The signal transducer encoded by *ampG* is essential for induction of chromosomal AmpC β -lactamase in *Escherichia coli* by β -lactam antibiotics and 'unspecific' inducers

Herbert Schmidt,¹† Gisela Korfmann,² Holger Barth¹ and Hans H. Martin¹

Author for correspondence: Herbert Schmidt. Tel: +49 931 2013901. Fax: +49 931 2013445.
e-mail: hschmidt@hygiene.uni-wuerzburg.d400.de

¹ Institut für Mikrobiologie,
Technische Hochschule,
64287 Darmstadt, Germany

² Bayer AG, 51368
Leverkusen, Germany

Chemical mutagenesis of the AmpC β -lactamase-hyperinducible *Escherichia coli* strain SN0301/pNu305 carrying the cloned *ampC* and *ampR* genes from *Citrobacter freundii* OS60 gave four independent mutants in which β -lactamase was no longer inducible, or was inducible only to a low level, by β -lactam antibiotics. The genes *ampC*, *ampR*, *ampD* and *ampE*, which were essential for β -lactamase induction, were functional in these mutants. In all four mutants, the sites of mutation were mapped to 9.9 min on the *E. coli* chromosome. Complementation with wild-type *ampG* restored inducibility of β -lactamase to wild-type levels. The nucleotide sequence of all four mutant *ampG* alleles (*ampG1*, *ampG3*, *ampG4* and *ampG5*) was determined. In three of the mutants, a single base exchange led to an amino acid change from glycine to aspartate at different sites in the deduced amino acid sequence. In the fourth mutant (*ampG4*), with low-level inducibility, the nucleotide sequence was identical to wild-type *ampG*. Spontaneous back-mutation of the chromosomal *ampG1* mutant resulted in restoration of wild-type inducibility and a return to the wild-type *ampG* sequence. Unspecific induction by components of the growth medium was also dependent on intact *ampG* function.

Keywords: β -lactamase induction, *ampG* mutants, unspecific induction

INTRODUCTION

The chromosomally encoded AmpC β -lactamase is present in many Gram-negative bacteria and can be induced to high levels by β -lactam antibiotics. This is recognized as a major factor of β -lactam resistance in these organisms. Molecular studies of the induction process have revealed many properties and functions of the structural and regulatory genes involved (Normark *et al.*, 1990, 1994). The more recently discovered gene *ampG* (Korfmann & Sanders, 1989; Lindquist *et al.*, 1993) appears to play a key role, presumably as membrane-bound transducer of the external induction signal to the intracellular β -lactamase regulon. The transducer is thought to relay the signal to

the *ampR*-encoded transcriptional regulator, which is thereby enabled to activate the transcription of the structural gene *ampC* (Honoré *et al.*, 1989; Lindberg *et al.*, 1985; Bartowsky & Normark, 1993). An additional interaction with the signal or the signal-binding regulator AmpR takes place with the *ampDE*-encoded negative modulator, which down-regulates induction in the absence of an inducing β -lactam. In *ampD*-defective mutants, AmpC β -lactamase is hyperinducible or semi-constitutively overproduced, causing high β -lactam resistance in these organisms (Lindberg *et al.*, 1987; Lindquist *et al.*, 1989b; Honoré *et al.*, 1989).

Still unknown features of the induction process are the physical nature of the induction signal resulting from the primary interaction of the inducer with a hypothetical induction sensor, and its transmission from sensor to AmpG transducer. Different mechanisms are considered in current models. The inducing β -lactam antibiotic itself may be the AmpG-transduced and AmpR-activating signal (Normark *et al.*, 1994). Alternatively, the signal may arise in an indirect pathway from the inactivating

† Present address: Institut für Hygiene und Mikrobiologie, Universität Würzburg, 97080 Würzburg, Germany.

Abbreviations: 6-Apa, 6-aminopenicillanic acid; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

The EMBL accession numbers for the sequences reported in this paper are X82158 (*ampG1*), X82159 (*ampG3*) and X82160 (*ampG5*).

binding of the inducing β -lactam to a penicillin-binding protein acting as induction sensor. The resulting disturbance of the synthesis and morphogenesis of cell wall peptidoglycan may create an indigenous peptidoglycan metabolite functioning as 'autoinducer' (Lindberg *et al.*, 1988; Normark *et al.*, 1990; Tuomanen *et al.*, 1991; Lindquist *et al.*, 1993; Tölg *et al.*, 1993). Thus, additional induction-specific gene functions may be involved and may be found by screening for mutation to non-inducibility in the presence of the functional genes *ampC* and *ampR*.

