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ORIGINAL PAPER

J. Basch · T. Franceschini · S. Tonzi · S.-J.D. Chiang

Expression of a cephalosporin C esterase gene in *Acremonium chrysogenum* for the direct production of deacetylcephalosporin C

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Abstract A recombinant fungal microorganism capable of producing deacetylcephalosporin C was constructed by transforming a cephalosporin C esterase gene from *Rhodosporidium toruloides* into *Acremonium chrysogenum*. The cephalosporin C esterase gene can be expressed from its endogenous *R. toruloides* promoter or from the *Aspergillus nidulans trpC* promoter under standard *Acremonium chrysogenum* fermentation conditions. The expression of an active cephalosporin C esterase enzyme in *A. chrysogenum* results in the conversion of cephalosporin C to deacetylcephalosporin C in vivo, a novel fermentation process for the production of deacetylcephalosporin C. The stability of deacetylcephalosporin C in the fermentation broth results in a 40% increase in the cephalosporin nucleus.

Keywords Antibiotics · Cephalosporin C · Deacetylcephalosporin C · Cephalosporin C esterase · *Acremonium chrysogenum* · *Rhodosporidium toruloides*

Introduction

Cephalosporin C is produced by fermentation of the fungal microorganism, *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*) [1]. For the commercial production of cephalosporins, cephalosporin C is chemically converted to 7-aminocephalosporanic acid (7-ACA), a β-lactam nucleus used in the manufacture of semisynthetic cephalosporins. In fermentation broth, non-enzymatic breakdown of cephalosporin C to 2-(D-4-amino-4-carboxybutyl)-thiazole-4-carboxylic acid (compound X) results in the loss of approximately 40% of the cephalosporin C produced (Fig. 1) [2]. Deacetylcephalosporin C, however, is far more resistant to this chemical reaction. In the β-lactam ring-opening hydrolysis of cephalosporins, the 3′-acetoxymethyl group is hypothesized to function as an electron-accepting leaving group [3]; and deacetylcephalosporin C is more stable because the remaining hydroxyl is less effective as an electrophile for this reaction. The insertional inactivation of the cephalosporin C acetyltransferase gene, the last step in the cephalosporin C biosynthetic pathway, results in the accumulation of deacetylcephalosporin C. However, a loss in the productivity of these transformants was observed [4]. Baldwin et al. [5] showed that deacetylcephalosporin C can be oxidized by the exandase/ hydroxylase enzyme, resulting in a subsequent hydrolysis of the β-lactam ring. Therefore, accumulation of intracellular deacetylcephalosporin C may result in the loss of the cephalosporin nucleus through this reaction [5].

A cephalosporin C esterase enzyme produced by the basidiomycetous yeast *Rhodosporidium toruloides* was identified which can deacetylate cephalosporin C to form deacetylcephalosporin C [6]. An 80-kDa glycoprotein with cephalosporin C esterase activity was purified from *R. toruloides* and the N-terminal amino acid sequence was determined [6]. The genomic cephalosporin C gene sequence was cloned using degenerate oligonucleotide probes designed to this N-terminal amino acid sequence. The presence of an N-terminal signal sequence in the cephalosporin C esterase gene and the observation that the enzyme activity could be released from the cells by treatment with EDTA suggest the enzyme is expressed on the extracellular surface of the *R. toruloides* cell membrane [6].

When *R. toruloides* cells are added to an *A. chrysogenum* fermentation, the cephalosporin C esterase enzyme can convert cephalosporin C to deacetylcephalosporin C, resulting in an increase in the

J. Basch · S. Tonzi
Analytical Biochemistry, Technical Operations,
Bristol-Myers Squibb Company,
Syracuse, NY 13221-4755, USA

J. Basch (✉) · T. Franceschini · S.-J.D. Chiang
Fermentation and Biocatalysis Development,
Technical Operations, Bristol-Myers Squibb Company,
Syracuse, NY 13221-4755, USA
Tel.: +1-315-4329578
Fax: +1-315-432-4785

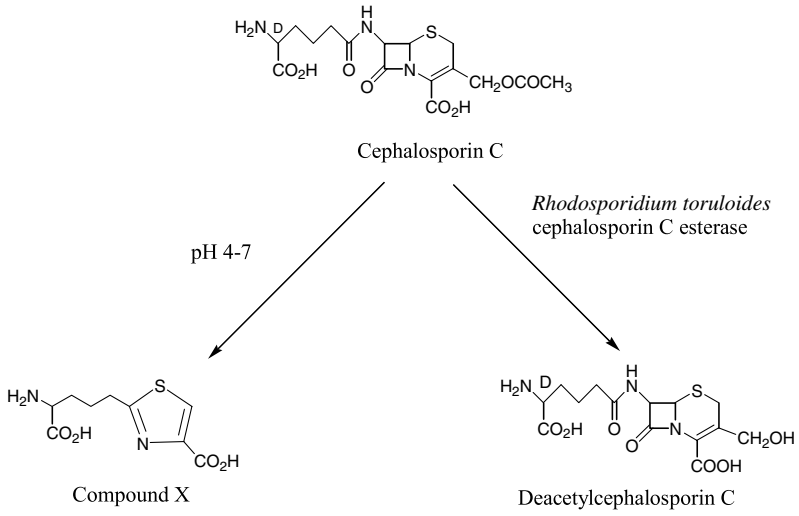


Fig. 1 Molecular structures of cephalosporin C, deacetylcephalosporin C and compound X

