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Genetic engineering approach to reduce undesirable by-products in cephalosporin C fermentation

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Deacetoxycephalosporin C (DAOC) is produced by *Acremonium chrysogenum* as an intermediate compound in the cephalosporin C biosynthetic pathway, and is present in small quantities in cephalosporin C fermentation broth. This compound forms an undesirable impurity, 7-aminodeacetoxycephalosporanic acid (7-ADCA), when the cephalosporin C is converted chemically or enzymatically to 7-aminocephalosporanic acid (7-ACA). In the cephalosporin C biosynthetic pathway of *A. chrysogenum*, the bifunctional expandase/hydroxylase enzyme catalyzes the conversion of penicillin N to DAOC and subsequently deacetylcephalosporin C (DAC). By genetically engineering strains for increased copy number of the expandase/hydroxylase gene, we were able to reduce the level of DAOC present in the fermentation broth to 50% of the control. CHEF gel electrophoresis and Southern analysis of DNA from two of the transformants revealed that one copy of the transforming plasmid had integrated into chromosome VIII (ie a heterologous site from the host expandase/hydroxylase gene situated on chromosome II). Northern analysis indicated that the amount of transcribed expandase/hydroxylase mRNA in one of the transformants is increased approximately two-fold over that in the untransformed host.

Keywords: *Acremonium chrysogenum*; cephalosporin C; deacetoxycephalosporin C; 7-ACA; 7-ADCA; expandase/hydroxylase

Introduction

Since the discovery of cephalosporin C in 1945 [20], cephalosporins have become one of the most important classes of antibiotics due to their low human toxicity combined with effectiveness against bacterial infection. The natural product, cephalosporin C, has only low activity against Gram-negative bacteria. More active cephalosporin C derivatives can be chemically synthesized by acylation of the nucleus of cephalosporin C, 7-aminocephalosporanic acid (7-ACA), which can be produced by hydrolysis of cephalosporin C (Figure 1) [4,12]. Following 40 years of development, chemical derivatives of cephalosporin C (semisynthetic cephalosporins) have become some of the most widely used antibiotics in the world; in 1995, the world market for cephalosporins was valued at $9.3 billion [1].

The cephalosporin C biosynthetic pathway in *Acremonium chrysogenum* is well characterized both biochemically and genetically (Figure 2) [4,8,10]. With the exception of the *cef*D gene encoding the isopenicillin N epimerase, all of the genes encoding the enzymes for cephalosporin C biosynthesis have been cloned [4]. As part of the fermentation process improvement program at Bristol-Myers Squibb, we are interested in improving the yield and quality of cephalosporin C produced by *A. chrysogenum* through genetic engineering.

Deacetoxycephalosporin C (DAOC) is formed as an intermediate in cephalosporin C biosynthesis. In this pathway, penicillin N (Pen N) is converted to DAOC then to deacetylcephalosporin C (DAC) by the bifunctional expandase/hydroxylase enzyme. During cephalosporin C fermentation, DAOC accumulates in the fermentation broth to a concentration of 1–2% of the cephalosporin C produced. When the cephalosporin C is extracted from the fermentation broth and hydrolyzed to 7-ACA, the DAOC present is converted to 7-aminodeacetoxycephalosporanic acid (7-ADCA), an undesirable contaminant of 7-ACA. In this report, we have demonstrated that it is possible to reduce the DAOC content in the cephalosporin C fermentation by genetically engineering strains for increased expression of the expandase/hydroxylase enzyme.

Materials and methods

Chemicals and enzymes

Unless otherwise noted, chemical compounds used were purchased from Sigma Chemical (St Louis, MO, USA). Reagent salts and organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA). Polyethylene glycol was purchased from Fluka Chemical (Milwaukee, WI, USA) and Novozyme 234 from InterSpex Products (Foster City, CA, USA). TRIzol® reagent, restriction enzymes and T4 DNA ligase were obtained from Life Technologies (Grand Island, NY, USA) and New England Biolabs (Beverly, MA, USA).

Microbial strains

Escherichia coli DH5α [F$^-$ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17($r_k^-m_k^+$) *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1] was purchased from Life Technologies

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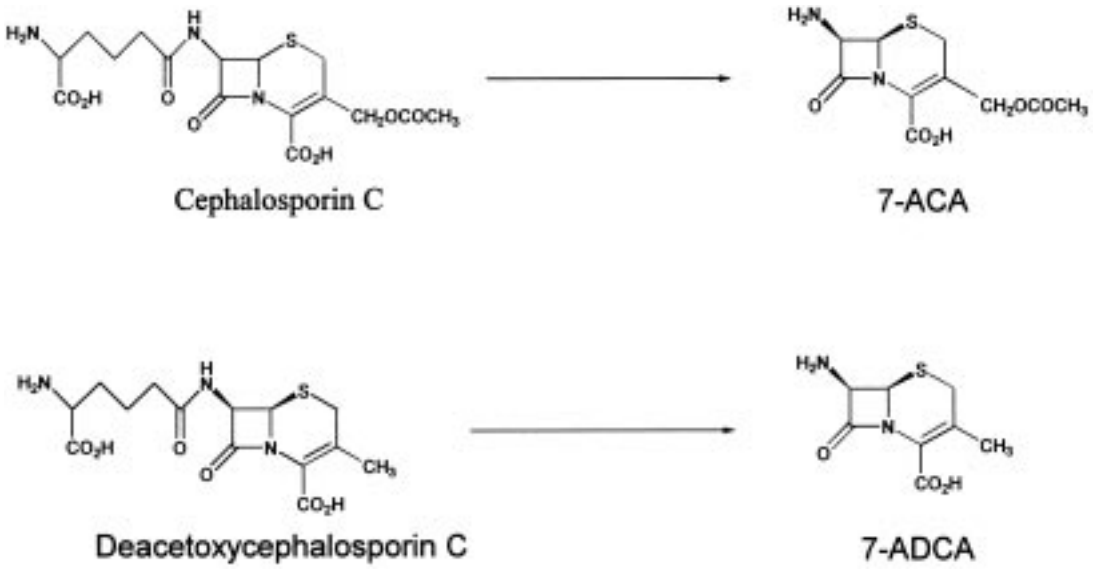

Figure 1 Structure of cephalosporins.

and used for all plasmid constructions and propagation. *A. chrysogenum* BC1385 and BC1386 were sequentially derived from ATCC 36225 (*A. chrysogenum* CW19) by multiple rounds of mutagenesis and screening for improved cephalosporin C production. BC1385 and BC1386 were used as a host for DNA transformation and as a control in the fermentation studies.