A search for such mutants described in the present paper showed that independently obtained, non-inducible mutants of *Escherichia coli* containing cloned *ampR* and *ampC* of *Citrobacter freundii* were all defective in *ampG*. On the other hand, and as indication of an indirect induction pathway, we also observed that intact *ampG* function is essential not only for induction of β -lactamase by β -lactams but also for so-called 'unspecific' induction by components of the growth medium (Gootz & Sanders, 1983; Cullmann *et al.*, 1984).

METHODS

Bacterial strains and plasmids. *E. coli* SN0301 is an *ampD1* mutant of SN03 (Lindberg *et al.*, 1987; Lindquist *et al.*, 1989a). *E. coli* strains SN0301-1, SN0301-3, SN0301-4 and SN0301-5 are β -lactam-sensitive and β -lactamase non-inducible mutants of SN0301 and are described in this study. Strains SN0301-11, SN0301-13, SN0301-14 and SN0301-15 are *recA*-positive derivatives of the mutants described above. HfrH, HfrC (Miller, 1972) and Hfr KL16 S1 (*srl::Tn10; recA*⁺) were used for mating experiments. The latter strain was derived from Hfr KL16 by mating with JC10240 (*srl::Tn10; recA56*) (Hantke, 1982). χ^{578} , KL723 and E5014 carry the the F-primes F254, F104 and F128

(Low, 1972), respectively (provided by B. Bachmann, Genetic Stock Center, Yale University, New Haven, CT, USA). H1785 (*fecA::Tn1732*), H1445 (*tsx*), H1469 (*tsx; lon::Tn10*), KB423 (*tsx*) and PCO135 (*purE*) were kindly provided by K. Hantke, Tübingen, Germany.

The recombinant plasmids used in this study are described in Table 1. Plasmids pUC19 (Vieira & Messing, 1982), pK18 (Pridmore, 1987) and pACYC184 (Chang & Cohen, 1978) were used as cloning vectors.

Growth media. *E. coli* mutants were routinely cultivated in L-broth with addition of 2 g glucose l⁻¹ and 1 mg thiamin l⁻¹ (Lindberg *et al.*, 1987), and M9CA medium (Lindberg *et al.*, 1987) supplemented with 2 g Casamino acids (Difco) l⁻¹, 50 mg uracil l⁻¹, 50 mg tryptophan l⁻¹ and 1 mg thiamin l⁻¹. Complex media and medium components for unspecific β -lactamase induction were Penassay Broth (Difco), Schaedler Anaerobic Broth (Oxoid), Peptone Soya and Peptone Special (Oxoid/Unipath).

Chemical mutagenesis with N-methyl-N'-nitro-N-nitroso-guanidine (NTG). Chemical mutagenesis was performed according to Adelberg *et al.* (1965) with slight modifications. Briefly, L-broth was inoculated with an overnight culture of *E. coli* SN0301/pNU305 to a density of 10⁶ cells ml⁻¹. The culture was agitated at 37 °C for 2.5 h. After washing the cells three times in TM buffer (0.05 M Tris base, 0.05 M maleic acid; pH 6.0), 4.5 ml of the bacterial suspension was mixed with NTG solution to give a final NTG concentration of 200 μ g ml⁻¹. After 7 min incubation at 37 °C on a slow rotary shaker the cells were rapidly separated from NTG by filtering the suspension through a 0.45 μ m membrane filter (Sartorius). The filter was washed twice in TM buffer; the cells were carefully suspended in L-broth and finally incubated for 2 h at 37 °C.

