Construction and analysis of $\beta$-lactamase-inhibitory protein (BLIP) non-producer mutants of *Streptomyces clavuligerus*

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The gene encoding BLIP, a beta-lactamase-inhibitory protein, was disrupted in wild-type *Streptomyces clavuligerus* and in a clavulanic acid non-producing mutant. The resulting BLIP mutant and BLIP/clavulanic acid double mutant showed no residual proteinaceous $\beta$-lactamase-inhibitory activity, indicating that only a single $\beta$-lactamase-inhibitory protein exists in *S. clavuligerus*. The lack of any proteinaceous $\beta$-lactamase-inhibitory activity in the *bli* and *bli*/*claR* mutants also indicates that BLP, the BLIP-like protein, encoded by *S. clavuligerus* does not possess $\beta$-lactamase-inhibitory activity despite its similarity to BLIP. The *bli* mutant and the *bli*/*claR* double mutant did not show any aberrant growth morphology, sporulation defects, or alterations in cephamycin C production or penicillin G resistance when compared to wild-type *S. clavuligerus* or to the *claR* single mutant. Mutants bearing the *bli* gene disruption did show an elevated level of production of clavam-2-carboxylate and hydroxymethyl clavam as well as clavulanic acid. This phenomenon was observed in the middle stages of production of these clavams but was not detected during maximum production. The production of BLIP was also determined to be down-regulated in a *ccaR* mutant, lacking the pathway-specific transcriptional regulator required for production of cephamycin C and clavulanic acid. Sequencing of the regions flanking the *bli* gene showed the presence of a partial open reading frame that encodes a DNA-binding protein, and several open reading frames apparently involved in the production of an ABC transporter.

**Keywords:** clavulanic acid, clavams, antibiotic resistance, exocellular protein

**INTRODUCTION**

*Streptomyces* spp. are filamentous Gram-positive soil bacteria known for their complex life cycle and their production of a large variety of biologically active secondary metabolites, degradative enzymes and enzyme inhibitors. *Streptomyces clavuligerus* has been the subject of extensive research in the last 30 years because of its ability to produce $\beta$-lactam metabolites with antibiotic, antifungal and $\beta$-lactamase-inhibitory activities. The major $\beta$-lactam compounds produced by *S. clavuligerus* are shown in Fig. 1. Penicillin N and cephamycin C are antibiotics while clavam-2-carboxylate, hydroxymethyl clavam, alanyl clavam and 2-formyloxymethyl clavam have weak antibacterial activity but have also been shown to have antifungal activities (Pruess & Kellett, 1983). In addition to these, *S. clavuligerus* is well known as the species used for the industrial production of clavulanic acid. Clavulanic acid differs from the other four clavams in that it has C-5 R stereochemistry, which is opposite to the C-5S stereochemistry of the other clavams. Furthermore, it has a carboxyl group at the C-3 position not seen in the other clavams. Although low in antibacterial activity, clavulanic acid has become a clinically important product of *S. clavuligerus* due to its potent inhibitory activity.

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Abbreviations: ABC, ATP-binding cassette; BLIP, $\beta$-lactamase-inhibitory protein; BLP, BLIP-like protein; MSD, membrane-spanning domain; MYM, maltose/yeast extract/malt extract medium; SSPE, sodium chloride/sodium phosphate/EDTA; TS, trypticase soy broth; TSBS, TSB-starch; TSBM, TSB-maltose; YEME, yeast extract/malt extract; YEMEM, YEME-maltose.

The GenBank accession number for the sequence determined in this work is M34538.
beta-protein. The protein, BLIP, is a beta-lactamase inhibitor from Streptomyces gedanensis characterized by Hata et al. (1972). The gene encoding BLIP (blp) has recently been cloned and sequenced, but the predicted amino acid sequence of BLIP-I does not show any significant similarity to BLIP from S. clavuligerus (Park & Lee, 1998). In contrast, the gene encoding BLIP-I (blpA) has recently been cloned and sequenced and BLP-I was shown to be similar in size (17.5 kDa) and sequence (38% identity) to BLIP from S. clavuligerus (Kang et al., 2000). Furthermore, blpA has been disrupted and shown to impose a bald phenotype on the mutant strain. Unlike S. clavuligerus, S. exfoliatus is not known to produce any beta-lactam metabolites.

Perez-Llarena et al. (1997a) have also reported the presence of a gene in S. clavuligerus that encodes a putative protein called BLP, for BLIP-like protein. The amino acid sequence of BLP shows significant similarity to BLIP, with 29.2% identity and an additional 27.6% functionally conserved residues. Interestingly, the BLP-encoding gene, blp, is located within the cephamycin C biosynthetic gene cluster but BLP does not appear to be required for production of beta-lactam antibiotics since deletion of the blp gene has no detectable phenotype, including no effect on cephamycin C production (Alexander & Jensen, 1998).

