidative enzyme systems are also involved in the cyclisation reaction of the tripeptide to isopenicillin N[18], the ring expansion of penicillin N to deacetoxycephalosporin C[1,2], and the hydroxylation of deacetoxycephalosporin C[8,10] to deacetylcephalosporin C.

The techniques described in this paper give high conversion rates of the ring expansion reaction by using cells and extracts of an appropriate mutant.

In most of the cephalosporin C producing wild strains, the proportion of cephem compounds accounts for more than 90% of the total β-lactam antibiotics formed, whereas the amount of penicillin N excreted into the culture broth is usually less than 10%. From this we might assume that the biosynthetic reaction sequence has been partially interrupted at the penicillin N stage causing a minor excretion of this precursor by normal cephem producers.

It seems likely that this minor interruption has become a complete block in our mutant M269. Consequently, penicillin N is formed as the only β-lactam compound and excreted in a tenfold higher concentration. Cephem negative, penicillin N producing mutants have already been described in a previous publication[19]. However, these mutants did not overproduce penicillin N, and they were more frequently isolated than mutants such as M269. It would be particularly interesting to check the ring expansion activity of these earlier mutants under the reaction conditions worked out for mutant M269. Unfortunately, these strains are no longer viable. New isolates are in preparation for these experiments. If they were impaired in their ability to carry out the ring expansion or totally incapable of doing so under the above described experimental conditions, this would support the assumption that a functional block has been introduced into the gene coding for the dioxygenase. Otherwise, the contradictory behaviour of M269 might be explained by the former hypothesis of subcellular mislocation[13,14] of an unknown component in a structured membrane bound enzyme complex.

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