GcvA, a LysR-type transcriptional regulator protein, activates expression of the cloned *Citrobacter freundii* *ampC* β -lactamase gene in *Escherichia coli*: cross-talk between DNA-binding proteins

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***Escherichia coli* JRG582 is an *ampD ampE* deletion derivative of strain HfrH and accordingly it is derepressed for expression of the cloned inducible β -lactamase gene of *Citrobacter freundii*, carried on plasmid pNU305. Following chemical mutagenesis of JRG582(pNU305) a cefotaxime sensitive mutant was isolated, CS51(pNU305), which produced low levels of β -lactamase due to a mutation in the host chromosome. Two recombinant plasmids containing genomic DNA from *E. coli* HfrH, namely pUB5608 and pUB5611, were isolated as a consequence of their ability to restore the β -lactam resistant phenotype to CS51(pNU305). This ability was due to direct transcriptional activation of the β -lactamase gene, *ampC*, rather than complementation of the CS51 mutation. Transposon mutagenesis and subcloning showed that restoration of ampicillin resistance to CS51(pNU305) was the function of a single gene, which maps at 60.3 min on the *E. coli* chromosome. The gene encodes a 33 kDa protein with significant homology to members of the LysR family of bacterial activator proteins, in particular the AmpR protein from *C. freundii*. Homology is especially strong over the N-terminal region which includes the helix-turn-helix DNA-binding motif. This gene was shown to complement the *gcvA1* mutation at 60.3 min on the *E. coli* chromosome, and the DNA sequence agrees exactly with the published sequence of *gcvA* which encodes the transcriptional activator of the inducible glycine cleavage enzyme system. It is suggested that GcvA can activate transcription of *ampC* by binding to the AmpR binding region upstream of *ampC* so as to mimic the activated state of AmpR and hence provides an example of cross-talk between DNA-binding proteins of different inducible enzyme systems.**

Keywords: β -lactamase, expression, DNA-binding protein, cross-talk

INTRODUCTION

Virtually all Gram-negative bacteria possess a chromosome-encoded Group I β -lactamase (Bush, 1989). In many of these, such as *Citrobacter freundii*, *Enterobacter cloacae* and

indole positive *Proteus* species, β -lactamase production can be induced many-fold by the addition of certain β -lactam antibiotics to the growth medium (Sanders & Sanders, 1987). In such organisms, expression of the β -lactamase *ampC* gene is regulated by a trans-acting protein designated AmpR (Honoré *et al.*, 1986; Lindberg *et al.*, 1985), which belongs to the LysR family of bacterial activators (Henikoff *et al.*, 1988). As with many of the genes that encode such transcriptional activators, the *ampR* gene is divergently transcribed and is immediately upstream of the gene that it controls, in this case *ampC* (Honoré *et al.*, 1986; Lindquist *et al.*, 1989b). It is thought

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that AmpR does not interact directly with the inducing β -lactam, as it has been shown that induction does not require the presence of an intact inducer within the cytoplasm (Everett *et al.*, 1989); rather, it is believed that disruption of cell wall biosynthesis by β -lactams causes the release of ligand from the peptidoglycan matrix, which, either through direct interaction, or via transmission of a transmembrane signal, causes AmpR to be converted from an inactive to an active form (Normark *et al.*, 1990; Tuomanen *et al.*, 1991). AmpR has been shown to bind to a 38 bp sequence within the intercistronic region between *ampR* and *ampC* (Lindquist *et al.*, 1989b). In the inactivated state, AmpR acts as a weak repressor of *ampC* transcription, whereas in the activated state it acts as a strong activator (Normark *et al.*, 1990). Like many similar regulatory loci, transcription of *ampR* is negatively autoregulated.

Escherichia coli also possesses a Group I β -lactamase; however, in this genus expression of the enzyme is very low-level and uncontrolled, due to the absence of both the *ampR* gene and the AmpR binding region upstream of *ampC* present in inducible strains (Honoré *et al.*, 1986). The *ampR* and *ampC* genes from both *C. freundii* and *Ent. cloacae* have been cloned and expressed in *E. coli* where they confer inducible β -lactamase phenotypes (Lindberg *et al.*, 1985; Honoré *et al.*, 1986). Several *E. coli* functions have been identified as necessary for the inducible phenotype, in particular the products of the genes *ampD* and *ampG*, located at 2.5 min and 10 min, respectively, on the *E. coli* map. Null mutations in *ampD* result in derepression of *ampC* expression with respect to the genes from both *C. freundii* and *Ent. cloacae*, providing the cognate *ampR* gene is also present (Honoré *et al.*, 1989; Lindquist *et al.*, 1989b). In contrast, null mutations in *ampG* override the high expression phenotype, both induced and genetically derepressed, to confer non-inducible basal-level expression and a β -lactam sensitive phenotype (Korfmann & Sanders, 1989). The functions of *ampD* and *ampG* are still speculative. In addition, several other functions have been reported to influence inducible expression of β -lactamase in *E. coli*, namely *ampE* (Honoré *et al.*, 1989; Lindquist *et al.*, 1989b), *phpA* (Oliva *et al.*, 1989) and *ftsZ* (Ottolenghi & Ayala, 1991) but little is known about their functions. Some of the products of these genes are implicated in cell wall metabolism, suggesting that regulation of both cell wall metabolism and inducible *ampC* expression have elements in common.