yield of the cephalosporin nucleus (Fig. 1). This conversion is observed with viable *R. toruloides* cells and with cells which have been killed by treatment with decanol. In order for this increase in cephalosporin nucleus to be realized as an increase in the final yield of 7-ACA, an effective chemical or enzymatic route from deacetylcephalosporin C to 7-ACA is required. The cephalosporin C esterase enzyme was found to acetylate deacetylcephalosporin C to form cephalosporin C in the presence of an acetyl donor [6]. Therefore, the deacetylcephalosporin C could be converted back to cephalosporin C by cephalosporin C esterase for the subsequent production of 7-ACA.

Expression of the cloned cephalosporin C esterase gene, both the cDNA [6] and genomic copy, in recombinant *A. chrysogenum* cells resulted in the in vivo conversion of cephalosporin C to deacetylcephalosporin C and a fermentation process for the direct production of deacetylcephalosporin C.

Materials and methods

Chemicals and enzymes

Unless otherwise noted, chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo.). Reagent salts and organic solvents were obtained from Fisher Scientific (Pittsburgh, Pa.). Polyethylene glycol was purchased from Fluka Chemical (Milwaukee, Wis.) and Novozyme 234 from InterSpex Products (Foster City, Calif.). Restriction enzymes and T4 DNA ligase were obtained from Life Technologies (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.).

Microbial strains

Escherichia coli DH5α-MCR [F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d *lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *sup*E44 *thi*-1 λ^- *gyr*A96 *rel*A1] was purchased from Life Technologies and used for all plasmid constructions and propagation, unless otherwise noted. *E. coli* XL1-blue cells [*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*(F'*pro*AB *lac*I^q ZΔ M15 *Tn*10)] were purchased from Stratagene (La Jolla, Calif.).

A cephalosporin C high-producing strain, BC1386, was derived from *A. chrysogenum* CW19 (ATCC 36225), obtained from the American Type Culture Collection (ATCC; Manassas, Va.) by multiple rounds of mutagenesis and screening for improved cephalosporin C production through a long-term strain improvement program at Bristol–Myers Squibb (BMS). BC1386 was used as a host for DNA transformation and as a control in the fermentation studies. The *R. toruloides* culture (ATCC 10657) was obtained from the ATCC. *R. toruloides* cells were killed by treatment with decanol prior to addition to shake-flask or to 14-l and 4,000-l tank fermentations.

Media and buffers

E. coli was grown in Luria Broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) or LB agar (LB supplemented with 1.5% agar). *A. chrysogenum* was propagated in PV media (2.4% malt extract, 2.7% yeast extract, 1% peptone, 0.1% $CaCO_3$) and YE agar (1% malt extract, 0.4% yeast extract, 0.4% glucose, 2% agar, pH 7.3). For all fermentation experiments in shake-flasks and fermentors, BMS proprietary seed and fermentation media, consisting of corn syrup, corn steep liquor, DL-methionine, soyflour and Pharmamedia (Trader Protein, Memphis, Tenn.), were used to evaluate the deacetylcephalosporin C productivity.

The following buffers were used for this study: TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), TAE electrophoresis buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) and 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0).

Preparation of chromosomal DNA of *R. toruloides*

Fifty milliliters of *Rhodosporidium* growth medium (2% glucose, 1% yeast extract, 1% Bacto-peptone, 0.5% KH_2PO_4, pH 6.0) in a 250-ml Erlenmeyer flask were inoculated at 4% with a frozen culture of *R. toruloides*. The culture was grown at 28°C in a shaker at 250 rpm for 24 h. Cells were harvested by centrifugation and washed once in buffer containing: 1 M sorbitol, 50 mM sodium citrate, pH 5.4. Cells were centrifuged again and resuspended in wash buffer containing 0.5% lysing enzymes (L-8757; Sigma Chemical Co.) at 37°C for 3 h. Spheroplasts were collected by centrifugation and digested (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1.0% SDS, 100 μg/ml proteinase K). The solution was then incubated at 50°C for 16 h. The mixture was extracted twice, first with phenol:chloroform:isoamyl alcohol mixture (25:24:1) and then with chloroform:isoamyl alcohol mixture (24:1); and the DNA was then precipitated with 70% ethanol. The DNA was recovered by centrifugation, washed with 70% ethanol and the DNA pellet was dissolved in TE with 100 μg/ml RNase A and incubated at 37°C for 16 h. The organic solvent mixture extractions and ethanol precipitations were repeated and the DNA was dissolved in TE. The DNA concentration was then determined spectrophotometrically.