Gene mapping. For rough mapping the non-inducible mutants were made *recA*⁺ by curing them of pNU305 (*ampR*⁺ *ampC*⁺), transforming them with pNU344 (*ampR*⁺ *ampC*⁺) and mating them with *E. coli* Hfr KL16 (*recA*⁺; *srl::Tn10*). Conjugative

Table 1. Characteristics of recombinant plasmids used in this study

Recombinant plasmid	Vector	Relevant genotype/origin of inserted DNA	Marker*	Reference
pNU305	pBR322	Wild-type <i>ampR</i> and <i>ampC</i> genes from <i>C. freundii</i> OS60	Tc ^r	Lindberg <i>et al.</i> (1985)
pNU344	pACYC184	Wild-type <i>ampR</i> and <i>ampC</i> genes from <i>C. freundii</i> OS60	Cm ^r	Lindberg <i>et al.</i> (1987)
pNU413	pACYC184	Wild-type <i>ampD</i> and <i>ampE</i> genes from <i>E. coli</i> SN03	Cm ^r	Lindquist <i>et al.</i> (1989a)
pGKS273-3	pACYC184	Wild-type <i>ampG</i> gene from <i>E. coli</i> JRG582	Cm ^r	G. Korfmann, unpublished
pMH203	pACYC184	<i>ampG1</i> mutant allele from <i>E. coli</i> SN0301-1	Cm ^r	This study
pMH203-3	pUC19	<i>ampG1</i> mutant allele subcloned from pMH203	Ap ^r	This study
pMH303-1	pUC19	<i>ampG3</i> mutant allele from <i>E. coli</i> SN0301-3	Ap ^r	This study
pMH305-1	pUC19	<i>ampG5</i> mutant allele from <i>E. coli</i> SN0301-5	Ap ^r	This study
pMH600	pK18	Wild-type <i>ampG</i> gene subcloned from pGKS273-3	Km ^r	This study
pMH601	pK18	<i>ampG1</i> mutant allele cloned from pMH203-3	Km ^r	This study
pMH603	pK18	<i>ampG3</i> mutant allele cloned from pMH303-1	Km ^r	This study
PMH605	pK18	<i>ampG5</i> mutant allele cloned from pMH305-1	Km ^r	This study
pMH601-R1	pK18	Wild-type <i>ampG</i> ; back-mutation of <i>ampG1</i> (pMH601)	Km ^r	This study

* Resistance to the respective antibiotic: Tc, tetracycline; Cm, chloramphenicol; Ap, ampicillin; Km, kanamycin.

mating in liquid culture was performed as described by Miller (1972), with a ratio of donor to recipient of 1:5. Tetracycline-resistant transconjugants were selected and tested for the presence of the *recA* gene by the method of Clark & Margulies (1965). Complementation of gene defects was tested by mating with F-primes (Low, 1972; Miller, 1972). Transconjugants which were able to complement the gene defects were selected on agar plates containing 256 μ g ampicillin ml⁻¹.

Fine-structure mapping was performed by cotransduction experiments with bacteriophage P1_{vir} as described by Miller (1972). To determine the relative position of the mutation in relation to the marker genes, three-point analysis was performed. The distance between the cotransduced markers was calculated from the equation of Wu (1966): $f = (1 - X/L)$, with $L = 2.0$.

Polymerase chain reaction (PCR). Amplification of the mutant alleles was performed in a total volume of 50 μ l containing 1 ng chromosomal DNA of the mutants, each deoxynucleoside triphosphate at 200 μ M, 50 pmol of primers HS1 (5'-CCCG-AATTCTGCCTCCTGGCCC-3') and HS2 (5'-CCCAAGCTTCGTAATAATTACGGCG-3'), 5 μ l tenfold-concentrated *Taq* DNA polymerase synthesis buffer and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Cetus). DNA was denatured at 94 °C for 60 s, the primers were annealed at 50 °C for 60 s and primer extension was conducted for 90 s at 72 °C. Thermal cycling was carried out in a Perkin-Elmer Cetus thermal cycler N801-0177. After 30 cycles the amplification products were subjected to a final extension step of 5 min at 72 °C.

Recombinant DNA methods. Standard procedures were used (Sambrook *et al.*, 1989).

Nucleotide sequence analysis. DNA sequencing was performed by the method of Sanger *et al.* (1977) with the T7 sequencing kit (Pharmacia LKB), following the manufacturer's instructions. Labelling was performed with [³⁵S]dATP α S (approx. 1 Ci mmol⁻¹; 37 GBq mmol⁻¹). Target DNA, cloned in pUC vectors, was used for the double-strand sequencing procedure. Sequencing products were separated on field gradient gels as described by Ansorge & Barker (1984) and visualized on X-ray film (Kodak XAR5) after exposure for 24 h.

