Regulation of class D β -lactamase gene expression in *Ralstonia pickettii*

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Ralstonia pickettii, an environmental bacterium that may also be responsible for human infections, produces two unrelated, inducible and chromosomally encoded oxacillinases, OXA-22 and OXA-60. In order to study the molecular basis of the induction process of these oxacillinase genes, the induction kinetics, the promoter/operator regions necessary for expression and induction, and the role of several ORFs located upstream and downstream of the *bla*_{OXA} genes were investigated. The β -lactamase production reached a maximal level after 1 h induction, returned to its basal level within the following 3 h and was then again inducible. Using 5' RACE experiments, the promoter sequences of both oxacillinases were determined. These sequences showed weak promoter activities, which could, however, be increased approximately 200-fold by mutating the –35 promoter sequence. Deletion of the sequences located upstream of the promoter regions did not modify the basal β -lactamase expression in *R. pickettii*, but resulted in the lack of induction. A minimum of 240 and 270 bp upstream of the transcription initiation sites was required for inducible expression of the *bla*_{OXA-22} and *bla*_{OXA-60} genes, respectively. Analysis of the genetic environment of both *bla*_{OXA} genes revealed several ORFs that were inactivated by homologous recombination. Disruption of ORF-RP3, located 190 bp upstream of *bla*_{OXA-60} and divergently transcribed, abolished induction of both β -lactamases. ORF-RP3, which encoded a polypeptide of 532 aa with an estimated molecular mass of 58.7 kDa, displayed no obvious sequence homology with known regulatory proteins. *Trans*-complementation of ORF-RP3 restored the basal and inducible expression of both oxacillinase genes, indicating that the induction of both enzymes was related to the presence of ORF-RP3. In addition to the loss of induction, inactivation of the ORF-RP3 in *R. pickettii* resulted in a complex pleiotropic phenotype, with increased lag phase and reduced survival after heat exposure, suggesting that ORF-RP3 might be a global regulator involved in unrelated regulatory pathways.

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INTRODUCTION

Ralstonia pickettii is a non-fermenting Gram-negative rod that is isolated from water, soil, plants, fruits and vegetables (Giligan, 1995). It is rarely involved in nosocomial septicaemia and tissue infections (Chen *et al.*, 1995; Verschraegen *et al.*, 1985). Most of the infections are traced to contamination of parenteral fluids or of medical equipment (Chetoui *et al.*, 1997; Dimech *et al.*, 1993; Kahan *et al.*, 1983; Raveh *et al.*, 1993).

All the tested *R. pickettii* strains possess two chromosomally located and inducible Ambler class D β -lactamases

(oxacillinases; Ambler *et al.*, 1991): OXA-22 (Nordmann *et al.*, 2000) and OXA-60 (Girlich *et al.*, 2004b). Oxacillinases (oxacillin-hydrolysing β -lactamases) usually hydrolyse oxacillin, methicillin and cloxacillin better than benzylpenicillin and their activity is inhibited by NaCl (Naas & Nordmann, 1999; Bush *et al.*, 1995). While most of the oxacillinases are plasmid-mediated, several chromosomally encoded oxacillinases have been reported in environmental species (Héritier *et al.*, 2004; Poirel *et al.*, 2004; Salanoubat *et al.*, 2002) and also in clinically relevant Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Aeromonas* sp. and *Acinetobacter baumannii* (Alksne & Rasmussen, 1997; Girlich *et al.*, 2004a; Rasmussen *et al.*, 1994; Héritier *et al.*, 2005).

Although most of the β -lactamase genes are not regulated, modulation of the expression of antibiotic-resistance genes can occur by insertion of insertion sequences (IS), which bring promoters located in or near their inverted-repeat

Abbreviations: MR, minimal region; RACE, rapid amplification of cDNA ends.