Media and buffers

E. coli was grown in Luria broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or Luria agar (Luria broth 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 the extraction of total RNA, a chemically defined fermentation medium containing 0.5% dl-methionine was used to cultivate cells [19]. All the other fermentation experiments in shake flasks and fermentors, BMS proprietary seed and fermentation media, consisting of corn syrup, corn steep liquor, dl-methionine and soyflour, were used to evaluate the cephalosporin productivity.

The following buffers were used for this study: TE buffer, TAE electrophoresis buffer, 20 × SSC, TBE electrophoresis buffer [16].

Cloning of the pcbC and cefEF genes

The isopenicillin N synthetase gene (*pcb*C) and expandase/hydroxylase gene (*cef*EF) of *A. chrysogenum* have been cloned and were previously characterized [17,18]. The *pcb*C and *cef*EF gene were cloned from *Bam*HI cleaved genomic DNA of *A. chrysogenum* strain BC1385 as a 3.3-kb fragment and a 7.5-kb fragment, respectively. In addition, the *pcb*C gene of *Penicillium chrysogenum* was cloned as a 5.4-kb *Bgl*II fragment according to Carr *et al* [3]. These gene fragments were used to construct the fungal transformation vectors described in the Results section.

Acremonium chrysogenum transformation

The *A. chrysogenum* DNA transformation was slightly modified from the method of Queener *et al* [14]. One milliliter of a frozen vegetative stock culture of *A. chrysogenum* strain BC1385 or BC1386 was used to inoculate 100 ml of PV media in a 500-ml Erlenmeyer flask. The culture was incubated in a Model G25 shaker incubator (New Brunswick Scientific, New Brunswick, NJ, USA) at 250 rpm for 64 h at 28°C. The mycelia were harvested by vacuum filtration through a 30-μm mesh nylon filter (Spectra/Mesh Nylon N, Spectrum Medical Industries, Houston, TX, USA) and washed with sterile H_2O. The mycelia were then weighed and each gram of mycelia was resuspended in 20 ml filter-sterilized Neutral McIlvaine's buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 10 mM dithiothreitol, pH 7.1). The mixture was incubated in an incubator shaker at 150 rpm for 90 min at 28°C. The mycelia were again harvested by filtration through a 30-μm mesh nylon filter and washed with sterile H_2O. Each gram of mycelia was resuspended in 10 ml filter-sterilized Isotonic and Acidic McIlvaine's buffer (0.1 M citric acid, 0.8 M NaCl, 20 mM $MgSO_4$, 0.2 M Na_2HPO_4, pH 6.35) containing Novozyme 234 (4 mg ml^{-1}). The mixture was incubated at 28°C in an incubator shaker at 100 rpm for 60 min. The mycelial clumps were dissociated by pipetting up and down with a glass pipette. Four volumes of washing buffer (0.8 M NaCl, 20 mM $MgSO_4$) were added and the entire preparation was filtered through a sterilized glass funnel loosely packed with glass fiber. The filtrate was collected and centrifuged at 850 × *g* for 8 min at room temperature. The supernatant was decanted immediately. The protoplast pellet was washed twice in $\frac{1}{2}$ volume of washing buffer at room temperature and re-centrifuged. The pellet was resuspended in 0.8 M NaCl to a concentration of 3–5 × 10^8 protoplasts ml^{-1}. To 1 ml of protoplasts, 5 μl dimethyl sulfoxide and 80 μl of 1 M $CaCl_2$ were added. Twenty micrograms DNA in 20 μl TE and 4 μl of heparin (10 mg ml^{-1}) were added to a 15-ml polypropylene tube (Falcon No. 2059, Becton