Doran et al. (1990) suggested that BLIP might function as a regulator of cell wall growth or morphogenesis in S. clavuligerus. More recently, Perez-Llarena et al. (1997b) reported the discovery of a beta-lactamase-encoding gene (bla) in S. clavuligerus. The beta-lactamase, when produced in a heterologous species, had poor catalytic activity, two to four orders of magnitude lower than other class A enzymes against penicillin G. When assayed against clavulanic acid, the S. clavuligerus beta-lactamase was found to be sensitive to inhibition by clavulanic acid although the inhibition was weak compared to other target enzymes. Due to its ability to bind penicillin but its weak hydrolysing activity, the authors proposed that this enzyme actually functions in cell wall morphogenesis like a penicillin-binding protein. This theory was also supported by the finding that an analogous beta-lactamase in Nocardioidactamidurans is involved in cell wall biosynthesis.

BLIP was also found to have an inhibitory effect on the S. clavuligerus beta-lactamase (Perez-Llarena et al., 1997b) and the authors further suggested that the activity of the S. clavuligerus beta-lactamase in cell wall synthesis and morphology may be regulated by BLIP and/or clavulanic acid. In this study, a BLIP non-producer mutant was constructed to assess the effects of the absence of BLIP on the development of S. clavuligerus. Furthermore, a BLIP and clavulanic acid double non-producer mutant was constructed to assess the effects of the absence of both of these beta-lactamase inhibitors on the growth of the organism.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>S. clavuligerus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRRL 3585</td>
<td>Wild-type</td>
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<tr>
<td><em>claR</em> mutant</td>
<td>Clavulanic acid production defective mutant; disruption in the <em>claR</em> gene</td>
<td>Northern Regional Research Laboratories, Peoria, IL, USA</td>
</tr>
<tr>
<td><em>ccaR</em> mutant</td>
<td>Cephamycin and clavulanic acid production defective mutant; disruption in the <em>ccaR</em> gene</td>
<td>Alexander &amp; Jensen (1998)</td>
</tr>
<tr>
<td><em>bli</em> mutant</td>
<td>BLIP non-producer generated by disruption of the <em>bli</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td><em>bli</em>/<em>claR</em> mutant</td>
<td>BLIP and clavulanic acid non-producer generated by disruption of <em>bli</em> in <em>claR</em> mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>S. lividans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK24</td>
<td>str-6; plasmidless cloning host (SLP2- SLP3-)</td>
<td>T. Kieser, John Innes Institute, Norwich, UK</td>
</tr>
<tr>
<td>E. coli MV1193</td>
<td>Δ(lac-proAB) rpsL thi endA spcB15 bsdR4 Δ(srl-recA)306::Tn10(tetR)</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80 lacZAM15) bsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>ESS</td>
<td>β-Lactam antibiotic sensitive strain</td>
<td>Aharonowitz &amp; Demain (1978)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUC119</td>
<td>E. coli cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Vieira &amp; Messing (1987)</td>
</tr>
<tr>
<td>pIJ2925</td>
<td>pUC18-based cloning vector, contains multiple cloning site flanked by BglII sites</td>
<td>Janssen &amp; Bibb (1993)</td>
</tr>
<tr>
<td>pJOE829</td>
<td><em>Streptomyces</em> cloning vector, contains hygromycin-resistance cassette</td>
<td>Aidoo et al. (1994)</td>
</tr>
<tr>
<td>pIJ702</td>
<td><em>Streptomyces</em> cloning vector, contains thiostrepton-resistance cassette</td>
<td>Katz et al. (1983)</td>
</tr>
<tr>
<td>pBLIP</td>
<td>pLAFR3-based cosmid containing a 13.5 kb DNA insert including <em>bli</em></td>
<td>Doran et al. (1990)</td>
</tr>
<tr>
<td>pBLIP2</td>
<td>Contains <em>bli</em> gene</td>
<td>Doran et al. (1990)</td>
</tr>
<tr>
<td>pBLIP3</td>
<td>Contains <em>bli</em> gene with BamHI linker in an internal ApaI site</td>
<td>This study</td>
</tr>
<tr>
<td>pIJ2925::tsr</td>
<td>pIJ2925 containing <em>bli::tsr</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJOE829::tsr</td>
<td>pJOE829 containing <em>bli::tsr</em></td>
<td>This study</td>
</tr>
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1. *Escherichia coli* cultures were grown as described in Sambrook et al. (1989). For sporulation, *S. clavuligerus* was grown on ISP-3 medium (Difco) while *Streptomyces lividans* was grown to produce spore stocks on R2YE without sucrose as described in Hopwood et al. (1985). Genomic and plasmid DNA was isolated from cultures of *Streptomyces* spp. grown in trypticase soy broth (TSB) supplemented with 1% starch (TSBS) with shaking at 280 rpm for 2–3 d until maximum growth was obtained. *S. clavuligerus* cultures were incubated at 28 °C while *S. lividans* cultures were grown at 30 °C in flasks equipped with steel springs. Plasmid-bearing cultures were supplemented with 50 µg hygromycin ml⁻¹ or 50 µg thiostrepton ml⁻¹ for *S. lividans*, and 5 µg thiostrepton ml⁻¹ or 250 µg hygromycin ml⁻¹ for *S. clavuligerus*, as appropriate to the plasmid.