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(IR) sequences (Aubert *et al.*, 2003). Inducible expression of the chromosomally encoded class C β -lactamases (cephalosporinases) of Gram-negative bacteria is usually regulated by LysR-type elements (Lindberg *et al.*, 1985; Lindberg & Normark, 1987; Lindquist *et al.*, 1989). These proteins act as negative regulators in the absence of β -lactam inducers and as positive regulators in the presence of β -lactam inducers (Bennett & Chopra, 1993). LysR-type elements are also involved in inducible expression of carbapenem-hydrolysing class A β -lactamases such as NmcA (Naas & Nordmann, 1994) and SmeA (Naas *et al.*, 1995). As for *ampC-ampR* systems of *Enterobacter cloacae* (Lindberg & Normark, 1987), *Citrobacter freundii* (Bartowsky & Normark, 1991; Lindberg *et al.*, 1985), *Yersinia enterocolitica* (Seoane *et al.*, 1992), *P. aeruginosa* (Lodge *et al.*, 1990), *Morganella morganii* and *Hafnia avei* (Poirel *et al.*, 1999; Girlich *et al.*, 2000), the regulatory genes are located immediately upstream of the structural β -lactamase genes and divergently transcribed. By contrast, in the *smeR-smeA* system from *Serratia marcescens* (Naas *et al.*, 1995), and *nmcA-nmcR* from *Ent. cloacae* (Naas & Nordmann, 1994), the LysR-like proteins act as positive regulators both in the absence and in the presence of a β -lactam inducer. Such LysR-like proteins acting as positive regulators are also involved in the regulation of β -lactamases in Gram-positive bacteria such as *Streptomyces cacaoi* (Urabe & Ogawara, 1992). BlaP and BlaZ β -lactamase secretion is also induced in *Bacillus licheniformis* and *Staphylococcus aureus*, respectively, in the presence of exogenous inducers (Joris *et al.*, 1994; Philippon *et al.*, 1998). In both strains, the regulatory genes *blaI*, *blaR1* and *blaR2* are involved in the derepression of *blaP* or *blaZ* genes. These regulatory genes encode a cytoplasmic repressor (BlaI) that maintains a low level of β -lactamase expression in the absence of antibiotic and a penicillin sensory transducer (BlaR1). The additional regulatory gene, *blaR2*, is not yet identified, but it is believed to play an essential role in the signal transfer process (Filee *et al.*, 2002). β -Lactamase synthesis regulatory genes *blaI* and *blaR1* in *B. licheniformis* are similarly located upstream of the β -lactamase gene and divergently transcribed (Kobayashi *et al.*, 1987).

The only known regulation system of chromosomally encoded class D β -lactamases is that of *Aeromonas* spp. (Alksne & Rasmussen, 1997; Iaconis & Sanders, 1990; Rasmussen *et al.*, 1994). Coordinated expression of multiple β -lactamases in *Aeromonas* spp. does not involve an AmpR-like regulator, but a two-component system (TCS) closely related to the CreBC TCS of *Escherichia coli* (Alksne & Rasmussen, 1997; Avison *et al.*, 2004). β -Lactamase induction in *Aeromonas* spp. depends upon the expression of the transcription regulator BlrA (related to the family of phosphorylation-dependent response regulators) and a sensor kinase BlrB. The genes *blrA* and *blrB*, encoding the corresponding proteins, are located immediately upstream of the oxacillinase-encoding *ampH* gene in *Aeromonas hydrophila* (Niumsup *et al.*, 2003; Avison *et al.*, 2004).

In the present study we investigated the molecular mechanism involved in the expression of the *bla*_{OXA-22} and *bla*_{OXA-60} β -lactamase genes. We compared the induction kinetics with those of two well-known regulation systems (AmpC from *C. freundii* and Amp, Cep and Imi from *Aeromonas* spp.). We characterized the promoters of the *bla*_{OXA-22} and *bla*_{OXA-60} genes and determined the DNA sequence necessary for induction and expression. Furthermore, as for other systems, we investigated the genetic environment of both β -lactamase genes in order to identify potential regulators. We characterized an open reading frame (ORF-RP3) that is involved in the expression and induction of both β -lactamase genes.

METHODS

Bacterial strains and plasmids. *R. pickettii* clinical isolate PIC-1 has been previously described (Girlich *et al.*, 2004b; Nordmann *et al.*, 2000). *R. pickettii* PIC-1 Δ OXA-22 and PIC-1 Δ OXA-60 isogenic mutants, lacking OXA-22 and OXA-60 respectively, were obtained as described previously (Girlich *et al.*, 2004b). *A. hydrophila* CIP76.14 and *C. freundii* P478 strains were used in induction kinetics experiments (Pasteur Institute). *E. coli* DH10B was used as a host for cloning experiments. The kanamycin-resistant pPCRBluntII-TOPO plasmid (Invitrogen) was used as cloning vector and as a suicide vector for gene inactivation in *R. pickettii* PIC-1 strain since its ColE1 origin of replication restricts its host range to *E. coli* and a few other enterobacterial species. The tetracycline-resistant multicopy plasmid pLAFR3 was used as cloning vector in *R. pickettii* (Staskawicz *et al.*, 1987). Bacterial cultures were grown in trypticase soy (TS) broth at 37 °C for 18 h unless indicated and were monitored by optical density at 600 nm using an Ultrospec 2000 (Amersham Biosciences). Plasmids were introduced in *R. pickettii* by electroporation (Gene pulser, Bio-Rad) with the same technique as that used for *E. coli* (Sambrook & Russell, 2001).