Construction of genomic DNA library of *R. toruloides*

The purification of cephalosporin C esterase and determination of the N-terminal sequence have been described [6]. From the N-terminal amino acid sequence (H_2N-Thr-Asn-Pro-Asn-Glu-Pro-Pro-Pro-Val-Val-Asp-Leu-Gly-Tyr-Ala- Ala), four 17-mer degenerate oligonucleotides were synthesized (5′-GGYTCRTTGGGRTTNGT-3′, 5′-GGYTCRTTAGGRTTNGT-3′, 5′-GGYTCRTTTGGRTTNGT-3′, 5′-GGYTCRTTCGGRTTNGT-3′). Four oligonucleotides were designed to reduce the degeneracy of each individual probe fourfold. The oligonucleotides were end-labeled with [$\gamma-^{32}$P]ATP, and used to probe a Southern blot of *R. toruloides* chromosomal DNA digested with restriction endonucleases *Bam*HI and *Pst*I . Hybridization was conducted in tetramethylammoniumchloride (Sigma Chemical Co.) buffer at 46.8°C for 48 h. A 3-kb *Bam*HI fragment hybridized to one of the probes; and a partial genomic library was constructed by digesting genomic DNA with *Bam*HI, isolating the 3-kb fraction and ligating the genomic fragments to pBluescript KS+ phagemid (Stratagene) cleaved with *Bam*HI. The ligation mixture was used to transform *E. coli* XL1-blue cells by electroporation at 2.5 kV, 200 Ω, 25 μF, using a Bio-Rad electroporator (Hercules, Calif.). The transformants were selected on LB agar containing 100 μg/ml ampicillin and screened by colony blot hybridization, using the oligonucleotide probe. A plasmid was identified containing a 3.0-kb insert which strongly hybridized to the oligonucleotide probe. DNA sequencing of the plasmid confirmed the presence of the N-terminal amino acid sequence. This plasmid was designated pBMesterase1.

Construction of the genomic cephalosporin C esterase with its endogenous *R. toruloides* promoter

The 2.2-kb *R. toruloides* genomic cephalosporin C esterase gene sequence was isolated from pBMesterase1 with *Pst*I and *Bam*HI cleavage and ligated to the vector pBluescriptII KS+. The ligation mixture was transformed to DH5α-MCR competent cells. The constructed plasmid was designated pBMesterase1a (Fig. 2). A DNA linker fragment generated by annealing the two oligonucleotides (5′-GATCACCCGGGT-3′, 3′-TGGGCCCACTAG-5′) was synthesized to convert the *Bam*HI site of pBMesterase1a to a *Sma*I site, useful for subsequent plasmid construction. The resulting plasmid was designated pBMesterase1b (Fig. 2).

A DNA fragment from the 5′ end of the cephalosporin C esterase gene was digoxigenin (Dig)-labeled and used to probe a Southern blot of *R. toruloides* chromosomal DNA cleaved with *Pst*I. A single hybridizing band at 1.6-kb was observed. The 1.6-kb *Pst*I genomic DNA fragment was isolated and ligated into the pBluescriptI KS+ vector. The ligation mixture was transformed to DH5α-MCR competent cells and plated on LB agar with 100 μg/ml ampicillin. The Dig-labeled esterase probe was used to screen colony blots of the partial genomic library. A plasmid with a 1.6-kb insert which strongly hybridized to the cephalosporin C esterase probe was identified and designated pBMesterase2 (Fig. 2). This cloned 1.6-kb fragment was isolated from *Pst*I-cleaved pBMesterase2 and ligated to the *Pst*I-cleaved plasmid pBMesterase1b containing the coding region of the esterase gene. The ligation mixture was transformed to DH5α-MCR cells. The ligated plasmids were screened for the correct orientation of the insert by a *Bam*HI/*Eco*RI double digest. The newly constructed plasmid, pBMesterase3 (Fig. 2) contained an intact cephalosporin C esterase genomic gene sequence and about 750 bp of the promoter region.

Cloning and sequencing of the cephalosporin C esterase cDNA from *R. toruloides*

The cDNA was cloned using 3′ rapid amplification of cDNA ends with a gene-specific primer designed from the genomic cephalosporin C esterase gene sequence, as previously described [6]. The sequence of the cDNA gene was submitted to GenBank (accession # AF025410).