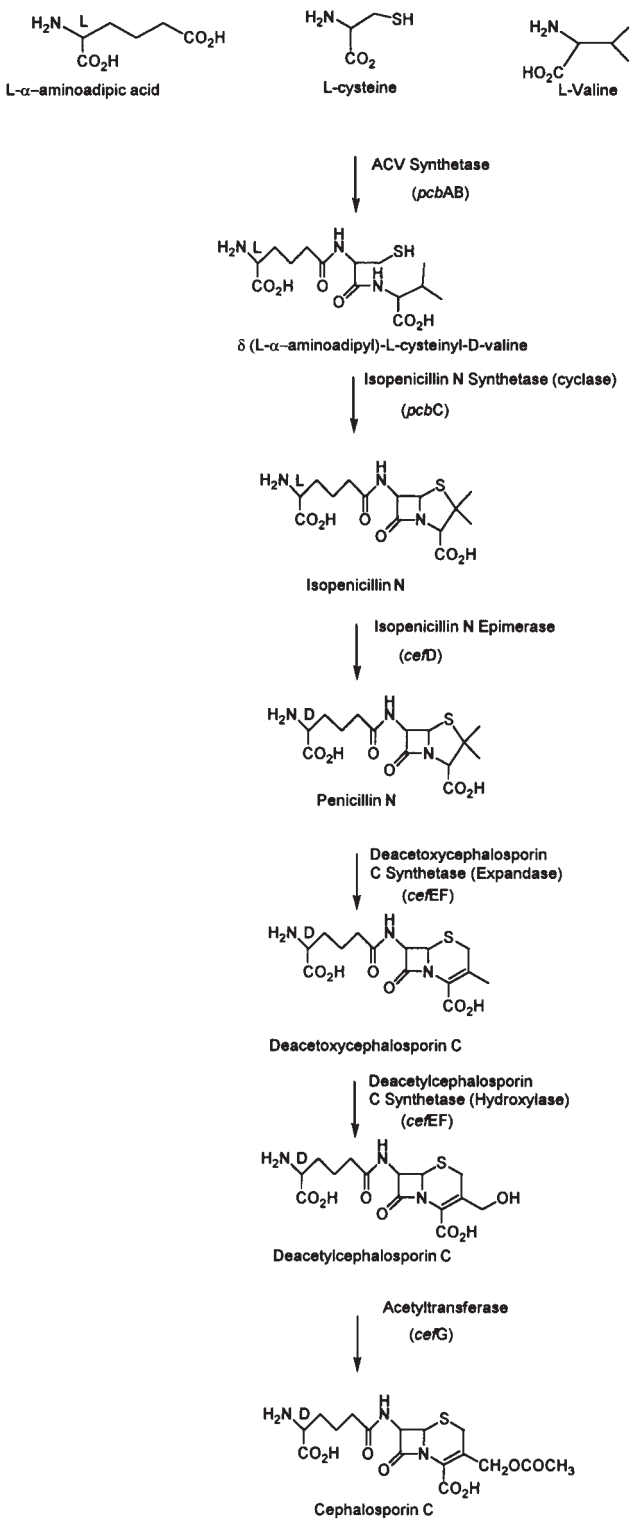

Figure 2 Biosynthetic pathway of cephalosporin C. The nomenclature used for the genes encoding these enzymes follows published recommendations [8].

Dickinson Labware, Lincoln Park, NJ, USA). Protoplasts (100 μl) were added to the DNA tube, followed by 20 μl of 40% polyethylene glycol-4000. The preparation was mixed gently and incubated for 10 min at room temperature. One milliliter of 40% polyethylene glycol-4000 was added, mixed gently, then 10 ml of molten (50°C) top agar (0.8 M NaCl, 0.7% agar) was added. Five milliliters of agar were pipetted to two plates pre-poured with 20 ml of regeneration agar (3% Trypticase Soy broth, 10.3% sucrose, 2% agar). The cultures were incubated at 28°C for 24 h, then overlayed with 5 ml of top agar containing phleomycin 12 μg ml^{-1} (final concentration 2 μg ml^{-1}). Transformants were observed after incubation for 10 days at 28°C.

Shake flask evaluation of transformants

After 2 weeks, transformants were transferred by sterile toothpick to YE agar plates containing 10 μg ml^{-1} phleomycin and incubated for 7 days at 28°C. For the subsequent culturing of the transformants, phleomycin was not incorporated in any growth or fermentation media. Colonies were inoculated onto slants containing 6 ml of YE agar and grown 7 days at 28°C. Two milliliters of sterile H_2O were used to resuspend the culture from each slant, 1 ml of the resuspended culture was then inoculated to 25 ml of seed media in a 125-ml Erlenmeyer flask. The seed cultures were cultivated in a shaker at 28°C for 48 h, 250 rpm. Two milliliters of the seed culture were then transferred to 20 ml of fermentation media in a 125-ml Erlenmeyer flask, grown 7 days at 24°C, 250 rpm. The concentration of cephalosporin C and deacetoxycephalosporin C in the whole broth was determined by high-performance liquid chromatography (HPLC) on a reverse-phase column [23]. Controls were untransformed BC1385 and BC1386.

Isolation of A. chrysogenum genomic DNA

One milliliter of a frozen vegetative stock culture from BC1386 and transformants BC1388 and BC1389 was inoculated in 30 ml of PV media. The mycelial cultures were grown, collected, and treated with Novozyme 234 to form protoplasts as described above. The protoplast pellet was resuspended in 3 ml lysis buffer (0.7 M NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) and incubated at 37°C for 5 min. Three tenth ml of 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl was added to the lysis mixture and incubated at 65°C for 10 min. The preparation was extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) to remove the CTAB-polysaccharide complex [13]. The aqueous solution was collected and DNA was precipitated with the addition of 6 ml of 100% ethanol. The DNA pellet was collected by centrifugation, washed with 70% ethanol, dried 5 min under vacuum and resuspended in 500 μl of TE. Ten microliters of RNase A (10 mg ml^{-1}) were added and incubated at 37°C for 1 h. Proteinase K solution (Boehringer Mannheim, Indianapolis, IN, USA) was added to the tube to a final concentration of 400 μg ml^{-1} and incubated at 50°C for 30 min. Sixty microliters of 3 M NaCl were added and the mixture was extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v). The DNA was precipitated with two volumes of 100% ethanol for 1 h at room temperature. The DNA was then pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in 400 μl of TE.

Gel electrophoresis and Southern hybridization

Genomic DNA (10 μg) was cleaved with the appropriate restriction enzymes and subjected to agarose gel electrophoresis. The DNA in the agarose was depurinated for 10 min with 0.25 N HCl then rinsed with H_2O. The DNA was transferred to a nylon membrane (Boehringer Mannheim) with 0.4 N NaOH using a Bio-Rad Model 785 Vacuum Blotter (Bio-Rad Laboratories, Hercules, CA, USA). The DNA was cross-linked to the nylon membrane by UV irradiation using a GS Gene Linker UV Chamber (Bio-Rad Laboratories) at an exposure of 125 mJ.

DNA probes labeled with [α-^{32}P]dCTP (Amersham Corporation, Arlington Heights, IL, USA) were prepared by nick translation with DNA polymerase I as described by Rigby *et al* [15]. DNA hybridizations were carried out at 65°C overnight [16]. Membranes were washed twice at room temperature for 30 min each with 2 × SSC/0.1% SDS, followed by two 30-min washes at 65°C with 0.1 × SSC/0.1% SDS. Membranes were dried and subjected to autoradiography.