Induction studies and β -lactamase assay. Inducibility of the β -lactamase content from each *R. pickettii* culture was tested in TS broth at 37 °C using the induction protocol with several concentrations of imipenem or cefoxitin as described (Poirel *et al.*, 1999). These β -lactam inducers are known to be good inducers of β -lactamase expression (Poirel *et al.*, 1999). For β -lactamase induction kinetics, β -lactam inducers were added to 200 ml TS broth cultures of *R. pickettii* PIC-1, *A. hydrophila* CIP76.14 and *C. freundii* P478 in exponential phase (OD₆₀₀ value of 0.7). Ten millilitres of the culture was collected by centrifugation during the growth and the β -lactamase activity in crude extracts was determined as described by Poirel *et al.* (1999). The β -lactamase activity was monitored over a period of 75 h. Eighteen hours after imipenem addition, cells were washed, diluted 1:100 in fresh medium, and the growth was continued for an additional 48 h. One unit of β -lactamase activity was defined as the amount of enzyme that hydrolysed 1 μ mol benzylpenicillin or nitrocefin per minute. The total protein content was measured using the DC Protein assay kit (Bio-Rad).

Plasmid extraction, cloning and PCR experiments. Recombinant plasmid DNA was prepared by using Qiagen midi columns (Cogen). All enzymes for DNA manipulations were used according to the recommendations of the supplier (Amersham Biosciences). Unless specified, standard molecular techniques were used (Sambrook & Russell, 2001). Whole-cell DNA of *R. pickettii* PIC-1 was extracted as previously described (Nordmann *et al.*, 2000) and used as template for PCR amplification. For each PCR experiment,

500 ng total DNA was used in a standard PCR reaction mixture supplemented with 10 % (v/v) DMSO (Girlich *et al.*, 2004b; Sambrook & Russell, 2001).

The recombinant plasmid pC2, containing the *bla*_{OXA-60} gene and surrounding sequences, has been previously described (Girlich *et al.*, 2004b). In order to obtain a recombinant plasmid with a flanking DNA sequence upstream of the *bla*_{OXA-22} gene in *E. coli* DH10B, a ligation-mediated PCR (LMPCR) was developed (Prod'homme *et al.*, 1998). Genomic DNA from *R. pickettii* PIC-1 was digested with *Sac*II restriction endonuclease, blunt-ended with *Pfu* polymerase and ligated into pPCR-BluntII TOPO (Invitrogen), used as a linker for subsequent amplification with a primer pair recognizing the linker, M13-40 universal primer and OXA-22 intINV-1, complementary to the *bla*_{OXA-22} gene sequence (Table 1).

Internal fragments of ORF-RP2, ORF-RP3, ORF-RP4, ORF-D and ORF-E were amplified by PCR with internal primers (Δ RP2-1, Δ RP2-2, Δ RP3-1, Δ RP3-2, Δ RP4-1, Δ RP4-2, Δ D-1, Δ D-2, Δ E-1, Δ E-2, Table 1). The amplified fragments (342 bp, 789 bp, 857 bp, 488 bp and 362 bp, respectively) were blunt-ended with *Pfu* polymerase and ligated into pPCR-Blunt TOPO (Invitrogen), resulting in recombinant plasmids p Δ ORF-RP2, p Δ ORF-RP3, p Δ ORF-RP4, p Δ ORF-D and p Δ ORF-E.

A PCR product of 1684 bp, including the complete sequence of the *rp3* gene, was generated using primers RP3A and RP3B (Table 1), located at each end of the *rp3* gene of *R. pickettii* PIC-1 (Girlich *et al.*, 2004b). PCR amplicon of the entire ORF-RP3 was then cloned into plasmid pPCRBluntII-TOPO, as recommended by the manufacturer (Invitrogen) and expressed in *E. coli* DH10B. The cloned insert was then removed by *Eco*RI (Amersham Biosciences) restriction and subcloned into the *Eco*RI-digested shuttle vector pLAFR-3 (Staskawicz *et al.*, 1987), which replicates in both *R. pickettii* and *E. coli*. The recombinant plasmid, named pLAF-RP3, was introduced into *R. pickettii* PIC-1 by electroporation as previously described (Girlich *et al.*, 2004b).

Gene inactivation. Recombinant plasmids p Δ ORF-RP2, p Δ ORF-RP3, p Δ ORF-RP4, p Δ ORF-D and p Δ ORF-E were used as suicide vectors for homologous recombination in *R. pickettii* PIC-1 as previously described (Girlich *et al.*, 2004b). Strains deficient in ORF-RP2, -RP3, -RP4, -D and -E and in the sequences upstream of the *bla*_{OXA-22} and *bla*_{OXA-60} genes obtained after a single recombination event were selected onto TSA plates containing kanamycin (Kan, 30 μ g ml⁻¹). The disruption of the targeted genes in *R. pickettii* was verified by PCR.