This article describes the cloning and characterization of an *E. coli* gene, the product of which has a stimulatory effect on expression of the cloned *ampC* gene from *C. freundii*. Evidence is presented which suggests that this gene encodes a LysR-type transcription factor which can mimic the activated form of AmpR, and so provides an example of cross-talk between DNA-binding proteins.

METHODS

Bacterial strains and plasmids. The following *E. coli* strains were used: JRG582 (*thi* Δ nadC-aroP) (Langley & Guest, 1977) is an *ampD ampE* deletion derivative of HfrH (*thi*); XL1-blue

(Bullock *et al.*, 1987) was used as a host strain for the *E. coli* gene bank; SN03 (*ampA1 ampC8 pyrB recA rpsL*) produces negligible amounts of its native *ampC* β -lactamase and was used as a suitable background for measuring expression of the cloned *C. freundii ampC* (Normark & Burman, 1977); C600 (*supE44 thi thr leuB6 lacY1 tonA21*) (Appleyard, 1954) was used for λ 467 propagation; LE392 (*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*) (Borck *et al.*, 1976) was used as host for the Kohara λ clones; DS410 (*minB lac rpsL*) (Davie *et al.*, 1984; Dougan & Kehoe, 1984) was used for minicell analysis; GS970 (*serA gcvA1 thi pheA905 Δ lacU169 araD129 rpsL150*) does not grow on glycine and is derived from GS958 (*serA25 thi pheA905 Δ lacU169 araD129 rpsL150*) (Wilson *et al.*, 1993).

The cloning vector pSU19 (Martinez *et al.*, 1988) has a P15 origin of replication and carries the *cat* gene, conferring chloramphenicol resistance, and the multiple cloning site from pUC19. Plasmid pNU305 is derived from pBR322, encodes tetracycline resistance and carries the *C. freundii ampR* and *ampC* genes; pNU307 is an *ampR* deletion of pNU305 but retains *ampC* (Lindberg *et al.*, 1985).

Media and growth conditions. Minimal medium used was M9 (Miller, 1972) supplemented with 0.2% (w/v) glucose and, where required, one or more of thiamin (20 μ g ml⁻¹), uridine (20 μ g ml⁻¹), nicotinic acid (50 μ g ml⁻¹), serine (200 μ g ml⁻¹), and glycine (300 μ g ml⁻¹). Lab M nutrient broth no. 2 (Amersham) was used for most experiments, including MIC and β -lactamase determinations. When required, one or more of chloramphenicol (Cm; 30 μ g ml⁻¹), tetracycline (Tc; 25 μ g ml⁻¹) or kanamycin (Km; 30 μ g ml⁻¹) was added as a selective agent. BHI broth (Difco) was used for the growth of *E. coli* DS410.

Isolation of cefotaxime (Ctx)-sensitive mutants. Mutagenesis of JRG582(pNU305) was performed using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to the method of Maniatis *et al.* (1982). Ctx-sensitive mutants were identified by replica-plating colonies on to nutrient medium containing 50 μ g Ctx ml⁻¹ and selecting those that failed to grow.

Creation of *E. coli* gene bank. *E. coli* HfrH chromosomal DNA was partially digested with *Sau3A* to give a random distribution of DNA fragments. Size-fractionated fragments of between 5 and 10 kb were ligated into vector pSU19, previously digested with *Bam*HI and treated with calf intestinal alkaline phosphatase to prevent re-annealing. Recombinant DNA was introduced into *E. coli* XL1-blue by electroporation (Gene Pulser, 2.5 V, 25 μ F, 400 Ω ; Bio-Rad) and transformed bacteria were selected on nutrient agar containing Cm. Nutrient broth (1 ml) was added to each plate and bacterial cells were rinsed off and the suspensions were pooled. Plasmid DNA was prepared from 200 μ l aliquots and stored at -40 °C. Dialysed gene bank DNA solution (2 μ l) was used to electroporate host cells in subsequent cloning experiments.

Subcloning. Subclone pUB5628 contains *orf*s 1, 2 and 3 and was constructed by ligation of the 2.7 kb *Kpn*I-*Cl*aI fragment of pUB5611 into pSU19, which had been cleaved with *Kpn*I and *Acl*I. Subclone pUB5632 contains *orf*1 and was constructed by cleaving pUB5611 with *Eco*RI and partially digesting the resulting fragments with *Bam*HI, followed by ligation of the 1 kb *Eco*RI-*Bam*HI fragment into pSU19 cut with *Eco*RI and *Bam*HI. pUB5636 contains *orf*1 under the control of the Ptac promoter and was constructed by introducing the Ptac promoter on an *Eco*RI fragment from pRU883 (Ubben & Schmidt, 1987) into the *Eco*RI site of pUB5632 immediately upstream of the *orf*1 gene.

DNA techniques. Enzymic manipulation of DNA and Southern

hybridization were performed using standard protocols as previously described (Maniatis *et al.*, 1982; Ausubel *et al.*, 1990).

β -Lactamase determinations. β -Lactamase assays were performed on either uninduced or induced cultures grown at 37 °C in Lab M broth. Cells from 10 or 20 ml samples as appropriate were pelleted and washed and resuspended in 10 mM phosphate buffer, pH 7.0, and disrupted by sonication. Cleared lysates were assayed for β -lactamase activity using the chromogenic cephalosporin, nitrocefin. Total protein in the samples was measured according to the Bio-Rad protein microassay procedure.