CHEF gel electrophoresis

A. chrysogenum chromosome isolation was performed by a modification of the method of Skatrud and Queener [21]. The *A. chrysogenum* protoplast pellet was resuspended in 0.8 M NaCl with 50 mM EDTA, pH 8.0 at a final concentration of 2×10^9 protoplasts ml^{-1}. One milliliter of the protoplast suspension was pre-warmed to 37°C and mixed gently with 1 ml of agarose (2% Bio-Rad chromosomal grade agarose in 0.8 M NaCl/50 mM EDTA, maintained at 50°C). This mixture was poured into a Bio-Rad Plug Mold and allowed to solidify at 4°C for 1 h. The protoplast plugs were transferred into a 15-ml Falcon tube and lysed by the addition of 10 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 1% sodium lauroylsarcosine and proteinase K 1 mg ml^{-1}). The tube was incubated at 50°C overnight. The lysis solution was then removed, replaced with 10 ml of 50 mM EDTA, pH 8.0, and stored at 4°C.

Electrophoretic separation of *A. chrysogenum* DNA was carried out in a Bio-Rad CHEF-DRII system (Clamped Homogeneous Electric Fields). Agarose plugs containing lysed protoplasts were inserted into the wells of a 0.8% Bio-Rad chromosomal grade agarose gel in 0.5 × TBE electrophoresis buffer and sealed with molten agarose. Conditions for separation of *A. chrysogenum* chromosomes were as follows: (1) 45 V, 60-min pulse interval for 82 h at 120° pulsing angle; (2) 45 V, 45-min pulse interval for 60 h at 120° pulsing angle. Temperature was maintained at 14°C with constant buffer circulation and the 0.5 × TBE electrophoresis buffer was replaced with fresh buffer at 72 h.

RNA extraction and Northern gel analysis

Fungal cultures were grown in a chemically defined medium [19] at 24°C, 250 rpm. Fungal mycelia were harvested at times indicated in the text, collected by filtration and washed with H_2O. The mycelia were transferred to the surface of a piece of aluminum foil, wrapped and frozen in dry ice. The frozen mycelial pad was ground in a mortar to a fine powder in the presence of liquid nitrogen, and transferred into a 15-ml Falcon tube. Total RNA was isolated in TRIzol® reagent following the manufacturer's instructions [5] and further purified by lithium chloride precipitation [7]. RNA was denatured with glyoxal in the presence of ethidium bromide and glycerol, then subjected to electrophoresis through a 1% agarose gel [2]. The RNA was vacuum-blotted to a nylon membrane in NaOH. The hybridization was carried out as described for Southern hybridization using a digoxigenin-labeled probe.

Digoxigenin-labeled DNA probes were prepared by random primed labeling (Boehringer Mannheim Dig System Users' Guide) and used for hybridization. The filter was prehybridized at 50°C for 30 min in 10 ml of Dig-Easy Hyb Buffer (Boehringer Mannheim). A digoxigenin-labeled probe (50 ng) was diluted in 1 ml of Dig-Easy Hyb Buffer and denatured in a boiling water bath for 10 min. The denatured probe was placed on ice for 2 min and added to the prehybridization solution. The filter was then hybridized at 50°C overnight. The filter was washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature and then washed twice in 0.5 × SSC, 0.1% SDS for 15 min at 65°C. Chemiluminescent detection of the digoxigenin probed blot was performed as described in the Dig System Users' Guide.

Results

Construction of fungal vectors

Plasmid pUT715 [9] is a promoter probe vector carrying a promoter-less phleomycin-resistant gene from *Streptoalloteichus hindustanus*. In order to construct a fungal vector for the selection of transformants of *A. chrysogenum*, a fungal promoter has to be inserted to direct the transcription of the phleomycin-resistant gene. The *P. chrysogenum pcb*C gene (encoding the isopenicillin N synthetase enzyme) was cloned at a 5.4-kb *Bgl*II fragment. The promoter sequence of the *pcb*C gene is contained in a 1140-base pair (bp) *Nco*I fragment. This 1140-bp fragment was isolated by agarose gel electrophoresis and recovered by electroelution [16]. The pUT715 plasmid was digested with *Nco*I and ligated to the 1140-bp *P. chrysogenum pcb*C promoter fragment. The ligation mixture was transformed into *E. coli* DH5α and the resulting plasmid was designated as pSJC43 (Figure 3).

To study the gene dosage effect of the *pcb*C and *cef*EF (encoding the expandase/hydroxylase enzyme) genes on the production of cephalosporin C, the following vectors were constructed. The *cef*EF gene of *A. chrysogenum* was initially cloned as a 7.5-kb *Bam*HI fragment. Further characterization of this fragment indicated that the promoter and coding sequence of the *cef*EF gene is located in a 3.2-kb *Bam*HI/*Bgl*II fragment. The 3.2-kb *Bam*HI/*Bgl*II fragment was inserted into the fungal vector pSJC43 at the *Bgl*II site to generate plasmid pBM17 (Figure 3). A 3.3-kb *Bam*HI fragment containing the *A. chrysogenum pcb*C gene was cloned and inserted into the *Bgl*II site of pBM17 to form pBM19, which contains both the *pcb*C and *cef*EF genes from *A. chrysogenum* (Figure 3).