Promoter sequence determination. Different sizes of DNA regions containing the promoter sequence were constructed to

Table 1. Nucleotide sequences of primers used for amplification and sequence analysis

Primer	Position	Sequence (5' → 3')	GenBank no.
OXA-22 C	1513–1491	TCTGCACGGTACGGTCGAGCATC	AF064820
OXA-22 intINV-1	1034–1015	ATCGTGACGATCGGGTGCTC	AF064820
OXA-22GSP1	1275–1252	CGAATACCAGACCACCGAATACTT	AF064820
OXA-22GSP2	1158–1138	TTTGAGGAAGCCGTGGTCGAA	AF064820
Δ D-1	207–227	TTTCCTGTCGGCACGAAGCTG	AF064820
Δ D-2	694–674	AGACACAGTCCCAAGCAGTTC	AF064820
Δ E-1	1801–1819	TTCGGCCTCGCTGCGCACC	AF064820
Δ E-2	2163–2144	AATCCCCATCCCACTTAGCC	AF064820
Am22mut-1*	843–852/869–887	TTGCGCCCCGCGCTGTGGCGGTCTGCAGGAC	AF064820
Am22mut-2*	887–869/852–843	GTCCTGCAGACCGCCACACGCGCGGGCGCAA	AF064820
OXA-60 E	3381–3361	TGCTGCTCGATCAACAGCATG	AF525303
Δ RP2-1	502–523	TCGCGGATGGCCTGCACCGTATC	AF525303
Δ RP2-2	844–822	AGCGCGAATGGACGGTCGCGTAT	AF525303
Δ RP3-1	1255–1272	AGGCTTCGGCAAACGGTC	AF525303
Δ RP3-2	2043–2023	TGCTGCATGCACTGAGCACC	AF525303
Δ RP4-1	4512–4531	ACTCGACCATGAACCTTGCGC	AF525303
Δ RP4-2	5366–5347	ACTGGAAGAAGAACGACTGG	AF525303
RP3-A	2589–2566	CTGCCCCTATGACACCGCCACGC	AF525303
RP3-B	908–934	TAGCGACGGATCATTAGGGATACAACG	AF525303
MR1	565–585	TTTGCTGGCGATTTCGCTGATG	AF064820
MR2	675–695	AACTGCTTGGGACTGTGTCTG	AF064820
MR3	756–775	TCTACCAATTCCGCCACCTG	AF064820
MR4	874–904	GCGGTCTGCAGGACGCGACCTCGCTGCTAC	AF064820
MR5†	874–904	GCGGT <u>TTGACAGACGCGACCTCGCTGCTAC</u>	AF064820
MR6	2355–2373	TTCTGCCAGTCGCGAAAGG	AF525303
MR7	2447–2467	TTGCTCCGCGACAGACGATG	AF525303
MR8	2542–2562	TCAGTTCCACATGAAGTGGCG	AF525303
MR9	2674–2697	GGCGCTGGCCGACGCGACGTCGG	AF525303
MR10†	2674–2697	GGCGCT <u>TGACACAGCGACGTCGG</u>	AF525303

*Primers used for site-directed mutagenesis: deletion of the hyphenated tandem repeat GTTAC-n₄-GTTAC.

†*E. coli* σ^{70} –35 promoter consensus sequence TTGACA is underlined.

dissect the regulatory region and to determine a DNA fragment that carries an active promoter and/or an active operator. *R. pickettii* PIC-1 total DNA was used as template in PCR experiments with primers located upstream of the *bla*_{OXA-22} gene on the one hand (MR1, MR2, MR3, MR4, MR5) and one internal primer, OXA-22C, located in the *bla*_{OXA-22} gene on the other hand (Table 1). The amplified fragments were blunt-ended with *Pfu* DNA polymerase and ligated into pPCR-BluntII TOPO (Invitrogen) in *E. coli* DH10B. The resulting recombinant plasmids, pMR1, pMR2, pMR3, pMR4 and pMR5, were used as suicide vectors for homologous recombination in *R. pickettii* PIC-1ΔOXA-60. All constructs contained the putative σ^{70} *bla*_{OXA-22} promoter intact or with an *E. coli* σ^{70} -35 promoter-consensus sequence TTGACA (pMR5) (Lisser & Margalit, 1993).

Similar constructs were made with DNA fragments located upstream of *bla*_{OXA-60}. *R. pickettii* PIC-1 total DNA was used as template in PCR experiments with primers located upstream of the *bla*_{OXA-60} gene on the one hand (MR6, MR7, MR8, MR9, MR10) and one internal primer, OXA-60E, located in the *bla*_{OXA-60} gene on the other hand (Table 1). All constructs contained the putative σ^{70} *bla*_{OXA-60} promoter, intact or with a *E. coli* σ^{70} -35 promoter-consensus sequence TTGACA (pMR10). The constructs with *bla*_{OXA-60} minimal regions (MRs) were introduced in *R. pickettii* PIC-1ΔOXA-22, an isogenic mutant of *R. pickettii* PIC-1 lacking *bla*_{OXA-22} gene expression. The level of β -lactamase expression from the different isogenic mutants was determined as described by Philippon *et al.* (1997).