Cells were induced for β -lactamase production by dilution of exponential-phase cells ($OD_{420} = 0.8$) into prewarmed broth containing twice the final inducer concentration. Samples were withdrawn after 40 min.

Mapping of cloned insert. The cloned insert within pUB5608 which carries *orfs* 1, 2 and 3, as well as the downstream region was mapped with relation to the *E. coli* genome using the method of Kohara *et al.* (1987). Each of the 476 λ clones were spotted on a lawn of *E. coli* LE932. After overnight incubation at 37 °C phage plaque lifts were performed using Nytran membranes. Prehybridization of filters, nick translation of pUB5608 probe and autoradiography were performed according to Maniatis *et al.* (1982).

Minicell analysis. Minicells were prepared from overnight cultures of *E. coli* DS410, containing recombinant plasmids, by removal of most whole cells by centrifugation followed by successive purifications on sucrose gradients (20%, w/v, sucrose in M9 medium) (Davie *et al.*, 1984; Dougan & Sherratt, 1977). Purified minicells were resuspended in 30% (v/v) glycerol in M9 medium, at a density of 2×10^{10} minicells ml^{-1} , and frozen in aliquots at -70 °C. Minicell suspension (100 μ l) was mixed with 5 μ l methionine assay medium (Difco) and incubated for 2 h. Protein was labelled using 2 μ l [35 S]-methionine (20 μ Ci; 740 kBq) and incubated for a further 2 h. Labelled minicells were washed three times in M9 medium and proteins were fractionated by SDS-PAGE according to the method of Laemmli (1970).

Tn5 mutagenesis. The Tn5-containing λ phage, 2467, was used to mutagenize pUB5628 using previously described methodology (de Bruijn & Lupski, 1984).

DNA sequencing. DNA was sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase 2.0 (United States Biochemical). M13 universal primer was used to sequence in from the end of clones and subclones, and internal synthetic primers were used to sequence across the gaps.

Sequence analysis. Analysis of nucleotide and amino-acid sequences was performed with the aid of the University of Wisconsin genetics computer group (UWGCG) package (Devereux *et al.*, 1984) accessed via a VAX mainframe system.

RESULTS

Isolation of Ctx-sensitive mutants of *E. coli* JRG582(pNU305)

E. coli JRG582 is a derivative of HfrH which carries a chromosome deletion encompassing the *ampD* and *ampE* genes (Lindberg *et al.*, 1985). Loss of these genes results in constitutive high-level expression of the inducible Group I β -lactamase from *C. freundii* encoded by pNU305 (Lindquist *et al.*, 1989a). Accordingly, JRG582 carrying

Table 1. Effect of recombinant plasmids pUB5608, pUB5611 and pUB5628 on β -lactamase expression and β -lactam resistance in *E. coli* strains harbouring pNU305

Strain + pNU305	Recombinant plasmid	MIC*		β -Lactamase activity†
		Ap	Ctx	
HfrH	—	64	1	0.2
JRG582	—	1024	16	36.8
CS51	—	32	0.5	0.2
CS51	pUB5608	512	8	7.9
CS51	pUB5611	512	4	4.2
CS51	pUB5628	512	8	4.2
CS51	pUB5632	512	128	+ + ‡

* μ g antibiotic ml^{-1} .

† μ mol nitrocefin hydrolysed min^{-1} (mg protein) $^{-1}$. Values represent the mean of duplicate experiments.

‡ Not determined quantitatively.

pNU305 exhibits high-level resistance to a number of β -lactam antibiotics, including ampicillin (Ap) and Ctx. A mutant of JRG582(pNU305) was isolated which is Ctx sensitive (see Methods). This mutant, CS51(pNU305), was shown to produce low levels of β -lactamase compared to JRG582(pNU305), equivalent to the uninduced basal level production in HfrH(pNU305) (Table 1). The MIC values of Ctx and Ap for CS51(pNU305) were also greatly reduced compared to JRG582(pNU305) (Table 1). Plasmid pNU305 isolated from CS51 was shown to confer high-level β -lactam resistance when introduced into JRG582, indicating that the mutation in CS51(pNU305) is on the chromosome, rather than on pNU305. Confirmation of the chromosomal nature of the CS51 mutation was obtained by curing CS51 of pNU305 and showing that the subsequent re-introduction of pNU305, from a new source, gave the same β -lactam sensitive phenotype shown by the original isolate (data not shown).

Cloning of a region of *E. coli* DNA that restores high level β -lactamase production in CS51(pNU305)

A gene bank of chromosomal DNA from *E. coli* HfrH was constructed in the cloning vector pSU19 and subsequently amplified in *E. coli* XL1-blue. The recombinant gene bank was used to transform the Ctx-sensitive mutant CS51(pNU305), and transformants were selected which were resistant to Ap (100μ g ml^{-1}). Plasmid DNA from the resistant transformants was isolated and was demonstrated to restore resistance to Ap when reintroduced into CS51(pNU305). Analysis by restriction endonuclease digestion showed that all the recombinant plasmids contained DNA inserts and all but one gave identical restriction patterns; the exception was shown to contain the same insert, but in the opposite orientation with respect to the multiple cloning site. Two clones, pUB5608 and pUB5611, representing the two orientations were

Fig. 1. Diagrammatic representation of (a) clones of pUB5608 and pUB5611 (drawn to scale), and (b) subclones pUB5628 and pUB5632 (not drawn to scale). Hatched regions represent DNA derived from vector pSU19; arrows show the position and direction of open reading frames encoded by subcloned insert DNA (see text for details).

chosen for further study. The restriction maps of both clones are shown in Fig. 1(a).