*S. lividans* was cultivated for the production of protoplasts as described in Hopwood et al. (1985), while *S. clavuligerus* was cultivated for the production of protoplasts according to a modification of the method of Bailey & Winstanley (1986). TSB + 1% maltose (TSBM) medium was inoculated with *S. clavuligerus* spores and incubated for 3 d at 25 °C and 250 rpm. in a flask containing a steel spring. This seed culture was used to inoculate (4%, v/v) a 25 ml amount of TSBM/YEMEM combination medium [10 ml TSB, 15 ml YEME (yeast extract/malt extract; Hopwood et al., 1985), 1% maltose and 0.005 M MgCl₂] and the culture was incubated with shaking at 25 °C for 18 h. Mycelium was harvested by centrifugation, washed twice with 10% Tween 80 and resuspended in 6 ml of a complex Soy fermentation medium (Paradkar et al., 1995). BLIP production and β-lactam antibiotic production by *S. clavuligerus* were assayed in TSB broth grown cultures inoculated from a spore suspension and incubated as described above. Penicillin G resistance studies in...
liquid medium were conducted using *S. clavuligerus* grown in TSBS broth. For resistance studies on solid medium, *S. clavuligerus* spores were grown on TSBS agar.

**Disruption of the bli gene.** pBLIP2 was linearized by digestion with *Apa*I, and an *Apa*I/ BamHI linker oligonucleotide (5'-GTGGATACCAACGGCC-3') was introduced. The *Apa*I site is located near the middle of the *bli* gene. A thiostrepton-resistance gene (*tsr*), released from pIJ2702 as a BstI fragment, was introduced into the BamHI site of the *bli* gene. The *bli::tsr* disrupted gene was removed as an EcoRI/BstI fragment, passed through pIJ2925 to introduce compatible restriction endonuclease sites and then inserted into the BigII site of pJOE829 to generate pJOEBl::tsr. pJOEBl::tsr was transformed into *S. lividans* and from there into wild-type *S. clavuligerus* and a clmA mutant strain of *S. clavuligerus*. Thiostrepton resistant (Thio<sup>+</sup>), hygromycin-resistant (Hyg<sup>+</sup>) transformants were transferred to ISP-3 medium plates and allowed to sporulate in the absence of antibiotic selection. The resulting spores were plated on ISP-3 medium to give isolated colonies, and then replica plated onto MYM medium to identify thiostrepton resistant, hygromycin sensitive (Thio<sup>-</sup> Hyg<sup>-</sup>) mutants.

**DNA isolation, transformation and Southern blot analysis.** Plasmid DNA was isolated from *E. coli*, and restriction enzyme digestion and ligation reactions were performed as described in Sambrook *et al.* (1989). Generation and transformation of *E. coli* competent cells was performed as described by Chung *et al.* (1989). Streptomyces plasmids were isolated and purified by the alkaline lysis method of Kieser, and *Streptomyces* genomic DNA was purified according the method of Fisher, both as described in Hopwood *et al.* (1985). *S. lividans* protoplast formation, transformation and regeneration were as described in Hopwood *et al.* (1985). *S. clavuligerus* protoplast formation, transformation and regeneration were as described by Bailey & Winstanley (1986). Southern blot analysis was performed as described in Sambrook *et al.* (1989). DNA probes were labelled with [α<sup>32</sup>P]dCTP by nick translation according to the method in Hopwood *et al.* (1985). Hybridization reactions were carried out at 65 °C for 16 h in 5 x SSPE, 0.5 % (w/v) SDS, 5 x Denhardt's solution (Sambrook *et al.*., 1989). Following hybridization, nylon blots were washed twice with 2 x SSPE + 0.1 % SDS for 10 min at room temperature, once with 1 x SSPE + 0.1 % SDS at 65 °C for 15 min and finally once with 0.1 x SSPE + 0.1 % SDS at 65 °C for 15 min.

**Quantitation of growth.** Growth of *Streptomyces* cultures was estimated by two methods. For TSBS-grown cultures, optical densities were measured at 600 nm. For cultures grown in Soy fermentation medium, growth was assayed by quantitation of DNA content by the method of Burton as described in Daniels *et al.* (1994), using salmon sperm DNA as a standard.

**Detection of BLIP by β-lactamase inhibition assay.** Culture supernatants were diluted 1:1 (v/v) with methanol or water, incubated at 4 °C for 30 min and centrifuged for 5 min at 13000 r.p.m. in a MicroCentaur bench-top centrifuge. Bacto-penase (Difco, 2 x 10<sup>8</sup> units in 0.3 ml 0.1 M sodium phosphate buffer pH 7.0), was preincubated at 21 °C for 5 min with 0.2 ml of the diluted culture supernatant. Penicillin G (500 µg of 0.3 mg ml<sup>-1</sup> solution) was then added and the rate of decrease in absorbance at 240 nm was monitored. The difference between the rate of penicillin G degradation obtained when a culture supernatant sample was diluted with H<sub>2</sub>O (inhibition due to both clavulanic acid and BLIP) and that obtained when it was diluted with methanol (inhibition due to clavulanic acid only) represents the amount of β-lactamase-inhibitory activity due to BLIP. BLIP activity is expressed as percentage inhibition of Bactopenate assayed under the standard conditions described above. The presence of methanol in the assays at the concentration described above has no effect on β-lactamase activity.