Mapping the transcription start sites. Reverse transcription and rapid amplification of cDNA ends (RACE) were performed with the 5'RACE system version 2.0 (Invitrogen). Five micrograms of total RNAs extracted from an imipenem-induced culture of *R. pickettii* PIC-1 (Qiagen) and the OXA-22GSP1 and OXA-22GSP2 antisense *bla*_{OXA-22} gene-specific primers were used to determine the transcription initiation site of the *bla*_{OXA-22} gene (Table 1).

Site-directed mutagenesis. Since the identified sequence upstream of the *bla*_{OXA-22} gene contained a tandem repeat GTTAC-n₄-GTTAC similar to the 'cre/blr-tag' TTCAC-n₆-TTCAC from *Aeromonas* sp. (Fig. 2, Avison *et al.*, 2001) at positions -62 to -49 relative to *bla*_{OXA-22} (+1), a site-directed mutagenesis protocol was used as described by the manufacturer (Quick Change site-directed mutagenesis kit; Stratagene) for a deletion experiment. Recombinant plasmid pMR2, containing the MR upstream of the *bla*_{OXA-22} gene necessary for the inducibility of the expression of the gene and a truncated copy of the *bla*_{OXA-22} gene, was used as the template with primers Am22mut-1 and Am22mut-2 to generate recombinant plasmid pAm22mut (Table 1). Introduction of this suicide plasmid by

electroporation into *R. pickettii* PIC-1ΔOXA-60 resulted in deletion of the tandem repeat GTTAC-n₄-GTTAC upstream of the chromosomal copy of the *bla*_{OXA-22} gene.

Stress assays. The susceptibility of wild-type strain *R. pickettii* PIC-1 and mutant PIC-1ΔORF-RP3 to osmotic and acidic stress was determined as described by Nishino *et al.* (2003). Overnight cultures in TS medium (pH 7.2) were diluted 1:1000 into pre-warmed TS (pH 7.2), TS (pH 2.0), TS (pH 5.2) or TS with a 2 M final concentration of NaCl for 1 h at 37 °C and then were plated on TSA. Viable cells were counted after 48 h incubation at 37 °C. The susceptibility of wild-type strain *R. pickettii* PIC-1 and mutant PIC-1ΔORF-RP3 to heat shock was determined as described by Suh *et al.* (1999). The assay for cell-survival after exposure to heat shock at 50 °C was done with stationary-phase cultures of PIC-1 and PIC-1ΔORF-RP3 grown in TS medium, washed in M9 medium and transferred to pre-warmed tubes. The number of viable cells in each suspension was measured by plating aliquots on TS plates and kanamycin-containing TS plates (30 µg ml⁻¹), respectively, at each time point and determining the number of c.f.u. after 48 h incubation. Viability is expressed as a percentage of the number of c.f.u. at time zero. Growth experiments were performed three times.

DNA sequencing, DNA and protein analyses. PCR-generated fragments, purified using Quiaquick PCR purification spin columns (Qiagen), and the inserts of the recombinant plasmids were sequenced on both strands on an ABI 3100 automated sequencer (Applied Biosystems). The nucleotide and the deduced protein sequences were analysed with software available over the Internet at the National Centre of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Multiple nucleotide and protein sequence alignments were carried out online by using the program CLUSTALW available over the Internet at the University of Cambridge (<http://www.ebi.ac.uk/clustalW/>).

RESULTS AND DISCUSSION

Induction kinetics

Preliminary studies on induction had suggested that the β -lactamase expression in *R. pickettii* was inducible and that both oxacillinases might be co-regulated (Table 2, Girlich *et al.*, 2004b). Imipenem and cefoxitin do not behave in the same way in the induction of both oxacillinases, probably because imipenem only is hydrolysed by OXA-60. In fact, imipenem (1 µg ml⁻¹) is the best inducer of OXA-22 (no

Fig. 1. β -Lactamase levels produced by cultures of *R. pickettii* PIC-1 (○), *A. hydrophila* CIP76.14 (●) and *C. freundii* P478 (■) cultures after induction. Imipenem (1 µg ml⁻¹) was used as the β -lactam inducer for *R. pickettii* PIC-1 and *A. hydrophila*. Cefoxitin (10 µg ml⁻¹) was used as the β -lactam inducer for *C. freundii* P478 cultures. Benzylpenicillin (100 µM) was used as substrate.