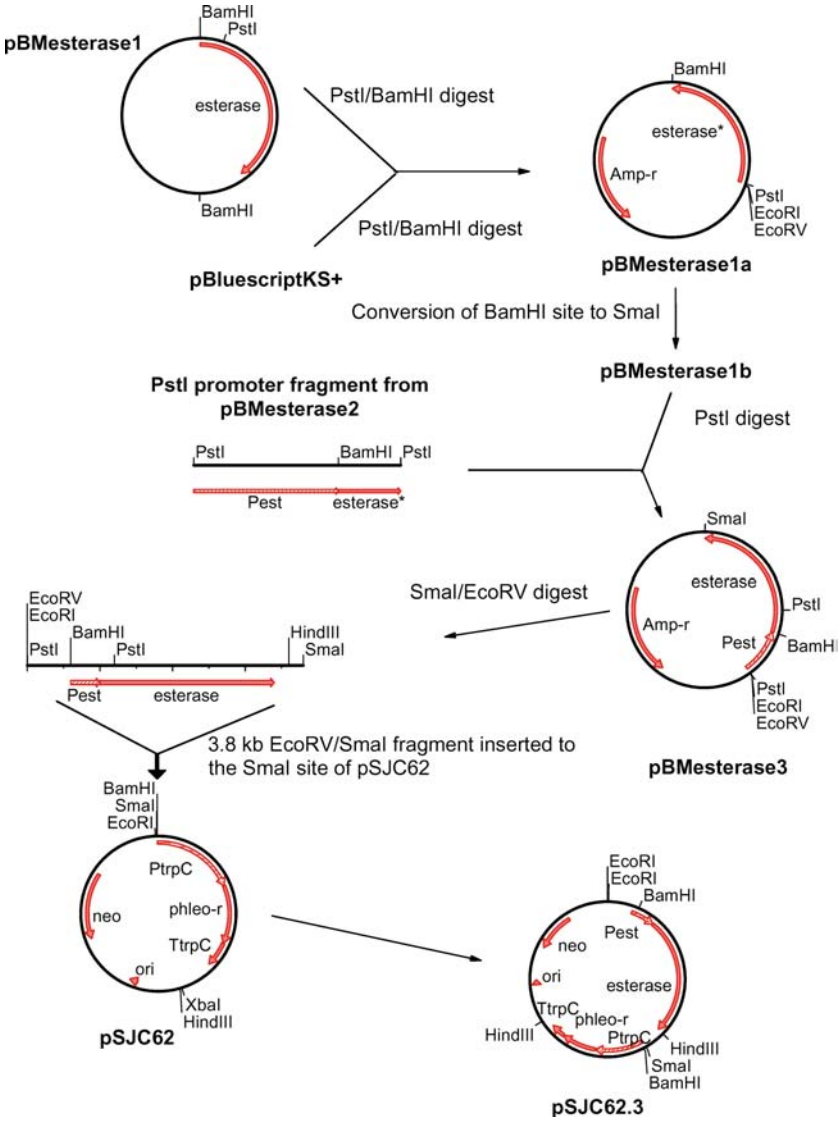


Fig. 2 Construction of plasmids pBMesterase3 and pSJC62.3. Plasmid pBMesterase3 was constructed by inserting a 2.2-kb *Pst*I/*Bam*HI genomic esterase gene fragment from plasmid pBMesterase1 into vector pBluescriptKS$^+$. The *Bam*HI site in the resulting plasmid, pBMesterase1a, was converted to a *Sma*I site and a 1.6-kb *Pst*I cephalosporin C esterase promoter fragment was inserted into the *Pst*I site of pBMesterase1a. Plasmid pBMesterase3 contains the genomic cephalosporin C esterase gene with its endogenous *R. toruloides* promoter. A 3.8-kb *Sma*I/*Eco*RV from vector pBMesterase3 was inserted into *Sma*I-digested pSJC26 to generate plasmid pSJC62.3. Plasmid pSJC62.3 contains the genomic cephalosporin C esterase gene with its endogenous *R. toruloides* promoter and the phleomycin resistance gene for selection of transformants

Construction of fungal vector pSJC62.3 with the endogenous *R. toruloides* promoter

Plasmid pSJC62.3 (Fig. 2) was constructed by inserting a 3.8-kb *SmaI*/*Eco*RV fragment from vector pBMesterase3 containing the genomic cephalosporin C esterase gene and its endogenous *R. toruloides* promoter into the fungal transformation vector pSJC62 [7], which had been cleaved with *Bam*HI and filled in with Klenow enzyme. The ligation mixture was transformed to DH5α-MCR cells. Plasmid pSJC62.3 contained the cephalosporin C esterase gene transcribed in the same direction as the phleomycin resistance gene (Fig. 2).

Construction of fungal vectors pBMesterase6, pBMesterase8 and pBMmat-esterase with the *Aspergillus nidulans trpC* promoter

Vectors containing the *R. toruloides* cephalosporin C esterase gene under the control of the *Aspergillur nidulans trpC* promoter were constructed to determine whether they would result in higher expression levels of the cephalosporin C esterase in *Acremonium chrysogenum*.

Plasmid pJB10 containing the *Aspergillus nidulans trpC* promoter and terminator for gene expression was constructed from plasmids pSCN43[8], pWB19N and pSJC62 [7].

The primers, Rc2a$^+$ (5′-ACTCGCCGCCATG *G*TCCTTAACCTCTTCAC-3′) and Rc4$^-$ (5′-GAAGGATC *C*CTAGAGACCCGCGTTCACCGA-3′) were used for PCR amplification of the cephalosporin C esterase gene for insertion into plasmid pJB10. The G and C bases in italics were modified in the primers to generate a *Nco*I site in primer Rc2a$^+$ and a *Bam*HI site in Rc4$^-$. The genomic and cDNA copies of the cephalosporin C esterase gene fragments, including the leader sequence, were amplified by PCR from vector pSJC62.3 and from a *R. toruloides* cDNA template, respectively [6]. The 2,048-bp PCR product from the genomic cephalosporin C esterase template and the 1.9-kb cDNA product were digested

with *Nco*I and *Bam*HI and gel-purified on 0.7% agarose. Each of the esterase gene fragments was then ligated to plasmid pJB10. For the cDNA gene insert, the resulting plasmid was named pBMesterase6 (Fig. 3). For the genomic gene insert, the resulting plasmid was further digested with *Eco*RI and *Xba*I, filled in with Klenow enzyme and a 4.0-kb fragment was isolated on a 0.7% agarose gel. Plasmid pSJC65, containing a phleomycin resistance cassette, was digested with *Bam*HI and filled in with Klenow enzyme. After ligation to the 4.0-kb fragment containing the *trpC* promoter and the genomic cephalosporin C esterase gene, the DNA mixture was transformed to DH5α-MCR cells. The resulting plasmid, named pBMesterase8, contained the phleomycin resistance gene and the cephalosporin C esterase genomic gene under the control of the *trpC* promoter (Fig. 3). Plasmid pBMmat-esterase was constructed as described for pMesterase8, but with the 5′ PCR primer designed to start at the beginning of the mature cephalosporin C esterase sequence (Fig. 3).