The 5.4-kb *Bgl*II fragment containing the *P. chrysogenum pcb*C gene was mapped and the *pcb*C gene was found to reside on a 2.3-kb *Bgl*II/*Eco*RI fragment. This fragment was isolated, modified by the addition of a *Bgl*II linker, and then cleaved with the *Bgl*II enzyme. Sub-

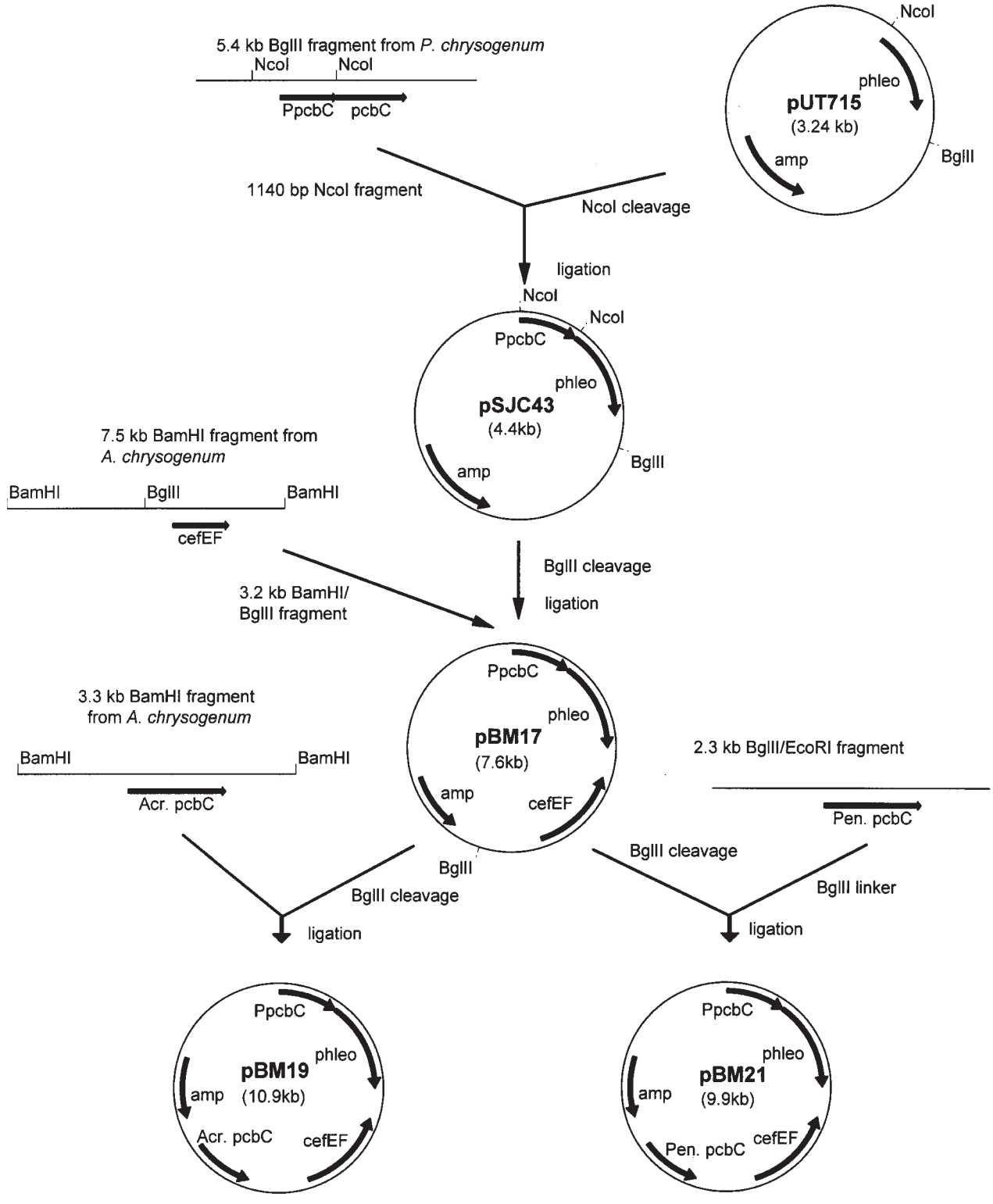

Figure 3 Construction of fungal transformation vectors.

sequently, this fragment was inserted into the *Bgl*II site of pBM17 and the resulting plasmid, pBM21, contains the *cef*EF gene from *A. chrysogenum* and the *pcb*C gene from *P. chrysogenum* (Figure 3).

A. chrysogenum transformation and selection of transformants

Twenty micrograms of plasmid pBM17 were used to transform the protoplasts of *A. chrysogenum* strain BC1385. Colonies resistant to phleomycin were observed after 10 days incubation. Genomic DNA was extracted from all of the phleomycin-resistant colonies and analyzed by DNA-dot blot hybridization [11] with an [α-^{32}P]dCPT-labeled plasmid probe. More than 95% of the phleomycin-resistant clones retained the transforming plasmid sequence. The transformation frequency observed in *A. chrysogenum* was 0.75 transformant per μg of ccc (covalently closed circular) plasmid DNA and 3.2 transformants per μg of plasmid DNA linearized by restriction enzyme *Bam*HI (Table 1). When 40 μg of heparin was added to the DNA prior to the addition of protoplasts, the transformation frequency was enhanced to 1.4 transformants per μg ccc DNA and 4.65 transformants per μg linear DNA (ie, a 1.5–1.9 fold increase in the presence of heparin). Plasmids pBM19 and pBM21 were also used to transform the BC1385 and BC1386 strain.

Shake flask and fermentor evaluation of the cephalosporin C productivity of transformants

A number of transformants were evaluated for cephalosporin C and DAOC production in shake flasks using BMS fermentation media. After 7 days of fermentation, fermentation broth was assayed. In the shake flask evaluation (Figure 4), one BC1385 derived transformant—BC1388 (generated by pBM21 vector), and two BC1386 derived transformants—BC1389 and BC1390 (generated by plasmids pBM19 and pBM17 respectively), produced a small increase in cephalosporin C titer and their DAOC content (as a percentage of cephalosporin C) was significantly reduced (50–75%). The transformant BC1389 was further characterized in 14-L and 30 000-L fermentors (Table 2). A 3% increase in cephalosporin C titer was observed in 30 000-L fermentors, but not in 14-L fermentors. When the DAOC content of the fermentation broth from both fermentors was analyzed, a significant reduction (approximately 40–70%) was observed in the transformant as compared with the BC1386 control. The reduction of DAOC content results in a reduction of contaminating 7-ADCA in the final 7-ACA product.