hydrolysis) and cefoxitin ($5 \mu\text{g ml}^{-1}$) is the best inducer of OXA-60 (no hydrolysis) (Table 2, Girlich *et al.*, 2004b). In order to investigate the kinetics of induction and to rule out *in vitro* selection of hyper-producing strains, induction experiments were performed over 75 h. After induction, a rapid increase of the β -lactamase activity was measured (maximal level reached after 1 h induction). Four hours after induction, the β -lactamase expression dropped significantly to reach almost its basal level. After 18 h, the basal level was reached and β -lactamase expression was still inducible upon reinduction (data not shown). These results showed that addition of a β -lactam inducer resulted in reversible β -lactamase expression in *R. pickettii* (Fig. 1). The OXA-60 and OXA-22 induction kinetics were similar to those observed with imipenem-induced ($1 \mu\text{g ml}^{-1}$) cultures of *A. hydrophila* CIP76.14 and cefoxitin-induced ($10 \mu\text{g ml}^{-1}$) cultures of *C. freundii* P478 (Fig. 1). This similarity in the induction behaviour suggested that these β -lactamase genes might be regulated by a system that could be related to any of those described in the two other species, but is not the result of selection of a derepressed mutant (Fig. 1).

Shotgun cloning with *Sau*3AI-restricted genomic DNA from *R. pickettii* PIC-1 yielded only a single *E. coli* DH10B

strain containing the *bla*_{OXA-22} gene (Nordmann *et al.*, 2000). No promoter sequence was present upstream of this ORF since only five nucleotides were present upstream of the β -lactamase gene on the 1220 bp insert of pSC13 (Nordmann *et al.*, 2000). In order to obtain larger inserts, several cloning experiments were attempted, but none yielded *bla*_{OXA-22}-containing inserts. In order to determine the sequence located upstream of the *bla*_{OXA-22} gene, an alternative technique of ligation-mediated PCR (Prod'homme *et al.*, 1998) was developed. The sequence obtained was used to design primers and to amplify the *bla*_{OXA-22} gene and surrounding sequences (895 bp upstream and 390 bp downstream). This PCR product was then introduced into pPCR-BluntII TOPO, resulting in recombinant plasmid pC14. *E. coli* DH10B harbouring pC14 expressed the OXA-22 β -lactamase at a low and non-inducible level.

Using 5'RACE PCR experiments, the site of initiation of transcription of the *bla*_{OXA-22} gene was mapped in *R. pickettii* PIC-1 and the deduced promoter region was compared with that of the *bla*_{OXA-60} gene (Girlich *et al.*, 2004b) (Fig. 2). The nucleotide sequence of the 5'RACE

β -Lactam inducer ($\mu\text{g ml}^{-1}$)	Specific activity [$\text{mU (mg protein)}^{-1}$]*					
	PIC-1		PIC-1 Δ OXA-22		PIC-1 Δ OXA-60	
	Basal level	Induced [†]	Basal level	Induced [†]	Basal level	Induced [†]
Imipenem (0.1)	1	24	< 1	44	1	0
Imipenem (1)	1	640	< 1	280	1	1028
Imipenem (5)	1	1200	< 1	420	1	0
Cefoxitin (5)	1	50	< 1	1200	1	80

†Induction was carried out for 3 h.

PCR product showed that transcription started at the A located 34 bp upstream of the *bla*_{OXA-22} translation start codon. Upstream of this transcriptional start point (TSP, +1), a -35 promoter sequence CTGCAG, was found, separated by 17 bp from a -10 promoter sequence, TACGCT (Fig. 2). For the *bla*_{OXA-60} gene, the transcription started at the cytosine located 55 bp upstream of the *bla*_{OXA-60} translation start site (Girlich *et al.*, 2004b). A putative σ^{70} promoter was identified at position 64–92 nucleotides (TGGCCG-n₁₇-TACGAT) upstream of the *bla*_{OXA-60} translation start site (Fig. 2). Promoter sequence analysis revealed in both cases a -35 promoter sequence that diverged from the *E. coli* σ^{70} promoter-consensus sequence TTGACA (Lisser & Margalit, 1993).

Characterization of the minimum promoter/operator region

Homologous recombination was used to identify the regulatory region and to determine a DNA fragment that carries an active *bla*_{OXA-22} and *bla*_{OXA-60} promoter (Fig. 3a). Different sizes of DNA regions containing the promoter sequence were constructed. The constructs carried either the largest portion of the *bla*_{OXA-22} regulatory region (pMR1) or 5' deletion derivatives, resulting in regions B (pMR2), C (pMR3) and D (pMR4) (Fig. 3b). All constructs contained the putative σ^{70} *bla*_{OXA-22} promoter. Since the expression of the *bla*_{OXA-22} and *bla*_{OXA-60} genes was inducible, the constructs with *bla*_{OXA-22} minimal regions (MRs) were introduced into *R. pickettii* PIC-1 Δ OXA-60, an isogenic mutant of *R. pickettii* PIC-1 lacking a *bla*_{OXA-60} gene. The level of β -lactamase activity from the different isogenic strains indicated low-level and inducible expression of OXA-22 only when the promoter sequence followed a minimal sequence of 240 bp. Shortening the promoter upstream region by 110 bp (from region A to region B, Fig. 3) resulted in the loss of 65 % of the β -lactamase activity after induction. Further shortening by 81 bp (from region B to region C or D, Fig. 3) resulted in loss of inducibility of *bla*_{OXA-22} gene expression.