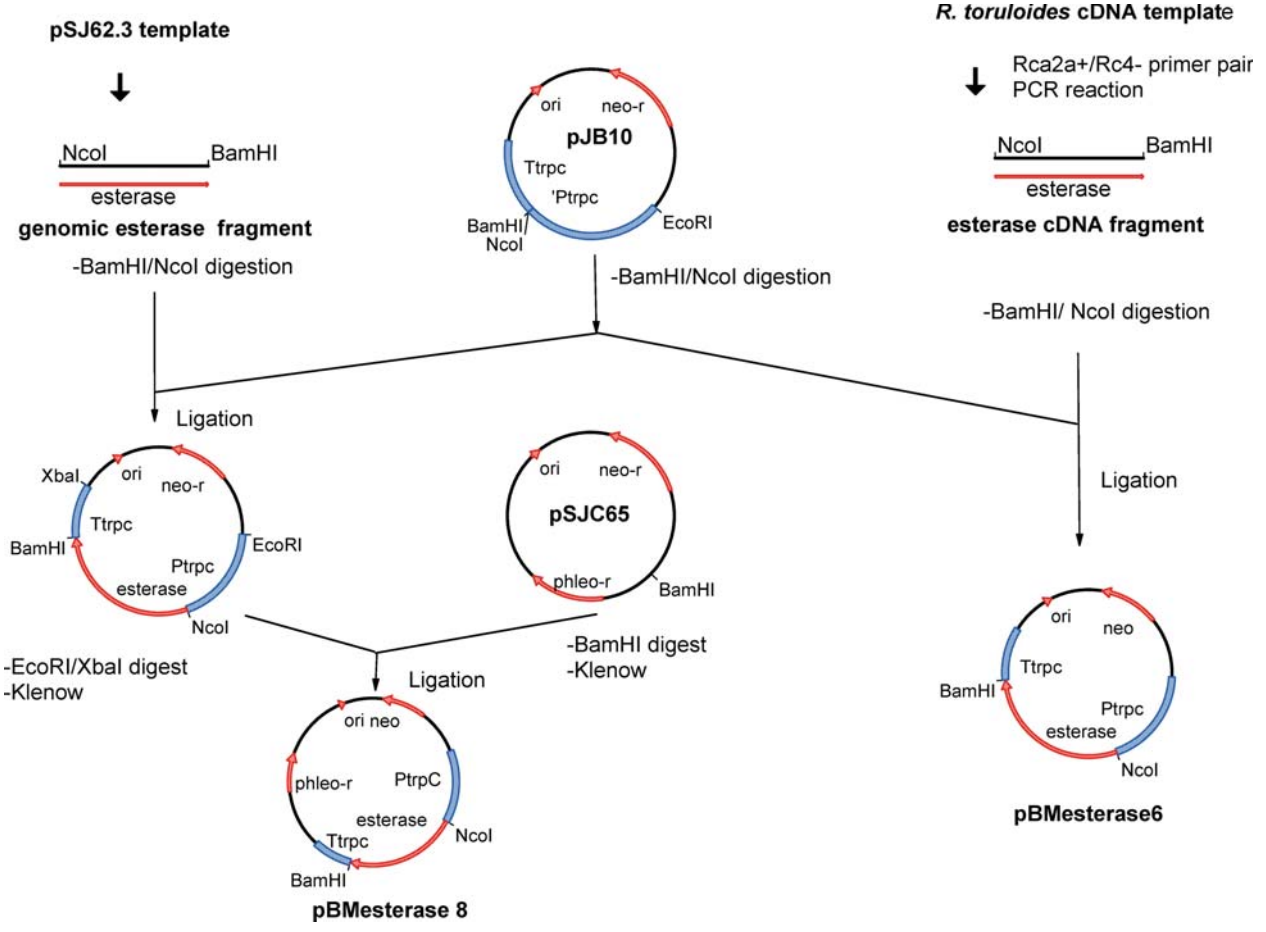
Acremonium chrysogenum DNA transformation

The procedure used to transform DNA into *Acremonium chrysogenum* cells was described previously [9]. Since plasmid pBMesterase6 does not contain a selectable marker, this plasmid was co-transformed with plasmid pSJC62 carrying the phleomycin resistance marker. Transformants were selected based upon phleomycin resistance and were subsequently evaluated for the integration of plasmid pBMesterase6 by dot blot hybridization.

Isolation of *A. chrysogenum* genomic DNA, gel electrophoresis and Southern hybridization

The procedures used to isolate DNA from *A. chrysogenum* cells and for gel electrophoresis and Southern blotting were described previously [9]. The filter was prehybridized at 50°C for 30 min in 10 ml of Dig-Easy Hyb buffer (Roche Molecular Biochemicals, Indianapolis, Ind.). Five microliters of a PCR-generated Dig-labeled probe (10 ng/μl) specific to the neomycin resistance gene was diluted in 1 ml of Dig-Easy Hyb buffer and denatured in a boiling water bath for 10 min. The denatured probe was then placed on ice for 2 min.