Table 1 Comparison of transformation frequency with different DNA sources

DNA source	Transformation frequency (transformants per μg DNA)
pBM17 (ccc)	0.75
pBM17 (ccc) + heparin	1.40
pBM17 (linear)	3.20
pBM17 (linear) + heparin	4.65

Analysis of transformant DNA by Southern hybridization

Large molecular weight genomic DNA was prepared from untransformed BC1386, and the transformants BC1388 and BC1389. The genomic DNA (10 μg) was digested with the restriction enzyme *Stu*I which does not cleave the transforming plasmids, fractionated by agarose gel electrophoresis and transferred to nylon membranes. Blots were hybridized with [α-^{32}P]dCTP-labeled pUT715, *A. chrysogenum pcb*C, or *cef*EF probes. When probed with the labeled pUT715 plasmid, Southern analysis showed that a DNA band isolated from the transformant, indicating that the transforming plasmids had integrated into the host chromosome (Figure 5a). In the Southern blot hybridized with the *cef*EF gene probe, a band of the same size as the one hybridizing to the pUT715 probe was observed; the 9.4-kb chromosomal *cef*EF gene band, however, was unchanged (Figure 5c). When the *A. chrysogenum pcb*C gene probe was used, the results indicated that in addition to the 8.7-kb *Stu*I cleaved chromosomal *pcb*C gene band, a larger molecular weight band appeared in BC1389 DNA but not in BC1388 DNA (Figure 5b). This result can be explained by the high stringency hybridization conditions which would not allow hybridization to occur between the *A. chrysogenum pcb*C gene probe and the integrated *P. chrysogenum pcb*C gene, which share a 74% DNA sequence similarity [3].

Another set of hybridizations was performed with *Bcl*I cleaved genomic DNA. The *Bcl*I enzyme does not cleave pBM21 and cleaves pBM19 once at the NT No. 993 position of the coding sequence of the *A. chrysogenum pcb*C gene [17]. In the Southern blot hybridized with the labeled pUT715 probe, two hybridization bands for BC1389 DNA with a molecular weight of 10 kb and 4.4 kb and one 11-kb hybridization band of BC1388 DNA demonstrated that the integration sites of pBM19 and pBM21, in BC1389 and BC1388, respectively, were different (Figure 5a). When hybridized with the *pcb*C or *cef*EF probes, the results also indicated that both pBM19 and pBM21 integrated into the host chromosome without altering the size of the hybridizing bands corresponding to the chromosomal *cef*EF and *pcb*C genes (Figure 5b, c).

In summary, these results demonstrated that the pBM19 and pBM21 plasmids had integrated into the host chromosome at a site other than the chromosomal *pcb*C and *cef*EF genes. The integration sites of the two transformants were different. The equal intensity of the hybridizing chromosomal and integrated plasmid bands suggests that only one copy of the plasmid had integrated into the host chromosome.

Mitotic stability of the integrated plasmid sequences in transformant BC1389

The genetic stability of the integrated plasmid DNA in transformant BC1389 was verified by collecting mycelia for genomic DNA preparation at several time points during a 30 000-L fermentation in the absence of phleomycin. Southern hybridization with this DNA revealed that the DNA banding pattern remained constant throughout the fermentation cycle, indicating that the integrated plasmid sequence is mitotically stable (data not shown). A similar

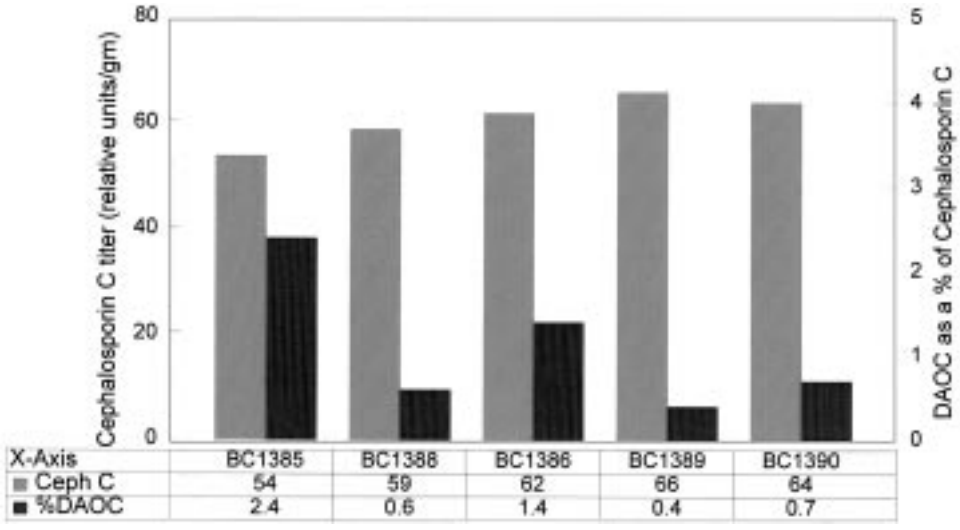

Figure 4 Shake flask evaluation of transformants for the yield of cephalosporin C and DAOC. Cephalosporin C titer is expressed in relative units per gram of fermentation broth. DAOC content is expressed as a percentage of cephalosporin C. The result of each strain is an average of three shake flasks.

Table 2 Comparison of cephalosporin productivity of untransformed BC1386 and transformant BC1389 in fermentors

Strain	Ceph C (Units g^{-1})[a]	DAOC (As % of Ceph C)	7-ADCA (As % of 7-ACA)
BC1386			
14-L fermentors	88.1	0.7%	0.3%
30 000-L fermentors	100.0	1.5%	0.8%
BC1389			
14-L fermentors	79.6	0.4%	0.2%
30 000-L fermentors	103.5 ± 7.5[b]	0.5–0.8%	0.3–0.4%

[a]Cephalosporin C titers were measured in relative units per gram of fermentation broth.
[b]Average of eight 30 000-L fermentors.

result has been described for hygromycin B-resistant transformants of *A. chrysogenum* [22].