β -Lactamase techniques. Procedures described by Lindberg *et al.* (1985, 1987) and Tölg *et al.* (1993) were used. Induction of β -lactamase was performed with 6-aminopenicillanic acid (6-Apa, 250 mg ml⁻¹). β -Lactamase activity was measured with the substrate cephalixin (200 μ M). One unit of β -lactamase hydrolyses 1 μ mol substrate min⁻¹. Total protein was determined as described by Bradford (1976).

RESULTS

Generation of β -lactamase non-inducible mutants in *E. coli* carrying cloned genes *ampR* and *ampC*

Mutagenesis with NTG was performed on the *ampD*-defective, β -lactamase-hyperinducible *E. coli* SN0301 (Lindquist *et al.*, 1989a) carrying cloned genes *ampR* and *ampC* from *C. freundii* OS60 on plasmid pNU305 (Lindberg *et al.*, 1985). Rare, non-inducible mutants were obtained only after heavy mutagenesis with 200 μ g NTG ml⁻¹, leaving 5–10% surviving bacteria. The presence of the multicopy plasmid pNU305 during mutagenesis enhanced the recovery of desired mutants whilst keeping the risk of isolating non-inducible *ampR*- or *ampC*-defective mutants low. The parent strain SN0301/pNU305 was resistant to 1024 μ g ampicillin ml⁻¹. Among

the surviving mutagenized bacteria, ampicillin-sensitive and presumably non-inducible mutants were detected at a frequency of 3×10^{-5} by their inability to grow on replica plates containing 256 μ g ampicillin ml⁻¹. Four independent, ampicillin-sensitive mutants were obtained. β -Lactamase was completely non-inducible in three of these mutants: SN0301-1, SN0301-3 and SN0301-5. In mutant SN0301-4 the enzyme was inducible to a low level. Values of β -lactamase activity and β -lactam sensitivity are shown in Table 2. Plasmid pNU305, purified from each of the mutants, did not transfer the non-inducibility when transformed into the parent strain SN0301. Nor was inducibility restored when the mutants were cured of resident plasmid pNU305 and retransformed with pNU305 isolated from the inducible strain SN0301/pNU305. Also, the mutants remained non-inducible after transformation with pNU413 (Lindquist *et al.*, 1989a). Therefore, genes *ampR*, *ampC*, *ampD* and *ampE* were not involved in the loss of inducibility.

Localization of the mutation site at 9.9 min on the *E. coli* chromosome

Matings of *recA*⁺ derivatives of the non-inducible mutants with different Hfr strains and with F-primes yielded recombinants inducible for β -lactamase and resistant to ampicillin only with HfrH and HfrC [the origin of transfer (*oriT*) sites of these donors are at 9.9 and 12 min, respectively] and with prime F254 (*lac-lip*, 8–15 min region of the *E. coli* chromosome). Cotransduction mapping was performed with P1_{vir} lysates of *E. coli* strains with the selectable markers *fecA*::Tn1732 (Km^r), *tsx*, *lon* and *purE* at 7, 9, 10 and 12 min, respectively. Frequencies of cotransduction of ampicillin resistance and calculated map distances were 52% and 0.41 min with *tsx*, and 83% and 0.12 min with *lon*. No cotransduction with the flanking genes *fecA* and *purE* was observed. The sequential order of the mutation site in relation to the closest markers *tsx* and *lon* was established by monitoring cotransduction of the three markers into mutant strain SN0301-1/pNU305 by a P1_{vir} lysate of *E. coli* H1469 (*tsx lon*). Among the transductants, strains with simultaneous resistance to ampicillin and phage T6 but devoid of *lon* were obtained. This excluded a position of *lon* between the site of mutation to non-inducibility and the *tsx* gene. Based on a distance of 0.58 min between *tsx* and *lon*, calculated from 35% cotransduction frequency, the site of mutation to non-inducibility in all four mutant strains, including the weakly inducible SN0301-4/pNU305, was identically localized at 9.9 min on the *E. coli* map, close to *lon*.