Fig. 3 Construction of plasmids pBMmat-esterase, pBMesterase6 and pBMesterase8. Plasmids pBMmat-esterase, pBMesterase6 and pBMesterase8 contain the mature esterase, esterase cDNA and esterase genomic gene, respectively, under the control of the *Aspergillus nidulans trpC* promoter



Five milliliters of the prehybridization solution was poured off the filters into a 14-ml polypropylene tube. The probe was added to this solution and pipetted back onto the filter. The filter was then hybridized at 50°C overnight. The filter was washed two times in 2× SSC, 0.1% SDS for 5 min at 25°C and two times in 0.5× SSC, 0.1% SDS for 15 min at 65°C and was then treated for the detection of Dig-labeled DNA hybridization.

Shake-flask evaluation of deacetylcephalosporin C fermentation

After 2 weeks of incubation of transformation plates, phleomycin-resistant transformants were transferred to YE agar plates containing 50 μg/ml phleomycin and incubated for 7 days at 28°C. Colonies were then used to inoculate slants containing 6 ml of YE agar and grown for 7 days at 28°C. Two milliliters of sterile H_2O were used to resuspend the culture from each slant and 1 ml of the resuspended culture was then inoculated into 25 ml of seed media in a 125-ml Erlenmeyer flask. The seed cultures were cultivated in a shaker for 48 h at 28°C, 250 rpm. Two milliliters of the seed culture was then transferred to 20 ml of fermentation media in a 125-ml bi-indented Erlenmeyer flask and grown for 7 days at 24°C, 250 rpm. Whole broth was used for a HPLC assay of the concentration of cephalosporin C and deacetylcephalosporin C. Sixty-eight transformants each were evaluated for the plasmids pSJC62.3 and pBMesterase8; and 35 and 29 transformants were evaluated for the plasmids pBMmat-esterase and pBMesterase6, respectively. The highest deacetlycephaslosporin C producers were then evaluated with a positive control of the *A. chrysogenum* BC1386 culture with the addition of 100 μl of decanol-treated *R. toruloides* cells.

Tank fermentation at 14 l and 4,000 l

The BC1386 parental strain and two transformants (DC3, containing plasmid pSJC62.3; DC11, containing plasmid pBMesterase8) were evaluated in 14-l tank fermentations for deacetylcephalosporin C and cephalosporin C titers. Decanol-treated *R. toruloides* whole cells were added to the fermentation broth of the BC1386 strain at a 1:200 dilution. The DC11 culture was evaluated at 4,000-l scale for deacetylcephalosporin C productivity as compared with BC1386 with decanol-treated *R. toruloides* whole cells.

Results

Identification of clones containing the cephalosporin C esterase gene

Colony blots of the 3.0-kb *Bam*HI partial genomic library were prepared and screened with a degenerate oligonucleotide probe designed to the N-terminal sequence. Colonies hybridizing to this probe were identified and the confirmed plasmid was named pBMesterase1 (Fig. 2). The sequence of the cephalosporin C esterase gene was submitted to GenBank (accession # BD005939). Translation of the cloned DNA sequence produced an amino acid sequence that matched the N-terminal protein sequence of the purified cephalosporin C esterase enzyme [6]. A total of five introns were found in the cephalosporin esterase gene. A comparison of the N-terminal sequence of the purified cephalosporin C esterase and the nucleotide sequence of the gene indicates that there is a 27-amino acid N-terminal leader sequence that is cleaved to produce the mature enzyme. Further analysis of the 3-kb *Bam*HI fragment by Southern blotting and DNA sequencing determined the location and orientation of the cephalosporin C esterase gene within the fragment. Using primer extension analysis [10] of the mRNA isolated from *R. toruloides* culture, it was determined that the translation start site is 77-bp from one end of the cloned *Bam*HI fragment and, therefore, the cloned upstream region is short of the entire endogenous promoter sequence. As illustrated in Fig. 2, a 1.6-kb *Pst*I genomic fragment containing the endogenous promoter sequence of the esterase was cloned separately. pBMesterase3 was constructed to contain a full-length cephalosporin C esterase genomic gene and its promoter.

Status of transforming plasmids in transformants

The transforming plasmids do not contain a fungal origin of replication. Previous characterization of stable, phleomycin-resistant transformants revealed that the transforming plasmid was forced to integrate into the host chromosome [9]. The use of integrative plasmids to deliver the gene is desirable for a commercial fermentation process, as no phleomycin selection is required after the initial identification of the transformants [11]. Removal of phleomycin from the fermentation broth would challenge the downstream purification steps and create a significant regulatory barrier to the use of the recombinant cultures. To confirm that the transforming plasmid was integrated into the chromosome of the phleomycin-resistant transformants selected in this study, genomic DNA was isolated from the pSJC62.3 transformants DC1, DC2 and DC3 and from the host culture BC1386. The DNA was cleaved with *Eco*RI, separated on an agarose gel and transferred to a Nylon membrane. The membrane was hybridized to a PCR-generated probe specific to the neomycin resistance gene. Plasmid pSJC62.3 is 9.8-kb with two *Eco*RI sites separated by 22-bp. Since a crossover event occurring between these two restriction sites upon integration is unlikely, each hybridizing band in the Southern blot was expected to correspond to integration at a distinct chromosomal location. The Southern hybridization