Molecular karyotyping and hybridization analysis of transformant chromosomes

In order to further explore the nature of plasmid integration in the *A. chrysogenum* transformants, CHEF gel electrophoresis was used to identify the chromosome containing the plasmid integration site. CHEF gel electrophoresis was used to separate the eight chromosomes of *A. chrysogenum* (Figure 6) [21]. After ethidium bromide staining of the DNA, comparison of the chromosomal banding between BC1386, BC1388, and BC1389 revealed that there is no significant difference of chromosome mobility among the three strains. After depurination in 0.25 N HCl, the DNA was transferred to a nylon membrane as described above. Hybridization was carried out with [α-^{32}P]dCTP-labeled

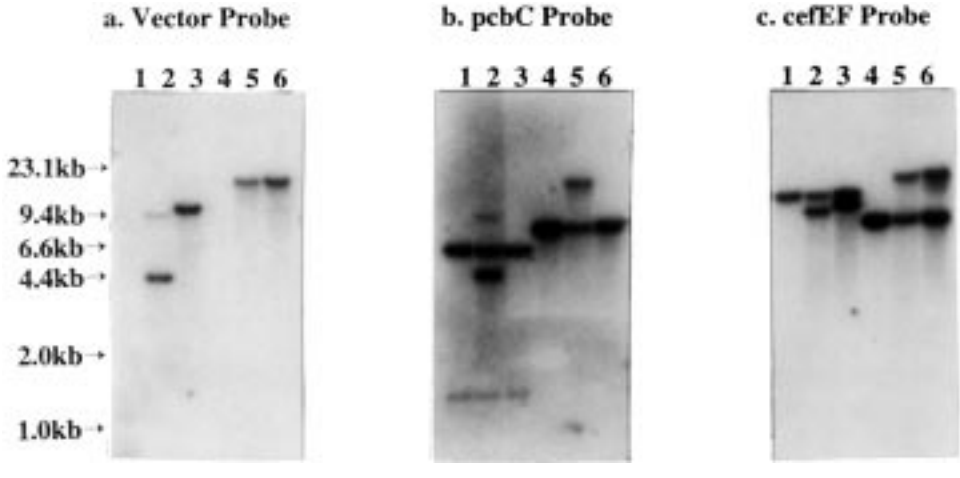

Figure 5 Southern hybridization of integrated plasmid sequences in *A. chrysogenum* transformants. Total genomic DNA (10 μg) was digested with *Bcl*I (lanes 1–3) or *Stu*I (lanes 4–6) enzymes, fractionated by agarose gel electrophoresis and transferred to nylon membranes. Blots were hybridized with ^{32}P-labeled pUT715 vector (panel a), or *A. chrysogenum pcb*C gene fragment (panel b), or *cef*EF gene fragment (panel c). Lanes 1 and 4, DNA from untransformed BC1386 strain; 2 and 5, DNA from transformant BC1388; 3 and 6, DNA from transformant BC1389. Positions corresponding to length (in kb) of *Hin*dIII-cleaved λ DNA markers are shown.

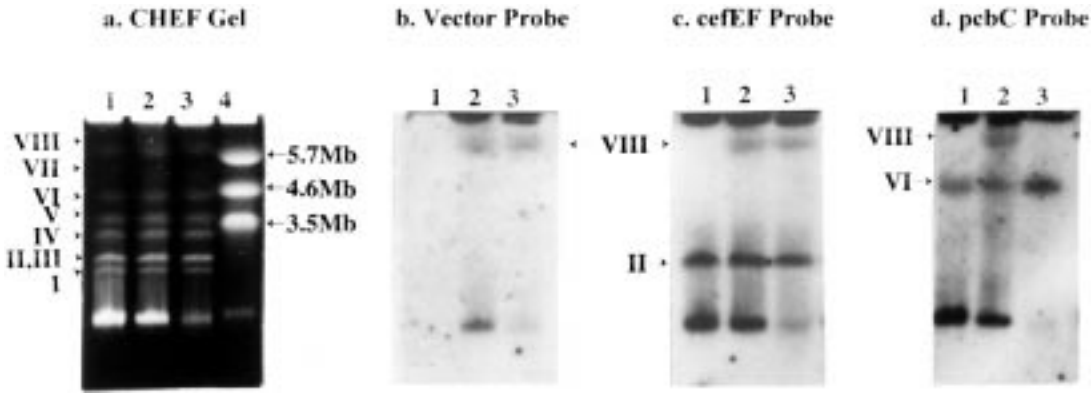

Figure 6 Molecular karyotyping and Southern hybridization of chromosomes from *A. chrysogenum* transformants. *A. chrosygenum* chromosomes were separated by CHEF gel electrophoresis and visualized after staining with ethidium bromide (panel a). Chromosomes were transferred to nylon membranes. Blots were hybridized with ^{32}P-labeled pUT715 vector (panel b), or *cef*EF gene fragment (panel c), or *A. chrosygenum pcb*C gene fragment (panel d). Lane 1, chromosomes isolated from untransformed BC1386 strain; 2, chromosomes from transformant BC1389; 3, chromosomes from transformant BC1388; 4, chromosomes from *Schizosaccharomyces pombe* (Bio-Rad Laboratories) as molecular weight marker.

pUT715, *A. chrysogenum pcb*C, or *cef*EF probes. Hybridization results confirm the observation of Skatrud and Queener [21] that the *cef*EF gene is located on chromosome II (Figure 6c) and the *pcb*C gene is located on chromosome VI (Figure 6d). The transforming plasmid, however, integrated into chromosome VIII (ie, a heterologous site). The DNA band at the top of the gel is composed of DNA aggregates which were too large to migrate into the gel. The DNA band below chromosome I consists of DNA degradation products.