Introduction of pUB5608 or pUB5611 into CS51(pNU305) promoted increased production of β -lactamase and a corresponding increase in β -lactam resistance, but not to the same levels exhibited by the parent strain, JRG582(pNU305) (Table 1). Plasmid pUB5608 was observed to have a greater stimulatory effect on β -lactamase expression than pUB5611.

Identification of the cloned region of DNA in pUB5608 and pUB5611

The 1.1 kb *Sal*I fragment from the insert of pUB5608 was radiolabelled and used as a probe against *Sal*I digests of pUB5608, pUB5611 and HfrH chromosomal DNA. In all

cases the probe hybridized to a 1.1 kb fragment confirming that the insert DNA in both pUB5608 and pUB5611 was derived from the same region of the HfrH chromosome. The map position of the cloned insert was determined using pUB5608 as a probe of the λ encyclopaedia developed by Kohara *et al.* (1987), representing the entire *E. coli* genome. Three positive signals were obtained corresponding to λ clones 8C5, 9A12 and 10B6. These three overlapping clones span the region from 59.9 to 60.6 min on the *E. coli* chromosome.

The *fuc* regulon, which encodes the genes required for fucose utilization, is located at 60.2 min on the *E. coli* chromosome (Zhu & Lin, 1988). The *fuc* regulon has been cloned and the insert DNA carrying the *fuc* genes mapped using restriction endonucleases. Comparison of this restriction map with that of the pUB5608 insert revealed

Fig. 2. Autoradiograph showing proteins encoded by pSU19 (vector), lane 1; pUB5608, lane 2; and pUB5628, lane 3; as determined by minicell analysis. The CAT protein is marked by an arrow. The positions of molecular mass standards (kDa) are shown. See text for details.

that one end of the *fuc* insert had a pattern almost identical to one end of the pUB5608 insert, the only difference being that pUB5608 possessed an additional *Pst*I site. This similarity suggested that the two inserts comprised overlapping segments of DNA, enabling the cloned insert to be localized specifically to 60.3 min. A 2.5 kb fragment from pUB5611, carrying all the DNA distal to the *fuc* regulon, was subcloned into pSU19. The resulting recombinant plasmid, pUB5628 (Fig. 1b), retained the ability to restore β -lactam resistance to, and to increase β -lactamase production by, CS51(pNU305) (Table 1).

During the course of this study the *E. coli* *ampG* gene was cloned and was reported to map at 10 min on the *E. coli* chromosome (Lindquist *et al.*, 1993) indicating that the cloned insert DNA in pUB5608/pUB5611 does not encode *ampG*. Strain CS51(pNU305), however, was found to be complemented to high-level β -lactamase production by an *ampG* clone (Everett, 1992) suggesting that CS51 carries an *ampG* mutation. This would explain the low-level non-inducible expression of the *C. freundii* *ampC* gene on pNU305 in CS51, and also indicates that the promotion of β -lactamase synthesis in this strain by

pUB5608 and pUB5611 occurs via a mechanism independent of AmpG.

Expression of recombinant plasmids in minicells

Plasmids pSU19, pUB5608, and pUB5628 were expressed in the minicell-producing *E. coli* strain, DS410. Proteins were radiolabelled and fractionated by SDS-PAGE (Fig. 2). The vector pSU19 expressed one protein of approximately 25 kDa, corresponding to the CAT protein which mediates Cm resistance in the plasmid host. Plasmid pUB5608 expressed five additional proteins of approximately 53, 44, 33, 30 and 16 kDa, three of which, 16, 33 and 44 kDa, were also expressed by the smaller subclone pUB5628. It is likely that the two proteins not expressed by pUB5628 are encoded by that portion of the *fuc* regulon present on pUB5608 but missing in pUB5628. It is interesting that the 14.4 kDa protein is much more abundant in lane 2 than in lane 3, suggesting that this protein is expressed more strongly in pUB5608 than in subclone pUB5628, which is derived from pUB5611 which carries the cloned insert in the opposite orientation.

Transposon mutagenesis and subcloning of pUB5628

Transposon mutagenesis was performed on pUB5628 to determine which areas were essential to activate expression of the *C. freundii* β -lactamase. Cells carrying pUB5628 were infected with λ 467, a λ phage derivative carrying the Tn5 transposon (de Bruijn & Lupski, 1984). Transductants were selected on agar containing Km and Cm. The colonies were pooled and plasmid DNA prepared. This was used to transform CS51(pNU305). Transformants were selected on agar containing Km and Cm, and screened for absence of Ap resistance. Plasmid DNA was prepared from those clones which exhibited an MIC of Ap $< 128 \mu\text{g ml}^{-1}$, and the position of each Tn5 insertion was mapped using restriction endonucleases. The Tn5 inserts were randomly distributed over a 1 kb region immediately distal to the *fuc* regulon (Fig. 3), suggesting that the integrity of this region is required for stimulation of β -lactamase activity.

A 1 kb *Eco*RI–*Bam*HI fragment from pUB5628, corresponding to this region, was subsequently ligated into vector pSU19. The resulting subclone, designated pUB5632 (Fig. 1b), was shown to confer raised β -lactamase levels and high-level β -lactam resistance in CS51 carrying pNU305, confirming the conclusion drawn from the transposon mutagenesis (Table 1).