Complementation of non-inducibility and β -lactam sensitivity of the mutants by the cloned *ampG* gene from *E. coli*

Recently, Lindquist *et al.* (1993) have mapped the new gene *ampG* to 9.6 min on the chromosomal map of *E. coli* by hybridization of the cloned wild-type *ampG* with the relevant phages from the Kohara lambda library (Kohara *et al.*, 1987), and by comparison of restriction maps

Table 2. Non-inducibility of β -lactamase and β -lactam sensitivity in NTG-generated mutants of *E. coli* SN0301/pNU305 (*ampR⁺ ampC⁺*), and restoration of inducibility and β -lactam resistance by transformation of mutants with pGKS273-3 (*ampG⁺*)

β -Lactamase was induced with 6-Apa. Values of specific enzyme activities are means of triplicate measurements.

Strain	β -Lactamase specific activity [U (mg protein) ⁻¹]		MIC (μ g ml ⁻¹)	
			Ampicillin	Cefotaxime
	Non-induced	Induced		
SN0301/pNU305	0.8	15	2048	64
SN0301-1/pNU305	0.5	0.5	32	4
SN0301-1/pNU305 + pGKS273-3	0.6	12	2048	64
SN0301-3/pNU305	0.35	0.3	32	4
SN0301-3/pNU305 + pGKS273-3	0.5	15	2048	64
SN0301-4/pNU305	0.5	5	64	8
SN0301-4/pNU305 + pGKS273-3	0.7	12	2048	64
SN0301-5/pNU305	0.3	0.3	32	4
SN0301-5/pNU305 + pGKS273-3	0.75	15	2048	64

Fig. 1. Restriction maps of recombinant *ampG* plasmids pGKS273-3 (*ampG⁺*), pMH203 (*ampG1*) and pMH203-3 (*ampG1*). The restriction sites for *EcoRI* (E), *XhoI* (X), *SalI* (S), *PstI* (P), *HindIII* (H), *EcoRV* (EV) and *BamHI* (B) are indicated.

indicating the immediate neighbourhood of *ampG* to the flanking genes *cyoA* and *bolA* (Lindquist *et al.*, 1993; Aldea *et al.*, 1988, 1989; Chepuri *et al.*, 1990). It was first shown by Korfmann & Sanders (1989) that cloned *ampG* restores the inducible synthesis of β -lactamase in a non-inducible mutant of *Enterobacter cloacae* in the presence of wild-type *ampC* and *ampR*.

The non-inducible *E. coli* mutants SN0301-1, -3, -4 and -5, each containing pNU305, were tested for possible defects in *ampG* by additional transformation with pGKS273-3, carrying wild-type *ampG* from *E. coli* JRG582 cloned into the *EcoRV* site of pACYC184 (Fig. 1). All four mutants were equally complemented by *ampG*

to inducibility of β -lactamase and resistance to cefotaxime and ampicillin, and were indistinguishable from parent strain SN0301 (Table 2). Thus, all mutants were defective in *ampG*.

Cloning of the *ampG* alleles from the non-inducible mutants

Chromosomal *EcoRI* fragments of *ampG1* mutant strain SN0301-1 were isolated and ligated into pACY184 linearized with *EcoRI*. Screening of 250 clones from this genomic library by hybridization with a pGKS273-3-derived *EcoRI*-*SalI* fragment carrying part of *ampG* revealed a single probe-positive recombinant plasmid. This plasmid carried a 5.3 kb chromosomal *EcoRI* fragment and was termed pMH203 (Fig. 1). Comparison of the restriction patterns of pGKS273-3 and pMH203 confirmed that the cloned 5.3 kb fragment of pMH203 contained the mutant allele *ampG1* (Fig. 1). Subcloning of the *ampG1* allele by ligating the 1.9 kb *EcoRI*-*XhoII* fragment from pMH203 into pUC19, which was linearized with *EcoRI* and *BamHI*, yielded plasmid pMH 203-3 (Fig. 1).