**Detection of BLIP by Western analysis.** Protein samples were separated on a 10 % SDS-PAG and then transferred to a PVDF membrane (Immobilon-P, Millipore). Transfer was carried out at 1.5 A for 2 h at 4 °C in 25 mM Tris/192 mM glycine at pH 8.3. Following transfer, the blot was blocked with 5 % (w/v) skim milk (Difco) and Western hybridization was carried out using the Chemiluminescence Western Blotting Kit (Roche Diagnostics) according to the manufacturer’s instructions. The membrane was probed with primary anti-BLIP antibodies prepared by D. Alexander as described by Alexander & Jensen (1998). Anti-BLIP antibodies were used at a dilution of 1:4000 while the sheep anti-rabbit secondary IgG coupled to horseradish peroxidase (Roche Diagnostics) was used at 1:5000.

**HPLC assay of clavams and clavulanic acid.** HPLC analysis was performed as described by Paradkar *et al.* (1998). Clavams-2-carboxylate and clavulanic acid were identified by comparison to authentic standards kindly provided by SmithKline Beecham, Worthing, UK. Hydroxymethyl clavam was identified according to its relative chromatographic mobility in comparison to clavam-2-carboxylate and clavulanic acid (Mosher *et al.*, 1999).

**Bioassay for β-lactam antibiotics.** Total β-lactam antibiotics were determined by a bioassay procedure using *E. coli* ESS as the indicator organism, as described by Aharonowitz & Demain (1978).

**Penicillin G resistance studies.** Penicillin G resistance on solid medium was examined using TSBS base agar plates. Each 100 x 15 mm plate containing 10 ml TSBS agar was overlaid with 5 ml soft TSBS agar containing 1 x 10<sup>9</sup> viable spores of *S. clavuligerus*. Paper bioassay disks of 0.5 in (~13 mm) diameter (Schleicher and Schuell) containing 50, 100 or 200 µg penicillin G were placed on the inoculated plates at 16 h post-overlay to examine the effect of penicillin G on germinated spores or 10 min post-overlay to examine the effect on ungerminated spores. The plates were then incubated at 28 °C and zones of inhibition were measured at 27 and 38 h.

Penicillin G resistance on liquid medium was examined by adding penicillin G to 12 h TSBS cultures to a final concentration of 0, 20 or 60 µg ml<sup>-1</sup>. At 24 h intervals, samples were removed, homogenized with a disposable pestle pestle mixer (Kontes) and growth was determined as OD<sub>690</sub>. Samples of each culture were also fixed with a drop of polyvinyl alcohol mounting medium on a microscope slide and examined by phase-contrast microscopy.

**DNA sequence analysis.** The DNA sequences of the regions upstream and downstream of the *bli* gene were determined using the plasmid pBIP as template and a series of custom oligonucleotide sequencing primers. Dideoxy chain-termination sequencing was performed using a *Taq* dye-deoxy terminator kit and model 373A sequencer (Applied Biosystems) by the University of Alberta Department of Biological Sciences DNA synthesis lab. Both strands of the template were sequenced in full for the regions reported.

Sequences were compiled using DNA Star software (DNASTAR, Inc.). ORFs were identified using the Codon Preference feature of GCG (Genetics Computer Group, Inc.)
RESULTS

Disruption of the bli gene in wild-type S. clavuligerus and in a clavulanic acid non-producer mutant

A BLIP non-producer mutant and a clavulanic acid/BLIP non-producer double mutant were constructed by gene replacement at the bli locus in wild-type S. clavuligerus and in the clavulanic acid production defective mutant, claR (Paradkar et al., 1998), respectively. To construct the bli single mutant, pJOEbliT::tsr was introduced into wild-type S. clavuligerus. The bli/claR double mutant was constructed by introducing the same pJOEbliT::tsr disruption construct into an existing claR mutant. The claR mutant is defective in clavulanic acid production and was generated by disruption of claR using an apramycin-resistance gene. claR encodes a transcriptional regulator required for the expression of genes involved in the late steps of clavulanic acid biosynthesis (Paradkar et al., 1998). Following sporulation of transformants on non-selective medium, and isolation of ThioR HygR colonies, seven putative bli single mutants and one bli claR double mutant were obtained.

Confirmation of the gene replacement event by Southern hybridization

Southern hybridization was carried out to confirm that the gene disruption events had taken place at the bli locus of the putative BLIP mutants. Genomic DNA of the wild-type, and the single and double mutants, was digested with KpnI, separated by agarose gel electrophoresis and then blotted onto nylon membranes. The membranes were probed with an 840 bp DNA fragment containing the bli gene and with a 1.09 kb fragment containing the tsr gene. A 5.5 kb bli-hybridizing fragment in wild-type genomic DNA was replaced with a 6.5 kb bli-hybridizing fragment in both the single bli mutant and the bli/claR double mutant, indicating that the KpnI fragment carrying the bli gene had taken up a 1 kb insert, presumably the tsr gene (data not shown). Probing with a labelled tsr gene gave no hybridizing signal in wild-type genomic DNA while a band of 6.5 kb was seen in the DNA digests of the bli mutant and the bli/claR double mutant. This confirmed that the expected gene replacement event had taken place in the bli and bli/claR mutants.