Sequencing and sequence analysis of the pUB5628 insert

The nucleotide sequence of the insert in pUB5628 was determined (Fig. 4). Three open reading frames, designated *orf1*, *orf2* and *orf3* were identified, the products of which are predicted to have molecular masses of 34.4, 14.3 and 42.0 kDa, respectively, values which correspond closely to those of the proteins encoded by pUB5628 in minicells. An initial search of the EMBL database using

Fig. 3. Diagrammatic representation of the insert from pUB5628 showing the position of Tn5 inserts as mapped by restriction analysis. Solid arrows indicate the sites of insertions which resulted in a loss of a β -lactamase stimulating activity; unfilled arrowheads indicate insertions which did not affect β -lactamase stimulating activity (see text for details).

the FASTA program identified a number of protein sequences with significant homology to that of the *orf1* gene product, ORF1. The best scores were obtained with proteins belonging to the LysR family of bacterial transcriptional activators, which includes AmpR, the activator of *ampC*. Furthermore, four of the five highest scores were obtained with AmpR proteins, suggesting that ORF1 is likely to be a transcription factor with similarities to AmpR. The results of sequence comparisons using the UWGCG GAP program, which inserts gaps where necessary to achieve optimal sequence alignment, are given in Table 2, with proteins listed in order of greatest similarity to the ORF1 protein. ORF1 showed between 32 and 38% identity with AmpR proteins when compared over the entire lengths of the sequences. These values increased to between 53 and 57% when only the first 70 amino acids were compared, indicating that the N-terminal ends are more similar than the proteins as a whole. Alignment of the ORF1 sequence and those of the AmpR proteins indicated a large number of conserved residues, particularly in the region of the helix-turn-helix binding motif proposed to comprise the DNA-binding site in such proteins; however, certain sequences also appeared to be conserved in the central and C-terminal regions (Fig. 5).

The other two open reading frames (*orf2* and *orf3*) encoded by pUB5628 showed no significant homology to any of the sequences in the database and are not discussed here further.

It was mentioned above that pUB5608 stimulated β -lactamase production more than did pUB5611; this was despite the fact that both inserts seemed very similar when analysed by endonuclease restriction and gel electrophoresis. Analysis of the DNA sequence upstream of the *orf1* translational start site in pUB5628 (derived from pUB5611) revealed a putative -10 promoter box within the cloned DNA; however, there was insufficient nucleotide sequence within the insert DNA to contain the -35 box. One possible explanation for the differences in phenotype conferred by pUB5608 and pUB5611 is that pUB5608, in contrast to pUB5611 (and its derivative pUB5628), carries the complete *orf1* promoter resulting in greater transcription of the *orf1* gene. Sequencing across the vector/insert junction of pUB5608, however, showed it to be identical to that in pUB5628 showing that both constructs lack a complete promoter. Transcription of *orf1* must, therefore, be initiated either from a promoter sequence within the vector or from a hybrid promoter composed of vector DNA sequences together with the

-10 box from the insert. Because the inserts from pUB5608 and pUB5611 are in opposite orientations in the vector, such a hybrid promoter would be different in each case and different rates of transcription of *orf1* might be expected. This finding agrees with the results of the minicell analysis which showed that the 14.4 kDa protein encoded by *orf1* was more abundant in minicells carrying pUB5608 than those carrying pUB5628.

Activation of *ampC* by *orf1* does not require the presence of the *ampR* gene

E. coli SN03 carrying either plasmid pNU305 or pNU307, which is an *ampR* deletion derivative of pNU305 (Lindberg *et al.*, 1985), was transformed with pUB5608, pUB5628 or pUB5632. β -Lactamase assays were performed on induced (500 μ g 6-aminopenicillanic acid ml^{-1}) and uninduced cultures and MIC values were determined for Ap and Ctx (Table 3). *E. coli* SN03 was used as the host strain because it produces negligible amounts of its native *ampC* β -lactamase (Normark & Burman, 1977). When plasmids carrying *orf1* were introduced into SN03-(pNU305) little effect on β -lactamase levels, whether induced or uninduced, or on MIC values was observed compared to the strain carrying only pNU305. This is in contrast to the results obtained with CS51(pNU305), where the introduction of *orf1* resulted in significantly raised β -lactamase levels (Table 1). Introduction of *orf1* into SN03(pNU307), however, resulted in a substantial increase in the level of β -lactamase activity, with corresponding increases in MIC values (Table 3).

These results demonstrate that, in *E. coli* SN03, ORF1 activates *C. freundii ampC* expression directly, without the requirement for AmpR, and support the evidence of sequence data analysis which indicates that ORF1 is a transcriptional activator. Furthermore, β -lactamase expression was found to be unaffected by the addition of inducer to the medium, indicating that ORF1 does not respond to a normal β -lactam induction stimulus. When AmpR was present, together with ORF1, e.g. in SN03-(pNU305, pUB5632), ORF1 did not stimulate expression suggesting that AmpR competes with ORF1 for binding to the operator site of the *ampC* promoter. The difference between the effect of ORF1 on expression of *ampC* from pNU305 in SN03 (Table 3) compared to CS51 (Table 1) may be due to differences in the genetic background of the two strains. In the latter strain, it is possible that the *ampG* mutation, which is as yet uncharacterized, may in some way impair the ability of AmpR to compete with ORF1.