The *ampG* mutant alleles of strains SN0301-3 (*ampG3*), SN0301-4 (*ampG4*) and SN0301-5 (*ampG5*) were amplified by PCR using primers HS1 (containing a 5'-*EcoRI* site and HS21 (containing a 5'-*HindIII* site). Amplification products with a size of 1.6 kb were obtained, spanning the open reading frames (ORFs) of *ampG3*, *ampG4* and *ampG5*. After double restriction with *EcoRI* and *HindIII*, the respective amplification products were cloned into pUC19 and independently transformed into *E. coli* DH5 α so that four independently cloned PCR products could be analysed by DNA sequencing from each mutant allele.

position:	5' ---	452	-----	803	-----	1118	---3'
	---	L G Y	---	L G L	---	A G W	---
<i>ampG</i>	---	CTG GGT TAC	---	CTT GGC TTA	---	GCG GGT TGG	---
	---	L G Y	---	L G L	---	A G W	---
<i>ampG1</i>	---	CTG GGT TAC	---	CTT GGC TTA	---	GCG GGT TGG	---
	---	L G Y	---	L G L	---	A G W	---
<i>ampG3</i>	---	CTG GGT TAC	---	CTT GGC TTA	---	GCG GGT TGG	---
	---	L G Y	---	L G L	---	A G W	---
<i>ampG5</i>	---	CTG GGT TAC	---	CTT GGC TTA	---	GCG GGT TGG	---

Fig. 2. Sites of point mutations in the nucleotide sequences (lower line) of different *ampG* mutant genes in *E. coli* SN0301-1 (*ampG1*), SN0301-3 (*ampG3*) and SN0301-5 (*ampG5*), and the respective amino acid changes (upper line) in the AmpG protein in comparison with the wild-type sequence (Lindquist *et al.*, 1993). The positions of the nucleotide sequence exchanges (boxed) are indicated in relation to the ORF of *ampG*. Base substitution at positions 452, 804 and 1118 led to amino acid exchanges G¹⁵¹ → D¹⁵¹ in AmpG1, G²⁶⁸ → D²⁶⁸ in AmpG3 and G³⁷³ → D³⁷³ in AmpG5, respectively.

Nucleotide sequences of the *ampG* mutant alleles

The 1.9 and 1.6 kbp inserts of the recombinant pUC plasmids carrying the *ampG* mutant alleles were sequenced by the dideoxy chain-termination method, using synthetic oligonucleotides as internal primers. From each of the genes *ampG3*, *ampG4* and *ampG5* at least two of the independent clones were sequenced, in order to identify any differences resulting from DNA copy errors caused by the *Taq* DNA polymerase. However, none were discovered.

DNA sequence analysis of the mutant *ampG* alleles derived from SN0301-1, SN0301-3 and SN0301-5 revealed single base changes at positions 452 (*ampG1*), 803 (*ampG3*) and 1118 (*ampG5*) in the respective ORFs. In each case the base change was confirmed by sequencing the complementary second DNA strand in the mutated region. These base changes all resulted in an exchange of glycine for aspartate at different sites in the presumptive AmpG1 (G¹⁵¹ → D¹⁵¹), AmpG3 (G²⁶⁸ → D²⁶⁸) and AmpG5 (G³⁷³ → D³⁷³) mutant proteins (Fig. 2). The nucleotide sequence of *ampG4* was identical to that of wild-type *ampG*.

Dependence of inducibility of β -lactamase on the intact *ampG* gene

For functional assays, the mutant *ampG* genes as well as wild-type *ampG* were cloned in plasmid pK18 (Pridmore, 1987) carrying a kanamycin-resistance gene. Genes *ampG1*, *ampG3* and *ampG5* were excised from plasmids pMH203-3, pMH303-1 and pMH305-1 with *EcoRI* and *HindIII* and ligated into pK18. Similarly, wild-type *ampG* was excised from pGKS273-3 with *EcoRI* and *XhoII*, and ligated into pK18. The resulting plasmids were pMH600 (*ampG*⁺), pMH601 (*ampG1*), pMH603 (*ampG3*) and pMH605 (*ampG5*).

β -Lactam resistance and inducibility of β -lactamase were tested after transforming these plasmids in the non-

Table 3. Functional assay of *E. coli* wild-type (in pMH600) and mutant alleles (in pMH601, pMH603, pMH604 and pMH605) of the *ampG* gene cloned in pK18 and transformed into *ampG*-defective *E. coli* SN0301-1/pNU344 (*ampR*⁺ *ampC*⁺)

Restoration of *ampG* function by spontaneous back-mutation in pMH601-R1 is also shown. For expression of *ampG* product and β -lactamase, transformants were induced with IPTG and 6-Apa. Values of specific activities of β -lactamase are means of triplicate measurements.