Comparison of β-lactamase-inhibitory protein activity in wild-type S. clavuligerus, the bli mutants, and the bli/claR double mutant

To determine if the mutants were defective in the production of BLIP, culture supernatants from the seven bli mutants and the bli/claR double mutant grown in TSBS were assayed for BLIP activity using the β-lactamase inhibition assay. Fig. 2 shows BLIP activity produced by the wild-type, one representative bli mutant and the bli/claR double mutant. Although high levels of BLIP activity were seen in wild-type culture supernatants, BLIP activity was not detected at any time point in either the single or double mutants. The absence of β-lactamase-inhibitory activity in culture supernatants of the bli mutant not only confirms that the expected mutation has taken place, but also shows that only one proteinaceous β-lactamase-inhibitory protein exists in S. clavuligerus.

Effects of bli disruption on morphology, sensitivity to penicillin G and cephemycin C production in S. clavuligerus

Mycelia from TSBS-grown cultures of wild-type S. clavuligerus and the bli and bli/claR mutants were examined by phase-contrast microscopy. No morphological differences were seen between the mutants and the wild-type organism. Sporulation on solid medium was also unaffected by the bli disruption. Resistance to penicillin G was also examined and no difference in penicillin G sensitivity was observed for liquid- or solid-medium-grown cultures of the different mutants and wild-type S. clavuligerus. Cephemycin production by wild-type S. clavuligerus, the bli mutant and the bli/claR double mutant was compared by bioassay against the indicator organism E. coli ESS. Again, no differences in the levels of antibiotic production in TSBS-grown cultures were found between the mutants and wild-type S. clavuligerus (data not shown).

Effects of bli disruption on clavam production in S. clavuligerus

Clavulanic acid and two of the other clavams identifiable by HPLC, clavam-2-carboxylate and hydroxymethyl clavam, are produced in abundance by 72 and 96 h in Soy fermentation medium grown cultures of wild-type

with a Streptomyces codon frequency table (Wright & Bibb, 1992). The sequence information obtained was analysed using the GCG/netBLAST program to search for similar protein sequences in the public databases.
S. clavuligerus. To determine if the bli disruption had any effect on clavulanic acid, clavam-2-carboxylate and hydroxymethyl clavam production, culture supernatants from triplicate Soy fermentation medium grown cultures of a representative bli mutant, the claR mutant, the bli/claR double mutant and wild-type S. clavuligerus were analysed by HPLC.

Clavam-2-carboxylate and clavulanic acid peaks were identified by comparison with authentic standards while hydroxymethyl clavam was identified by its known chromatographic properties relative to those of clavam-2-carboxylate and clavulanic acid (Mosher et al., 1999). The levels of clavam-2-carboxylate produced by wild-type S. clavuligerus, and by the bli, claR, and bli/claR mutants, are shown in Fig. 3. No differences in the levels of clavam-2-carboxylate produced by the bli mutant and wild-type S. clavuligerus were evident at 48 h although production levels were quite variable in the replicate cultures of the bli mutant (Fig. 3a). At 72 h, the bli mutant produced levels of clavam-2-carboxylate that were two to five times higher than those seen in the wild-type samples (Fig. 3b). This difference was transient and was no longer evident in the 96 h samples (Fig. 3c). At 48 h, the bli/claR double mutant appeared to produce lower amounts of clavam-2-carboxylate than either the claR single mutant or wild-type cultures (Fig. 3a). Despite this initial lag, by 72 h the bli/claR double mutant showed levels of clavam that were higher than either the wild-type or the claR mutant (Fig. 3b). By 96 h (Fig. 3c), the bli/claR and claR mutants were comparable, as was true for the bli and wild-type cultures. Therefore a transient elevated level of clavam-2-carboxylate was detected in the mid-production stages of strains bearing the bli disruption.

A similar pattern of production of hydroxymethyl clavam was seen in the wild-type and mutant cultures. At 72 h bli mutant cultures produced two- to almost tenfold higher levels of hydroxymethyl clavam than the wild-type culture, but by 96 h the difference was no longer statistically significant (data not shown). Despite considerable variation in clavam content between replicate cultures of the same strains, these temporal differences in clavam levels between the mutants and the wild-type culture were observed consistently, and the higher clavam levels were always seen in the bli mutants. Furthermore this variation in clavam production was seen even though DNA measurements indicated very similar extents of growth in the replicate cultures. This variability in replicate cultures despite rigorous attempts to produce cultures as near to identical as possible suggests that clavam production is highly sensitive to growth conditions and growth stages.

When the level of hydroxymethyl clavam produced by the bli/claR double mutant and the claR mutant was compared, no difference was detected at 72 h or 96 h despite an initial lag in production shown by the bli/claR double mutant at 48 h (data not shown).

Comparisons of clavulanic acid production by the wild-type and bli mutant showed that higher levels of clavulanic acid were seen in the bli mutant compared to wild-type cultures at 48 h when clavulanic acid was just beginning to be detected in cultures. Similar amounts of clavulanic acid were detected at 72 or 96 h for both bli and wild-type cultures (data not shown).