[illegible]

Fig. 4. Nucleotide sequence of the *E. coli* chromosome insert carried in pUB5628 showing the amino-acid translations of *orf1* (43–960), *orf2* (979–1374) and *orf3* (1367–2467). Putative Shine–Dalgarno sequences (SD) and –10 and –35 promoter regions are indicated. A putative transcriptional terminator structure is indicated by (****).

Table 2. Comparison of ORF1 and LysR-type proteins using GAP analysis

Protein	Organism	Percentage identity	Percentage similarity	Reference
AmpR	<i>Pseudomonas aeruginosa</i>	45.2	65.2*	Lodge <i>et al.</i> (1990)
AmpR	<i>Rhodobacter capsulatus</i>	37.4	52.6	Campbell <i>et al.</i> (1989)
AmpR	<i>Citrobacter freundii</i>	33.2	52.9	Lindberg <i>et al.</i> (1985)
AmpR	<i>Enterobacter cloacae</i>	32.3	52.2	Honoré <i>et al.</i> (1986)
TrpI	<i>Pseudomonas aeruginosa</i>	30.7	53.1	Chang <i>et al.</i> (1989)
TfdS	<i>Alcaligenes eutrophus</i>	28.3	48.6	Streber <i>et al.</i> (1987)
LysR	<i>Escherichia coli</i>	26.3	44.2	Stragier & Patte (1983)
YfeB	<i>Escherichia coli</i>	26.0	48.4	Brun <i>et al.</i> (1990)
MetR	<i>Salmonella typhimurium</i>	24.2	48.7	Plamann & Stauffer (1987)
MetR	<i>Escherichia coli</i>	24.1	46.4	Maxon <i>et al.</i> (1990)
CatM	<i>Acinetobacter calcoaceticus</i>	24.0	46.3	Neidle <i>et al.</i> (1989)
CatR	<i>Pseudomonas putida</i>	23.6	49.3	Rothmel <i>et al.</i> (1990)
TdcA	<i>Escherichia coli</i>	23.3	47.7	Schweizer & Datta (1989)
AntO	<i>Escherichia coli</i>	23.3	41.6	Mackie (1986)
LeuO	<i>Escherichia coli</i>	21.8	44.4	Haughn <i>et al.</i> (1986)
GltC	<i>Bacillus subtilis</i>	21.8	45.3	Bohannon & Sonenshein (1989)
NahR	<i>Pseudomonas putida</i>	20.9	45.3	Schell & Sukordhaman (1989)
MkaC	<i>Salmonella typhimurium</i>	17.0	40.1	Pullinger <i>et al.</i> (1989)

* Only partial sequence available (= 135 amino acids).

The level of *orf1* expression affects the level of expression of *ampC*

Expression of the *orf1* gene was placed under the control of the Ptac promoter by inserting the Ptac/*lacI*^q cassette from pRU883 (Ubben & Schmidt, 1987) immediately upstream of the N-terminus of *orf1* in pUB5632. The resulting construct, pUB5636, was checked by restriction analysis to ensure the cassette was in the correct orientation as regards *orf1* expression. pUB5636 was introduced into SN03(pNU307) and the MICs of Ap and β -lactamase levels were determined in the presence or absence of IPTG, which induces expression from the Ptac promoter. Induction of *orf1* by IPTG caused β -lactamase levels to increase about 30-fold compared to that of the non-induced strain (Fig. 6). Correspondingly, the MIC value for Ap increased from 64 $\mu\text{g ml}^{-1}$ to > 2000 $\mu\text{g ml}^{-1}$ upon induction with IPTG (60 $\mu\text{g ml}^{-1}$).

Complementation of the *gcvA1* mutation by *orf1*

A novel *E. coli* gene, recently described by Wilson *et al.* (1993) and which encodes a positive-acting regulatory protein required for expression of glycine cleavage enzyme genes, has been mapped to 60.3 min in the *E. coli* chromosome. This is the same location as that found by us for *orf1*. Given the similarities between the two genes, i.e. both encode transcription factors, we decided to investigate whether the *orf1* gene would complement the *gcvA1* mutation in *E. coli* GS970 (Wilson *et al.*, 1993). This strain is unable to grow on minimal media lacking serine but supplemented with glycine (300 $\mu\text{g ml}^{-1}$) due to the *gcvA1* mutation; however, transformation with pUB5632, which carries *orf1*, restored the ability to grow on medium containing glycine but lacking serine, indicating that *orf1*

and *gcvA1* are the same gene. Transformation of GS970 with pNU305 (*ampR*–*ampC*) did not permit growth on the same medium suggesting that AmpR, under the control of its natural promoter, is unable to activate expression of the glycine cleavage genes by substituting for GcvA.

DISCUSSION

In normal circumstances expression of the *C. freundii ampC* gene in *E. coli* above the basal level depends on the cognate *C. freundii* AmpR factor (Lindberg *et al.*, 1985). The work reported in this paper describes an *E. coli* transcription factor that can, in part, substitute for the *C. freundii* AmpR in that it activates expression of the *C. freundii ampC* gene. It is not however, responsive to β -lactam induction. The gene for the alternative transcription factor, temporarily designated *orf1*, maps at 60.3 min on the *E. coli* chromosome.