Plasmid	<i>ampG</i>	β -Lactamase specific activity [U (mg protein) ⁻¹]		MIC of cefotaxime (μ g ml ⁻¹)
		Non-induced	Induced	
pMH600	<i>ampG</i> ⁺	0.8	13	64
pMH601	<i>ampG1</i>	0.2	0.15	2
pMH603	<i>ampG3</i>	0.15	0.5	8
pMH605	<i>ampG5</i>	0.15	0.15	2
pMH601-R1	<i>ampG</i> ⁺	5	13.5	128
pK18	—	0.3	0.25	2

inducible *E. coli* mutant SN0301-1/pNU344 (Table 3). Upon induction of the expression of the *ampG* gene product and β -lactamase with IPTG and 6-Apa, transformants were resistant to cefotaxime and inducible for β -lactamase in the presence of wild-type *ampG*, but not with *ampG1* and *ampG5*. Slight leakiness of mutant *ampG3* was recognizable in transformants with pMH603 by a moderately elevated inducibility of β -lactamase and lower cefotaxime sensitivity. Nevertheless, it was evident that the loss of inducibility was the result of each of the identified point mutations.

Restoration of inducibility of β -lactamase by back-mutation of *ampG1* to wild-type

Back-mutation of chromosomal *E. coli* mutant *ampG* to wild-type with concomitant restoration of inducibility has been reported (Lindquist *et al.*, 1993). A search for spontaneous back-mutation of a cloned mutant allele to the wild-type *ampG* phenotype was carried out in plasmid pMH601 (*ampG1*). Individuals demonstrating β -lactam resistance, presumably due to regained inducibility of β -lactamase, were selected from *E. coli* transformants SN0301-1/pNU344 + pMH601 by their ability to grow on LB-agar plates containing cefotaxime (16 μ g ml⁻¹), IPTG, chloramphenicol and kanamycin. From 2.6×10^{10} plated transformants, 29 cefotaxime-resistant colonies were obtained. In order to distinguish back-mutations of *ampG1* in pMH601 from those in chromosomal *ampG1* in the host bacterium SN0301-1, plasmid pMH601 was purified from all cefotaxime-resistant isolates and again tested for the ability to mediate cefotaxime resistance after retransformation into SN0301-1/pNU344. Five indepen-

Table 4. *ampG*-dependent stimulation by complex growth media of the expression of β -lactamase from cloned *ampR*, *ampC* of *C. freundii* on pNU344 in *E. coli*

Specific β -lactamase activity was determined in strains grown to late exponential phase in supplemented M9 medium (M9⁺), Penassay Broth (PB) or Schaedler Anaerobic Broth (SAB). Values are means of triplicate measurements.

Strain	<i>ampG</i> allele	β -Lactamase specific activity [U (mg protein) ⁻¹] after growth in:		
		M9 ⁺	PB	SAB
SN0301/pNU305	<i>ampG</i> ⁺	2.1	2.1	9.2
SN0301-1/pNU305	<i>ampG1</i>	0.13	0.16	0.12
SN0301-1/pNU305 + pGKS273-3	<i>ampG1</i> + <i>ampG</i> ⁺	0.49	0.53	1.8

dent plasmid-borne back-mutants were identified, which were termed pMH601-R1 to -R5. Surprisingly, and for reasons so far unknown, constitutive expression of β -lactamase in the presence of all revertant plasmids R1-R5 was much higher than in the control with *ampG* wild-type plasmid pMH600. Typical values for inducibility of β -lactamase and β -lactam resistance are shown for transformants containing pMH601-R1 in Table 3. Nucleotide sequencing of pMH601-R1 revealed the exchange of dA for dG at position 452 of the ORF of mutant gene *ampG1* and thus return to the wild-type *ampG* sequence.