**Examination of the DNA sequence flanking the bli gene**

The gene encoding the BLIP-like protein, blp, is located in the cephamycin biosynthetic gene cluster (Perez-Llarena et al., 1997a) in close proximity to ccaR, a pathway-specific regulator of cephamycin C and clavulanic acid production. Since the bli mutation had effects, albeit transient, on clavam production, it seemed possible that BLIP could be involved in the regulation of clavam production. Therefore, it was of interest to determine whether a regulatory gene might be located near bli, just as ccaR is located near to blp.

The regions upstream and downstream of bli were sequenced using pBIP, the cosmids from which the bli gene was initially cloned (Doran et al., 1999), as template. The resulting 3876 bp sequence can be found in GenBank, accession number M34538. The blp gene is flanked on the 5' end by the ccaR and orf11 and on the 3' end by the genes encoding early enzymes of cephamycin biosynthesis (Perez-Llarena et al., 1997a). In contrast, the bli gene is flanked on the 5' end by an ORF, bliup1, located 388 bp upstream and oriented diver-
BLIP mutants of *S. clavuligerus*

Fig. 4. Alignment of the deduced amino acid sequences of the AbcA and AbcA' proteins with the known or predicted ATP-binding proteins of ABC transporters from *S. coelicolor* A3(2) and *Neisseria meningitidis*. + indicates conservative substitutions. Amino acid numbers for AbcA' are shown as a continuation of AbcA, but marked with a prime (') to indicate the frameshift. Corresponding nucleotide numbers are also given for the abcA and abcA' amino acid sequences to indicate their location in the deposited sequence (GenBank accession number M34538).

Gently to the bli gene. The bliup1 gene is 527 bp in length and encodes a predicted protein of 175 amino acids with a GTG start codon at nt 1009 and a possible Shine–Dalgarno sequence (G AAA GG) 7 bp upstream of the start codon. BLASTX analysis of Bliup1 shows some similarity to a hypothetical protein from Streptomyces coelicolor (24% identical, 39% similar amino acids) but there is no indication of possible function.

The translational stop codon of the bliup1 gene (TGA at nt 484) overlaps the translation start codon (GTG at nt 485) of a second ORF, bliup2. Although the sequence of bliup2 is incomplete, BLASTX analysis of the first 161 amino acids of the predicted protein showed similarity to a number of putative DNA binding proteins from *S. coelicolor*.

On the 3' end, bli is flanked by a pair of overlapping genes which encode proteins showing homology to the ATP-binding domain of an ABC transporter, and a third ORF showing no homology to known proteins. The first of these ORFs, *abcA*, is located 68 bp downstream from the bli gene and encodes a protein of 150 amino acids. A potential ribosome-binding site (GAA CCA GG) is located 4 bp upstream of the ATG start codon. A glycine-rich Walker A motif which typifies all ATP-binding proteins and is believed to interact with ATP (Mendez & Salas, 1998) is evident at amino acids 42–50. The second ORF, *abcA'*, overlaps the *abcA* gene. No typical start codon (ATG, GTG, TTG) can be found for the *abcA'* gene. A possible ribosome-binding site (GGG AAC GG) can be identified 6 bp upstream of an ATT codon in-frame with the *abcA'* ORF; however, ATT is an extremely rare start codon (Butler et al., 1987). Since no likely start codon could be found for the *abcA'* gene, and to ensure that the unusual arrangement of the *abcA'/A'* gene was not an artifact that had occurred during the construction and analysis of the bli-containing cosmids, the corresponding region was amplified by PCR from the genome of *S. clavuligerus* and sequenced. The PCR-amplified region of the genome was exactly the same size and had the same sequence as the cloned region (data not shown).

Starting from the ATT codon, the predicted *abcA'* peptide is 106 amino acids in length and includes the Walker B motif. The Walker B motif extends from amino acids 43 to 48 and encodes a stretch of hydrophobic amino acids including the conserved aspartic acid (D) residue thought to interact with magnesium in this class of proteins. Taken together, the overlapping *abcA* and *abcA'* ORFs show all of the motifs known to be conserved in ATP-binding proteins, but they are present on two separate ORFs. The on-line version of this paper (available at http://mic.sgmjournals.org) contains a supplementary figure showing the overlap of the *abcA* and *abcA'* genes. Alignment of the ORFs with ATP-binding proteins of a *S. coelicolor* ABC transporter (CAB48895, GenBank) and of a *Neisseria meningitidis* ABC transporter (AAF41615, GenBank) shows extensive sequence identity and similarity throughout much of the predicted amino acid sequence of both *abcA* and *abcA'* (Fig. 4). The *AbcA* protein shows homology to the N-
The third ORF downstream from bli is incomplete, extending for 1220 bp from a start codon (GTG) that overlaps the translational stop codon of the abcA' gene to the end of the sequenced region. This partial ORF shows no homology to known proteins although at least six regular stretches of hydrophobic amino acid residues that could represent the membrane-spanning domains (MSDs) of a transmembrane protein can be identified by Kyte–Doolittle hydropathy analysis (Kyte & Doolittle, 1982).