Wilson *et al.* (1993) recently reported the discovery in *E. coli* of an activator function that regulates production of enzymes of the glycine cleavage system. The activator gene, *gcvA*, was mapped to 60.3 min on the chromosome. When tested, *orf1* was found to complement a mutation in *gcvA*. More recently the *gcvA* gene has been sequenced (Wilson & Stauffer, 1994). The sequence is identical to that of *orf1*, thus confirming that *orf1* and *gcvA* are the same gene. Accordingly, we will refer to this locus as *gcvA*.

Our results show that the *gcvA* gene product promotes an increase in the expression of the *C. freundii ampC* β -lactamase gene independently of the normal activator, AmpR. An analysis of the predicted amino acid sequence of GcvA indicates that it is likely to belong to the LysR

	1				50
ORF1	MskrlpPLNa	LRVFDAAARH	LSFTTrAAeEL	fvtTqaAVShq	IKsLEdfLgl
Amprpseae	MvRphLPLNa	LaAFEAsARH	LSFTTrAAIEL	cVTqaAVShq	VKsLEerLgv
Amprrhoca	MdRpdLPLNa	LRvFEvAmRq	gsFTkAAIEL	rVTqaAVShq	VarLEdlLgt
Amprcitfr	MtRsyiPLNs	LRAFEAAARH	LSFTTrAAIEL	nVThsAISqh	VKsLEqqLnc
Amprentcl	MtRsyLPLNs	LRAFEAAARH	LSFTThAAIEL	nVThsAISqh	VKtLEqhLnc
Consensus	M-R--LPLN-	LRAFEAAARH	LSFT-AAIEL	-VT--A-S--	VK-LE--L--

	51				100
ORF1	kLFrRrnRsL	LLTEEGqsyF	ldikeiFsqL	teatrKlQar	sakgaLtVsl
Amprpseae	aLFkRlPRGL	MLThEGEsLL	PVLcDSFDRI	AGLLERFegg	hyrDvLtVGa
Amprrhoca	aLFrltSqGL	ipTdeGrllF	PVLehgFDam	srVLDRlQgr	rdiEvLKVGv
Amprcitfr	qLFvRqSRGL	MLTtEGEsLL	PVLnDSFDRm	AGMLDRFatk	qtqEkLKIGV
Amprentcl	qLFvRvSRGL	MLTtEGEnLL	PVLnDSFDRI	AGMLDRFanH	raqEkLKIGV
Consensus	-LF-R-SRGL	MLT-EGE-LL	PVL-DSFDR-	AGMLDRF---	---E-LK-GV
	101				150
ORF1	lpsFAihwLv	PrLssFnsay	PgIDvriqav	drqeDklaDd	vDvaIfYGrG
Amprpseae	VGTFtvGwLL	PrLEDFqarh	PfIDLrLSTH	NNRVD....	
Amprrhoca	ntTFamcwLM	PrLEaFrqah	PqIDLrLSTn	NNRVEilrEG	LDmaIRFGtG
Amprcitfr	VGTFaIGcLF	PlLsDFkrSy	PhIDLhLSTH	NNRVDpaaEG	LDytIRYGGg
Amprentcl	VGTFATGvLF	sqLEDFrgrY	PhIDLqLSTH	NNRVDpaaEG	LDytIRYGGg
Consensus	VGTF-A-G-LF	P-LEDF----	P-IDL--STH	NNRVD---EG	LD--IRYG-G
	151				200
ORF1	nWpglrvekL	yaeyLlPvCs	Pllltgekpl	ktPeDlakht	LLhdasrrrDW
Amprrhoca	gWtghDAipL	aeApMaPLCa	Pgl...Asrl	lhPsDlqgvt	LLRSYRsaEW
Amprcitfr	aWhdtDAQYL	csAlMsPLCs	Ptl...Asqi	qtPaDilkfp	LLRSYRrdEW
Amprentcl	aWhgtEAeLF	chApLaPLCt	Pdi...Aasl	hsPaDilrft	LLRSYRrdEW
Consensus	-W---DA--L	--A-M-PLC-	P-- A---	--P-D-----	LLRSYR--EW
	201				250
ORF1	qtYtrqlGln	hinVqggpif	shsamVlqaa	ihgqGvalAn	nv..Maqsei
Amprrhoca	pgWfeAAGvp	cPPv...tgp	VFDSSVaLaE	lAtsGaGVAL	lPIsMFesyi
Amprcitfr	alWmqAAGea	.PPspthnm	VFDSSVtMlE	aAqgGmGVAi	aPvMrMFthll
Amprentcl	taWmqAAGeh	.PPspthrm	VFDSSVtMlE	aAqaGvGIAi	aPvdmMFthll
Consensus	--W--AAG--	--PP-----	VFDSSV-M-E	-A--G-GVA-	-PV-MF----
	251				300
ORF1	eagRlvcPFn	dvlVsknaFY	lvchdsqael	gkiaaFrqWi	lakaaaegek
Amprrhoca	aqgRlaQPFG	vT.VsvGrYY	laWpsdRpaT	sAMstFSRWL	tgqsae...
Amprcitfr	sseRivQPFL	.TqIdlGsYW	itRlqsRpeT	pAMreFSRWL	tgvlhk...
Amprentcl	aseRivQPFF	aTqIelGsYW	ltRlqsRaeT	pAMreFSRWL	vekmkk...
Consensus	---R--QPF-	-T-I--G-YW	--R---R--T	-AM--FSRWL	-----
	301				
ORF1	frfryeq				
Amprrhoca					
Amprcitfr					
Amprentcl					
Consensus	-----				

Fig. 5. Multiple sequence alignments of ORF1 and AmpR proteins. The consensus sequence shows those residues conserved throughout the AmpR family (plurality = 3) and take no account of the sequence of ORF1. The N-terminal helix-turn-helix motif is identified by (****). Conserved C-terminal sequences are underlined.