Unspecific, *ampG*-dependent induction of AmpC β -lactamase by components of the growth medium

Elevated expression of chromosomal β -lactamase during growth in certain complex growth media has been described in *Ent. cloacae* (Gootz & Sanders, 1983), and highly effective 'unspecific induction' of this enzyme by Schaedler Anaerobic Broth (SAB) has been observed (Cullmann *et al.*, 1984). In *E. coli* SN0301 (*ampG*⁺)/pNU305 we confirmed this stimulating activity of SAB, in contrast to Penassay Broth and M9⁺ medium supplemented with complex additives (Table 4). Moreover, the unspecific induction by SAB was *ampG*-dependent. In *E. coli* SN0301-1 (*ampG1*)/pNU305, expression of β -lactamase was much decreased and equally low in all growth media. Transformation of pGKS273-3 (*ampG*⁺) into SN0301-1 (*ampG1*)/pNU305 led to elevated β -lactamase expression but only to partial restoration of inducibility by SAB.

Preliminary screening for unspecific inducing factors was undertaken by determining the β -lactamase activity in *E. coli* SN0301/pNU305 grown in Penassay Broth with individual addition of the components of SAB at concentrations as described by the manufacturer. Haemin, cysteine hydrochloride and Tris base were without effect. However, with added Peptone Soya (15 g l⁻¹) and Pep-

tone Special (5 g l⁻¹) specific enzyme activities were 9.9 U and 12.2 U (mg protein)⁻¹, respectively.

DISCUSSION

The search for mutants with non-inducibility of AmpC β -lactamase yielded only mutants defective in *ampG* and provided no evidence of new genes involved in this process in addition to those already known. Presumably, therefore, the complete list of genes engaged in the expression and regulation of AmpC β -lactamase comprises *ampC*, *ampR*, *ampD*, *ampE* and *ampG*, whose product probably fulfils the key function of transducer of the external induction signal. Several observations indicate that the AmpG-interacting induction signal may be a metabolite of bacterial cell wall peptidoglycan and may exert its function by interfering in different ways with the synthesis of this polymer. Induction of AmpC β -lactamase in *E. coli* by β -lactam compounds has been shown to depend on the function of the product of the cell-division gene *ftsZ* (Ottolenghi & Ayala, 1991) and the cell-shape-determining penicillin-binding protein 2 (Oliva *et al.*, 1989).

β -Lactamase induction was also found to occur in *E. coli* SN0301/pNU305 (*ampR*⁺ *ampC*⁺) during the mechanistically quite different inhibition of peptidoglycan synthesis by glycine and D-methionine. Absence of the glycine/D-methionine-mediated induction in the *ampG*-defective mutant SN0301-1/pNU305 suggests the essential function of a peptidoglycan-derived and AmpG-interacting signal in this process also (Martin & Schmidt, 1993). In the presence of all required *amp* genes, AmpC β -lactamase was expressed constitutively but was not inducible in cell wall-less L-form protoplasts (Tölg *et al.*, 1993).

The observed 'unspecific' but also *ampG*-dependent induction of β -lactamase by specific peptones in complex growth medium may be due to so far unknown components with modulating and signal-producing activity on peptidoglycan synthesis. Alternatively, signal production may be the result of a general shift-up in polymer synthesis and recycling during growth in the respective rich medium.

The *ampG* gene has been shown to lack its own promoter. Instead, it is cotranscribed as part of an operon together with a 579 bp upstream ORF of still unknown function (Lindquist *et al.*, 1993). Lindquist *et al.* (1993) have demonstrated a polar effect on *ampG* function by insertion mutagenesis in the upstream ORF. Consequently, the partial defect in *ampG* function in strain SN0301-4 could be due to mutation in the operon region upstream of *ampG*.

In the mutants SN0301-1, SN0301-3 and SN0301-5 described in this study the inactivation of *ampG* function is caused by single point mutations at widely separated positions within the gene. Obviously, the central function of AmpG depends on the correct amino acid sequence in the major part of the signal-transducer protein. It should therefore also be highly sensitive to the action of inhibitors

at different positions in the molecule. Treatment with β -lactams in combination with antibiotic action directed at AmpG could be expected to overcome bacterial resistance due to β -lactamase induction. It should also eliminate the hyperinducibility or semi-constitutive overproduction of Amp β -lactamase due to mutational defect in *ampD*, which is a major cause of high resistance to extended-spectrum β -lactam antibiotics in Gram-negative bacteria (Lindberg & Normark, 1987; Lindquist *et al.*, 1989b).

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