Northern hybridization analysis of RNA produced in the cephalosporin C fermentation

Mycelia were collected at 48, 72, 96, and 120-h time points during a shake flask fermentation of the transformant BC1389 and the untransformed BC1386. Total RNA samples were prepared as described above. Total RNA (10 μg) was denatured with glyoxal, electrophoresed, and blotted onto a nylon membrane. Hybridization was performed with a digoxigenin-labeled *cef*EF gene probe. The results indicate the *cef*EF transcript is increased approximately two-fold in the transformant over the untransformed BC1386 control at all four time points (Figure 7). From the intensity of the integrating plasmid band in Southern hybridization analysis, it is estimated that only one copy of the plasmid integrated into the host chromosome. Therefore, the Northern analysis is consistent with the expectation that the amount of *cef*EF mRNA corresponds to the copy number of the *cef*EF gene present in the genome.

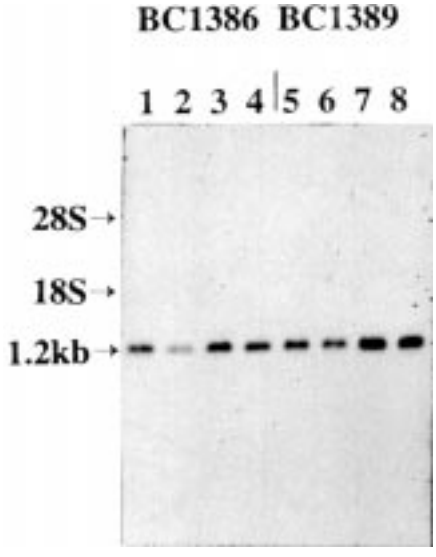

Figure 7 Northern hybridization analyses of the expandase/hydroxylase mRNA production. Total RNA (10 μg) was fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was hybridized with the digoxigenin-labeled *cef*EF gene fragment. Lanes 1–4, total RNA isolated from untransformed BC1386 strain at 48, 72, 96, and 120-h time points during a shake-flask fermentation; lanes 5–8, total RNA isolated from transformant BC1389 at 48, 72, 96, and 120-h time points during fermentation.

Discussion

By increasing the copy number of the *cef*EF gene in *A. chrysogenum*, a 40–70% reduction in DAOC level and a modest increase in cephalosporin C titer was observed in shake flasks and 30 000-L fermentors. When cephalosporin C was converted to 7-ACA, a significant reduction of contaminating 7-ADCA was also observed in the final 7-ACA product. The increase in cephalosporin C titer observed in the transformants appears to be dependent upon the level of Pen N and DAOC present in the fermentation [22]. In shake flasks and 30 000-L fermentors, the Pen N and DAOC levels (as a percentage of cephalosporin C) are higher than in 14-L fermentors, consequently a measurable increase in titer can be observed from the additional expandase/hydroxylase enzyme activity. The catalytic activities of the expandase/hydroxylase enzyme requires α-ketoglutarate, Fe^{+2}, and O_2 [6]. Any one of these factors or their combination could play a major role in determining the levels of Pen N and DAOC and consequently the effect of increased expandase/hydroxylase enzyme activity on cephalosporin C titer. Skatrud *et al* [22] demonstrated that the expandase/hydroxylase activity is rate-limiting for cephalosporin C synthesis in their production strains. Transformants with an extra copy of the *cef*EF gene exhibited two-fold more expandase/hydroxylase activity than the untransformed strain, a reduction of Pen N levels and an

increase in cephalosporin C titer. This increase in titer varied from 47% observed in laboratory scale fermentations to only 15% observed in 150-L fermentors [22].

In two of the transformants, BC1388 and BC1389, analyzed by CHEF gel electrophoresis, one copy of the transforming plasmid integrated into a heterologous site on chromosome VIII. Southern hybridization analysis of the genomic DNA of these two transformants revealed different banding of the integrated plasmids suggesting that each transforming plasmid DNA integrated into a different site on chromosome VIII. Skatrud *et al* [22] reported that in a similar experiment the transforming plasmids integrated into a heterologous site on chromosome III. Since Skatrud and co-workers only characterized one of the transformants and we studied two of the transformants, the significance of the heterologous integration site remains to be evaluated. Both of the transformants analyzed in this work were first screened for improved cephalosporin C titer. It is possible that homologous recombinants led to a decrease in titer and therefore were not selected for further analysis.

When total RNA was extracted from transformant BC1389 and probed with a *cef*EF specific probe, the transformant was found to produce approximately twice as much expandase/hydroxylase transcript as the untransformed host. This increase in transcription correlates with the doubling of the gene copy number, observed by Southern hybridization. Skatrud *et al* [22] reported a two-fold increase of expandase/hydroxylase activity in the *cef*EF transformants as well. It is not known if this relationship would hold true for higher copy number transformants or if it is dependent upon the integration site. Again, since the transformants were first screened for improved cephalosporin titer and only the best one was extensively characterized, it is not valuable to generalize all of the transformants with the limited available data.

The success of a genetic engineering approach to strain improvement relies upon the interaction between the introduced DNA, the recipient host and the fermentation process. In our current example of the *cef*EF gene, the integration site probably is significant as no more than approximately 10% of the transformants demonstrated improved cephalosporin C titers in shake flasks. In this study, we utilized the endogenous *cef*EF promoter for expression, so the introduced *cef*EF gene copy would be subject to the same regulation as the extant copy. In fact, in our mRNA studies the transformant BC1389 produced two-fold more *cef*EF transcript at all time points, indicating the same pattern of transcriptional regulation. The host utilized for transformation, BC1386, was known to accumulate DAOC to an undesirable level at industrial scale fermentation, indicating an opportunity to use genetic engineering to reduce the DAOC content. Finally, the dissimilar results observed at different fermentation scales with respect to the DAOC content and cephalosporin C titer demonstrate the importance of the fermentation process on the results achieved with the genetically engineered strains.

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