Expression of BLIP in a ccaR mutant

Since disruption of bli showed transient effects on the production of clavulanic acid and the other clavam compounds, it was of interest to determine whether the expression of bli was under the control of CcaR, the pathway-specific regulator of both clavulanic acid and cephamycin C production. Fig. 5 compares levels of BLIP in the culture supernatants of wild-type S. clavuligerus, the bli single mutant and a ccaR deletion mutant (Alexander & Jensen, 1998). Culture supernatants were analysed by Western analysis using anti-BLIP antibodies. Although optical densities indicated comparable levels of growth, the amount of BLIP detected in the supernatant of the wild-type cultures was noticeably greater than that detected in the ccaR mutant culture supernatant, at all time points studied. While the effect of ccaR disruption on BLIP production was not absolute, the significant decrease in BLIP production levels seen in the ccaR mutant does suggest that CcaR may regulate expression of BLIP.

The absence of any anti-BLIP-reactive material in the bli mutant suggests that all of the reactive bands observed in the wild-type are related to BLIP and not to cross-reacting material. Furthermore, the absence of any anti-BLIP-reactive material in the bli mutant also indicates that BLIP, if it is produced under these conditions, must be sufficiently different from BLIP that it does not cross-react with BLIP-specific antibodies.

**DISCUSSION**

The production of β-lactamase-inhibitory proteins has been characterized in two Streptomyces species, S. clavuligerus (Doran et al., 1990) and S. exfoliatus (Kim...
& Lee, 1994). However little is known of the function of these proteins in the producer organisms. In *S. clavuligerus*, clavulanic acid is produced in addition to a β-lactamase-inhibitory protein (BLIP), raising the question of why two β-lactamase inhibitors are required. BLIP non-producer mutants were generated in *S. clavuligerus* to investigate the role of BLIP in growth and β-lactam metabolite production. A double mutant, defective in the production of both BLIP and clavulanic acid, was also constructed to determine the relevance of β-lactamase inhibitors to *S. clavuligerus*. The isolation of a bli mutant indicates that BLIP is not an essential gene product, while the isolation of a bli/claR mutant also suggests that the production of β-lactamase inhibitors is not essential for the survival of *S. clavuligerus*.

The bli mutant showed no indication of proteinaceous β-lactamase inhibitors when culture supernatants were assayed for BLIP activity. This result shows that no second β-lactamase-inhibitory protein exists in *S. clavuligerus*, in contrast to the situation in *S. exfoliatus*, where two different β-lactamase-inhibitory proteins are produced. Furthermore, since *S. clavuligerus* contains a gene encoding a BLIP-like protein (BLP; Perez-Llarena et al., 1997a), the absence of proteinaceous β-lactamase inhibitors in the bli mutants indicates that BLIP has no β-lactamase-inhibitory activity despite its similarity to BLIP. No β-lactamase-inhibitory activity of any type was detected in the culture supernatant of the bli/claR double mutant.

Both the bli mutant and the bli/claR double mutant were analysed for aberrant growth phenotypes and were assayed for BLIP activity. This result shows that no second β-lactamase-inhibitory protein exists in *S. clavuligerus*, in contrast to the situation in *S. exfoliatus*, where two different β-lactamase-inhibitory proteins are produced. Furthermore, since *S. clavuligerus* contains a gene encoding a BLIP-like protein (BLP; Perez-Llarena et al., 1997a), the absence of proteinaceous β-lactamase inhibitors in the bli mutants indicates that BLIP has no β-lactamase-inhibitory activity despite its similarity to BLIP. No β-lactamase-inhibitory activity of any type was detected in the culture supernatant of the bli/claR double mutant.

The resistance of the *S. clavuligerus* mutants to penicillin G in liquid and surface-grown cultures was also studied. No apparent differences in resistance were found among the wild-type, claR mutant, bli mutant and bli/claR double mutant strains in either spores or germinated mycelia. Although BLIP is active against the β-lactamase of *S. clavuligerus*, it does not seem to affect the innate resistance of *S. clavuligerus* to penicillin G either in surface-grown or in liquid cultures. This result is in agreement with previous findings that the resistance of *S. clavuligerus* cultures to β-lactam antibiotics is a result of the production of penicillin-binding proteins having low affinity for β-lactams (Ogawara & Horikawa, 1980; Paradkar et al., 1998).

Comparison of cephamycin C production showed no apparent differences among the wild-type and the various mutants, supporting the conclusion that BLIP does not play a role in the production of antibiotic. It also suggests that BLIP does not play a role in the protection of *S. clavuligerus* against its own antibiotic products.

The disruption of the bli gene had little effect on most aspects of *S. clavuligerus* production of secondary metabolites with the exception of the production of the clavams. Both the bli mutant and the bli/claR double mutant showed transiently elevated levels of clavam-2-carboxylate production when compared to the wild-type and the claR mutant. A similar pattern was seen for both hydroxymethyl clavam and clavulanic acid. Although the effect of bli disruption on secondary metabolite production was limited to a transient effect on clavulanic acid and the clavams, the observation that bli expression was reduced in a ccaR mutant does suggest some kind of regulatory association between bli and β-lactam metabolite production. ccaR is located within the cephamycin/clavulanic acid gene supercluster, flanked on its downstream side by two genes of unknown function, orf11 and blp (Perez-Llarena et al., 1997a). Since blp resembles bli and is located near ccaR, it was of interest to determine if a regulatory gene resembling ccaR might also be located near bli. DNA sequence information was obtained for a 1396 bp region upstream of bli. Within that region, one complete ORF, bliup1, and a partial ORF, bliup2, were evident. Database searches indicated that the protein encoded by bliup1 shows no similarity to proteins of known function while the partial bliup2 gene product resembled several DNA-binding proteins from *S. coelicolor*. Although it remains to be determined whether Bliup2 is a regulatory protein involved in clavam production, it does not belong to the SARP (*Streptomyces* antibiotic-regulatory protein) family of pathway-specific transcriptional activators, which includes CcaR (Wietzorrek & Bibb, 1997).