The *bla*_{OXA-60} promoter region was similarly determined. The constructs with *bla*_{OXA-60} MRs were introduced into *R. pickettii* PIC-1 Δ OXA-22, an isogenic mutant of *R. pickettii* PIC-1 lacking *bla*_{OXA-22} gene expression. The level of β -lactamase activity from the isogenic strains indicated low-level and inducible expression of OXA-60 only when the

promoter sequence followed a minimal sequence of 269 bp. Shortening this upstream-located sequence by 92 bp (from region F to region G, Fig. 3c) resulted in the loss of 30 % of the β -lactamase activity after induction. Further shortening by 94 bp (from region G to region H, Fig. 3c) resulted in total loss of inducibility of *bla*_{OXA-60} gene expression.

These results suggested that *bla*_{OXA-22} and *bla*_{OXA-60} gene expression was not repressed and that induction required an upstream-located DNA sequence, probably binding a positive regulator.

Sequence analysis of the promoter regions of the three β -lactamase genes *cepH*, *ampH* and *imiH*, encoding CepH (cephalosporinase), AmpH (oxacillinase) and ImiH (carbapenemase), from *A. hydrophila* revealed sequence similarities (TTCAC motifs) that have been proposed to be consensus sequences for binding of regulators such as the BlrA protein (Avison *et al.*, 2004). Comparison of the *bla*_{OXA-22} and *bla*_{OXA-60} upstream sequences revealed a highly homologous region of 17 bp including a tandem repeat of (GTTAC-n₄-GTTAC) similar to the 'cre/blr-tag' (TTCAC-n₆-TTCAC) from *Aeromonas* sp. (Avison *et al.*, 2004). These tandem repeats were located at position -62 to -49 relative to *bla*_{OXA-22} (+1) and at position -80 to -67 relative to *bla*_{OXA-60} (+1) (Fig. 2). In addition, a third copy of this GTTAC motif was found further upstream of the *bla*_{OXA-22} (+1), at position -188 to -184 (data not shown). However, these repeats were not involved in the regulation of *bla*_{OXA-22} and *bla*_{OXA-60} since: (i) the region essential for induction lies between -240 and -159 , while the GTTAC-n₄-GTTAC repeat lies at -80 bp, and (ii) deletion of the tandem repeat GTTAC-n₄-GTTAC upstream of the chromosomal copy of the *bla*_{OXA-22} gene did not significantly change the induction properties of OXA-22.

Site-directed mutagenesis of -35 promoter sequences

In order to determine the involvement of the -35 promoter sequences CTGCAG and TGGCCG, respectively, in the expression of the *bla*_{OXA-22} and *bla*_{OXA-60} genes, this sequence was replaced by the *E. coli* σ^{70} -35 promoter-consensus sequence TTGACA (pMR5, pMR10) (Fig. 3). Increased expression of both *bla*_{OXA-22} and *bla*_{OXA-60} was obtained in, respectively, *R. pickettii* PIC-1 Δ OXA-60 and *R. pickettii* PIC-1 Δ OXA-22 with the *E. coli* σ^{70} -35

Fig. 3. β -Lactamase expression in *R. pickettii* PIC-1 isogenic strains. Schematic representation of the insertion-inactivation of *R. pickettii* sequences. Homologous recombination was used to analyse the regulatory region and to determine a DNA fragment that carries active *bla*_{OXA-22} and *bla*_{OXA-60} promoter. The open arrow indicates the direction of transcription of the non-modified *bla*_{OXA} gene. In *R. pickettii* PIC-1 isogenic mutants, harbouring inserted plasmids pMR1 to 10, which conferred resistance to kanamycin (Kan^r), the sequence of pPCR-Blunt TOPO is grey and the cloned fragment is hatched. Upon insertion, recombination yields two copies of the *bla*_{OXA} gene, one truncated and the other intact, downstream of a restricted promoter region (a). The cloned fragments consisted of different sizes of DNA regions containing the promoter sequence of the *bla*_{OXA-22} (b) or *bla*_{OXA-60} (c) gene and the corresponding truncated *bla*_{OXA} gene. β -Lactamase expression was determined with each construction in *R. pickettii* PIC-1 Δ OXA-60 (b) and in *R. pickettii* PIC-1 Δ OXA-22 (c) before and after imipenem induction. Specific activity was measured with nitrocefin (100 μ M) as the substrate.

promoter-consensus sequence (Fig. 3b, c). The level of expression was comparable to that obtained upon induction (Fig. 3b, c). These results suggested that the weak basal expression of the *bla*_{OXA} genes was the result of a weak activity of their promoters, due to inefficient -35 promoter sequences, and that their increased expression upon induction relies on the binding of an activator to the upper DNA sequence, which may contribute to the stabilization of the RNA polymerase on the promoter sequences.