analysis (Fig. 4) indicated that the neomycin resistance gene probe hybridized to the transformant DNA, but not to that of the untransformed host DNA. The neomycin resistance gene is adjacent to the *Eco*RI sites in the plasmid, so integration of the plasmid just outside of the neomycin gene could result in a hybridizing band smaller than the plasmid, as is observed in transformants DC1 and DC2. The transformants have multiple copies of the neomycin resistance gene, indicating multiple integration sites for the plasmid. Multiple integration events are often observed in the transformation of filamentous fungi. Multiple site integration may occur via independent heterologous integration events, recombination between plasmids prior to integration, or subsequent recombination after integration [12]. In *A. chrysogenum*, transformation with circular plasmid forms, as was employed in this study, was found to result in more complex hybridization patterns than transformation with linearized vectors [13].

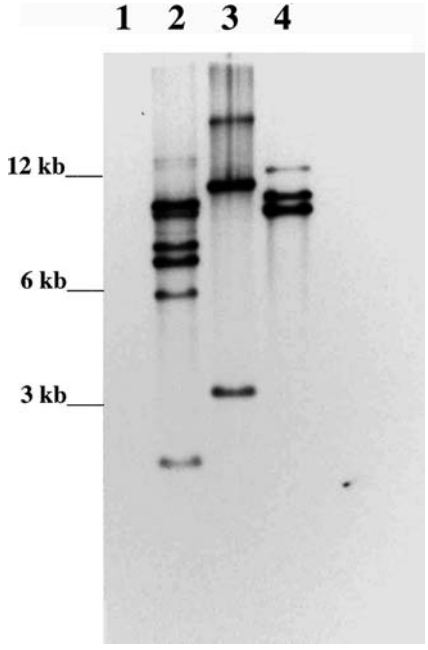


Fig. 4 Southern hybridization analysis of three pSJC62.3 transformants. *Lane 1* 5 μg *Eco*RI-digested BC1386, *lane 2* 5 μg *Eco*RI-digested DC1, *lane 3* 5 μg *Eco*RI-digested DC2, *lane 4* 5 μg *Eco*RI-digested DC3. The blot was hybridized at 50°C overnight with 50 ng of a probe specific to the neomycin resistance gene in 5 ml of Dig-Easy Hyb buffer. The filter was washed two times, for 5 min at 25°C, with 2× SSC, 0.1% SDS and two times, for 15 min at 65°C, with 0.5× SSC, 0.1% SDS. The filter was then treated for detection of Dig-labeled DNA hybridization. A 1-kb size standard was used to determine the size of the hybridizing bands

Shake-flask evaluation of deacetylcephalosporin C fermentation

Of the 68 pSJC62.3 transformants evaluated, 49 (72%) were found to produce primarily deacetylcephalosporin C. Of the 68 pBMesterase8 transformants evaluated, 36 (52%) were found to produce primarily deacetylcephalosporin C. Of 29 pBMesterase6 transformants screened, 7 (24%) were found to produce primarily deacetylcephalosporin C. The results of repeated shake-flask evaluation of selected transformants indicated that the pSJC62.3 (DC3), pBMesterase6 (DC11) and pBMesterase8 (DC14) transformants were found to produce only deacetylcephalosporin C under standard shake-flask fermentation conditions. The pBMmat-esterase (DC15) transformants lacking the 27-amino acid leader sequence and untransformed BC1386 produced predominantly cephalosporin C. BC1386 with and without the addition of 100 μl of decanol-treated *R. toruloides* cells was included as positive and negative controls for the production of deacetylcephelosporin C. The cephalosporin C and deacetylcephalosporin C titers from representative transformants are reported in Table 1. No significant differences in deacetylcephalosporin C titers or residual cephalosporin C were observed at the shake-flask scale between the transformants containing the genomic and cDNA cephalosporin C esterase genes and between the *Aspergillus nidulans trpC* promoter and the endogenous *R. toruloides* promoter constructs.

Tank evaluation (14 l) of deacetylcephalosporin C fermentation

The deacetylcephalosporin C titers of BC1386, with the addition of *R. toruloides* whole cells, reached a maximum value of 150 units at 168 h of fermentation. DC11 and DC14 reached maximum deacetylcephalosporin C titers of 166 units and 153 units, respectively, at 196 h. DC3 reached a maximum titer of 116 units at 192 h. The DC3 transformant, unlike BC1386, DC11 and DC14,

Table 1 Shake-flask evaluation of the recombinant *Acremonium chrysogenum* strains. Titers are expressed in relative units/gram of fermentation broth and are an average of four shake-flasks for each strain

Strain	Vector	Average cephalosporin C titer (units/g)	Average deacetylcephalosporin C titer (units/g)	Standard deviation
DC3	pSJC62.3	< 0.1	90	9.0
DC11	pBMesterase6	< 0.1	94	3.9
DC14	pBMesterase8	< 0.1	96	5.0
DC15	pBMmat-esterase	81	8	1.6[a]
BC1386	–	82	11	1.7[a]
BC1386 + *R. toruloides*	–	< 0.1	100	7.9

[a] Standard deviations of DC15 and BC1386 are those of the cephalosporin C values

accumulated some residual cephalosporin C in the fermentation broth (Fig. 5).