Table 3. Effect of subclones on inducible β -lactamase expression and β -lactam resistance in *E. coli* SN03 carrying pNU305 or pNU307

Plasmids	MIC ($\mu\text{g ml}^{-1}$)		β -Lactamase activity*	
	Ap	Ctx	Uninduced	Induced
pNU305	64	0.5	0.5	3.4
pNU307	128	2	2.8	1.6
pNU305 + pUB5608	128	2	0.9	3.9
pNU307 + pUB5608	512	8	21.8	23.7
pNU305 + pUB5628	128	2	0.7	2.2
pNU307 + pUB5628	512	8	19.7	23.4
pNU305 + pUB5632	64	0.25	0.3	2.9
pNU307 + pUB5632	512	0.5	22.4	25.4

* μmol nitrocefin hydrolysed min^{-1} (mg protein^{-1}). Values represent the mean of duplicate experiments.

Fig. 6. Effect of IPTG induction on β -lactamase production in SN03(pNU307, pUB5636). Specific activity is measured in μmol nitrocefin hydrolysed min^{-1} (mg protein^{-1}).

family of bacterial transcriptional activators (Henikoff *et al.*, 1988). Homologies between GcvA and other members of the family were found to be particularly strong in the N-terminal region which accommodates the helix-turn-helix motif reported to be necessary for binding to DNA. As might have been predicted, the greatest similarities between GcvA and other members of the LysR family are with AmpR proteins, the N-terminal regions of which are almost identical to that of GcvA (Fig. 5). It is, therefore, reasonable to propose that GcvA binds to the promoter region of the *C. freundii ampC* gene so as to mimic the bound, activated form of AmpR sufficiently closely to activate expression of *ampC*. This view is supported by the observation that GcvA-mediated *ampC* expression is higher in the absence of AmpR than in its presence, suggesting that the two proteins compete for the same, or overlapping, sites.

Transcription of *ampR* and similar regulatory genes has been shown to be negatively autoregulated. This is facilitated by the gene arrangements whereby the regulator gene and the first of the genes regulated are adjacent, with divergent transcription from the common intercistronic region. This allows the regulator binding site and the regulator gene promoter to overlap (Honore *et al.*, 1986; Lindquist *et al.*, 1989b). However, *gcvA* and the gene(s) it controls map to different locations on the *E. coli* chromosome (Wilson *et al.*, 1993), hence, it is not possible to predict if *gcvA* expression is self-regulating. Preliminary data suggest that *gcvA* is not highly expressed (Wilson & Stauffer, 1994; T. R. Walsh, unpublished results), and from this respect it is interesting to note that the original *gcvA* clones were recovered on DNA fragments that appear not to accommodate the normal *gcvA* promoter. In these cases we believe expression of the *gcvA* gene is from artificial promoters created by the cloning and which probably comprise the -10 box 36 bp upstream of the gene and a -35 box provided by vector sequences. If this is a correct interpretation, then the cloned *gcvA* gene will have resulted in constitutive and possibly enhanced expression. This may have facilitated recovery of the constructs by promoting, in turn, good expression of the *C. freundii ampC* β -lactamase gene and hence resistance to Ap. Wilson & Stauffer (1994) identified the transcriptional start of the *gcvA* mRNA as 72 bp upstream of the *gcvA*

start codon. The promoter was shown to have a -10 box with a perfect match to the consensus sequence (i.e. TATAAT) but an unusual -35 region. This suggests that the putative -10 region identified by ourselves is probably not part of a natural *gcvA* promoter, whether or not it forms part of a hybrid promoter in our particular constructs.

It is not known if, under normal conditions, expression of *gcvA* influences the level of *C. freundii ampC* expression. In SN03(pNU305) constitutive expression of *gcvA* from a multi-copy vector has little effect on β -lactamase levels; thus, given a single copy of the gene, when *gcvA* expression is predicted to be much lower, one would expect GcvA-mediated expression of *ampC* to be low or non-existent. However, this may not be the case in strains carrying pNU307 which lacks the *ampR* gene. It has always been assumed that increased *C. freundii ampC* expression from pNU307, compared to pNU305, is due to a loss of a repressor activity associated with AmpR in its unactivated state. An alternative explanation is that, in the absence of AmpR, a low level of the GcvA protein can effect low expression of the *ampC* gene.

It might be expected that if GcvA is able to substitute for AmpR, and thereby promote expression of *ampC*, then AmpR might similarly be able to substitute for GcvA allowing expression of the glycine cleavage system. The *C. freundii ampR* gene carried on pNU305, however, was shown not to complement the *gcvA1* mutation in *E. coli*, suggesting that the 'cross-talk' between the two systems is of a one-way nature. It should be pointed out, however, that pNU305 contains the autoregulatory site in the *ampR* promoter and so produces low-levels of AmpR. Whether overexpression of *ampR* from an artificial promoter would result in complementation of the *gcvA1* mutation has yet to be determined. Further studies to elucidate exactly which elements are necessary for transcriptional activation of the *ampC* promoter by AmpR and GcvA should provide an insight into the molecular basis of DNA-protein interactions in this important class of prokaryotic regulatory proteins.

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