Analysis of 1874 bp of DNA sequence obtained from downstream of bli showed the presence of a pair of ORFs, abcA and abcA', both having high similarity to ATP-binding proteins of ABC-type transporters. The abcA and abcA' ORFs each contain one of the two Walker motifs which typify ATP-binding proteins of ABC-type transporters. Although all nucleotide-binding proteins identified to date contain these conserved motifs in a single polypeptide, in the current study they were found on separate and overlapping ORFs. In other known *Streptomyces* ABC transporters, two absolutely conserved K and D residues in Walker A and B motifs respectively range from 116 to 149 amino acids apart (Mendez & Salas, 1998). The same residues in the ATP-binding proteins in this system are located on separate polypeptides. Although an ATG start codon and a potential ribosome-binding site can be identified for the abcA gene, no typical start codon was found for the abcA' gene. An in-frame ATT codon and potential ribosome-binding site could be seen upstream of the highly conserved region in the abcA' gene; however, it is unlikely that this is a translational start for the abcA' gene, since there is only one protein known which has an ATT start codon. The translation initiation factor IF(3) protein uses an ATT start codon as a translation in the autoregulation of its own translation (Butler et al., 1994).
The effect of the bli gene disruption on the time course of production of clavams, and the identification of genes encoding an ABC transporter immediately downstream of the bli gene, together raised the question of whether BLIP and the ABC transporter might be involved in the secretion of clavams into the culture media. It seems unlikely that the ABC transporter is involved in the secretion of BLIP itself because it is not clear if abcA and abcA′ encode a functional two-part ATP-binding protein of an ABC transporter, or whether they represent a defective gene. However, the existence of another pair of atypical ABC transporter genes has been reported in the gene cluster encoding production of the antibiotic epidermin by *Staphylococcus epidermidis*. In this organism, the epit gene encodes a 148 amino acid polypeptide; with a frame shift a second gene, epit′, follows. Together the Epit and Epit′ proteins are similar to one-component ABC transporters and are implicated in epidermin transport. The authors propose that these genes may compose a two-part ABC exporter, or that they may form a single exporter through a shift in reading frame (Fath & Kolter, 1993).

ABC transporters consist of both ATP-binding domains and membrane-spanning domains (MSDs), either as separate polypeptides or combined in various arrangements. A third incomplete ORF was identified immediately downstream of the abcA/AbcA′ ORFs. This partial ORF shows no similarity to known proteins, yet the presence of six hydrophobic MSDs can be predicted by the hydrophobic analysis method of Kyte & Doolittle (1982). Possibly this ORF encodes the MSD of the ABC transporter whose ATP-binding domains are encoded by abcA′/AbcA′. Although the presumptive MSD-encoding ORF has not been completely sequenced, the lack of similarity to other MSDs is consistent with the observation that little sequence conservation exists among the MSDs of bacterial ABC transporters (Fath & Kolter, 1993).

The effect of the bli gene disruption on the time course of production of clavams, and the identification of genes encoding an ABC transporter immediately downstream of the bli gene, together raised the question of whether BLIP and the ABC transporter might be involved in the secretion of clavams into the culture media. It seems unlikely that the ABC transporter is involved in the secretion of BLIP itself because bli encodes a protein with a typical signal peptide which is processed during secretion of the mature protein. Furthermore, the cloned bli gene expresses functional mature BLIP when expressed in *E. coli* (data not shown). Alternatively, BLIP may be involved together with the ABC transporter in the secretion of some other product. In addition to the ATP-binding protein and MSD, ABC transporters can also contain proteins described as accessory factors. In Gram-negative organisms, accessory factors are believed to aid in export through the inner and outer membrane by spanning the periplasm, connecting these two membranes. It is not clear what role accessory proteins play in Gram-positive organisms; however, accessory factors linked to ABC transporters of Gram-positive bacteria such as *Bacillus subtilis*, *Streptococcus pneumoniae* and *Lactococcus lactis* have been described (Fath & Kolter, 1993). Genes encoding accessory factors are invariably found to be linked to the ABC transporter genes (Fath & Kolter, 1993). Perhaps BLIP functions as an accessory protein for this ABC transporter that in turn plays some role in the excretion of clavam metabolites. However, disruption of bli would then be expected to impede rather than accelerate the production of clavam metabolites.

It remains unclear how the bli mutation results in the observed transient effects on clavam production. However, if the mutation in bli does exert its effects indirectly through the ABC transporter system, it cannot be due to polar effects of the bli mutation on the transcription of the genes, since previous studies have shown that BLIP is produced from a monocistronic transcript (Paradkar et al., 1994).

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