Tank evaluation (4,000 l) of transformant DC11

The deacetylcephalosporin C titers of DC11 and BC1386 with the addition of *R. toruloides* whole cells both reached similar maximum values: 235 units and 238 units, respectively. The BC1386 strain produced 164 units of cephalosporin C when fermented without the addition of *R. toruloides* whole cells (Table 2). The yield of cephalosporin nucleus was increased 40% by the direct fermentation of deacetylcephalosporin.

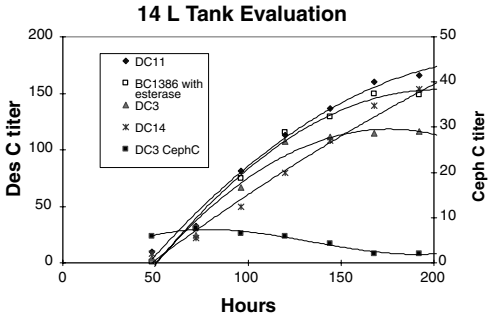


Fig. 5 Tank evaluation (14 l) of transformants. Two transformants (DC3, DC11) expressing the cephalosporin C esterase enzyme and a control (host strain BC1386 with *R. toruloides* whole cells added) were fermented for 200 h in 14-l tanks. The results presented are the average of two tank runs with each culture

Discussion

Deacetylcephalosporin C is the last intermediate in the cephalosporin C biosynthetic pathway and is present in the *A. chrysogenum* fermentation broth. The biosynthetic origin of this extracellular cephalosporin C is not due to secretion of this intermediate, but is most likely due to the presence of a cephalosporin C esterase produced by *A. chrysogenum* [14] . We have taken advantage of the stability of deacetylcephalosporin C to produce a recombinant *A. chrysogenum* strain capable of producing 40% more antibiotic nucleus under standard fermentation conditions.

A comparison of the N-terminal sequence of the purified cephalosporin C esterase and the nucleotide sequence of the cDNA indicates that there is a 27-amino acid N-terminal leader sequence that is cleaved to produce the mature enzyme [6]. This signal sequence was found to be required for cephalosporin C esterase activity in *A. chrysogenum*, as no increase in deacetylcephalosporin C was observed in *A. chrysogenum* transformants when this leader sequence was not present in the transforming plasmid (Table 1, DC15). The mature protein expressed without the leader sequence would not be targeted to the membrane.

The heterologous expression of the active cephalosporin C esterase in an *A. chrysogenum* host from the genomic gene and promoter indicates that: (1) the *Rhodosporidium* promoter is recognized by the *A. chrysogenum* RNA polymerase, (2) the five introns are correctly spliced, (3) the leader sequence correctly targets the protein and (4) the leader sequence is cleaved to produce the mature enzyme. In the shake-flask experiments, the transformants with either the endogenous *R. toruloides* esterase promoter or the *Aspergillus nidulans trpC* promoter yielded equivalent results. At the 14-l scale, however, higher deacetylcephalosporin C titers were achieved with the transformants utilizing the *A. nidulans trpC* promoter. A small quantity of cephalosporin C was also observed in the fermentation broth of DC3, indicating a lower level of cephalosporin C esterase activity in the transformants generated from plasmid pSJC62.3. The higher productivity of cephalosporin C in 14-l tanks as compared with shake-flasks may make differences in esterase expression levels between the constructs with the endogenous *R. toruloides* promoter and the *trpC* promoter more significant. It is also possible that the fermentation conditions resulted in changes in the levels of enzyme expression from the two promoters.

The stability of deacetylcephalosporin C in the recombinant *Acremonium chrysogenum* cultures is most likely due to localization of the cephalosporin esterase in the membrane [6]. The cephalosporin C produced does not have to be converted to deacetylcephalosporin C within the cell prior to release from the cell, but can be hydrolyzed during or after the secretion of cephalosporin C into the culture media. In transformant DC3, some cephalosporin C was present at the early timepoints of the 14-l tank fermentation, but this reduced as the fermentation progressed, suggesting that extracellular cephalosporin C is converted to deacetylcephalosporin C. The expression of esterase in the cytoplasm would result in the loss of deacetylcephalosporin C via oxidation by the expandase/hydroxylase enzyme and subsequent opening of the β-lactam ring, as was observed previously with insertional disruption of the acetyltransferase gene [4, 5].

Table 2 Tank evaluation (4,000 l) of transformant DC11. Titers are expressed in relative units/gram of fermentation broth and are an average of at least three tanks for each strain

Strain	Vector	Average cephalosporin C titer(units/g)	Average deacetylcephalosporin C titer (units/g)	Standard deviation
DC11	pBMesterase6	<0.1	235	9.2
BC1386	–	164	–	6.7
BC1386+ *R. toruloides*	–	<0.1	238	15.1

The fermentation of deacetylcephalosporin C by the recombinant expression of a cephalosporin C esterase was scaled-up to 4,000-l fermentors and proved to be an effective method for increasing the yield of the cephalosporin nucleus from *A. chrysogenum* fermentations.

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