Taken together, these results indicate that both β -lactamases are not repressed in the absence of inducer and are positively regulated upon induction. These findings argued for the presence of a specific activator-binding sequence near the promoter of each β -lactamase gene as for *Aeromonas* spp. (Avison *et al.*, 2004).

Genetic environment of *bla*_{OXA-22} and *bla*_{OXA-60}

Since genes encoding a bacterial transcription factor are often linked to one of the regulated genes (Niumsup *et al.*, 2003), the sequences upstream and downstream of the *bla*_{OXA-22} and *bla*_{OXA-60} genes were further examined. Analysis of the DNA sequence revealed several ORFs. ORF-D, which encoded a putative protein of 198 aa that shared no significant identity with known protein sequences available in the GenBank database, was found 229 bp upstream of the *bla*_{OXA-22} gene. Downstream of it, the 3' end of another ORF was identified, ORF-E, which encoded a protein that shared 35 % amino acid identity with a putative transcription activator of the LysR family from *Streptomyces coelicolor* (Redenbach *et al.*, 1996). Its function remains

unknown in *R. pickettii*. Several ORFs were identified in the DNA sequences surrounding the *bla*_{OXA-60} gene that shared sequence identity with chromosomally encoded genes of *Ralstonia solanacearum* (Salanoubat *et al.*, 2002) and *Chromobacterium violaceum* (Brazilian National Genome Project Consortium, 2003; Girlich *et al.*, 2004b), but their function remains unknown in *R. pickettii*. In order to test whether any of the proteins encoded in these ORFs might be involved in the regulation of expression of the oxacillinases in *R. pickettii*, these ORFs were knocked out. *R. pickettii* PIC-1 Δ ORF-D, PIC-1 Δ ORF-E, PIC-1 Δ ORF-RP2, PIC-1 Δ ORF-RP3 and PIC-1 Δ ORF-RP4 were analysed for β -lactamase expression and induction (Table 3). Among these *R. pickettii* PIC-1 isogenic mutants, PIC-1 Δ ORF-RP3 was the only one for which a modification of β -lactamase expression was observed (Table 3). The *R. pickettii* PIC-1 Δ ORF-RP3 strain presented a single copy of p Δ ORF-RP3 integrated into ORF-RP3, thus disrupting this ORF. The *R. pickettii* PIC-1 Δ ORF-RP3 strain was more susceptible to all β -lactams than wild-type *R. pickettii* PIC-1 although it expressed a sixfold higher constitutive β -lactamase level than that of the parental strain (Table 3). Furthermore, *R. pickettii* PIC-1 Δ ORF-RP3 lacked inducible β -lactamase expression, thus indicating that ORF-RP3 encoded a protein probably involved in β -lactamase expression (Table 3). *Trans*-complementation of *R. pickettii* PIC-1 Δ ORF-RP3 with recombinant plasmid pLAF-RP3, expressing ORF-RP3 from a multicopy plasmid pLAFR3 (Staskawicz *et al.*, 1987), resulted in recovery of a wild-type β -lactam susceptibility profile and an inducible β -lactamase expression in *R. pickettii*, although at a lower level (Table 3). Both β -lactamase genes were inducible, as revealed by IEF results

Table 3. β -Lactamase activity of *R. pickettii* PIC-1 and isogenic strains, deficient in ORFs surrounding both *bla*_{OXA} genes and that of *R. pickettii* PIC-1 Δ ORF-RP3, deficient in RP3, before and after transformation with a plasmid carrying ORF-RP3

<i>R. pickettii</i>	Specific activity [mU (mg protein) ⁻¹]*	
	Basal level	Induced†
PIC-1	1	100
PIC-1 Δ ORF-D‡	1	85
PIC-1 Δ ORF-E	1	78
PIC-1 Δ ORF-RP2	1	97
PIC-1 Δ ORF-RP4	1	110
PIC-1 Δ ORF-RP3	6	6
PIC-1 Δ ORF-RP3 + pLAF-RP3§	11	33
PIC-1 + pLAF-RP3	7	35

*Nitrocefin (100 μ M) was used as substrate. Data are means of three independent experiments. Standard deviations were within 10 % of the geometric means.

†Cefoxitin (0.5 μ g ml⁻¹) was used as β -lactam inducer.

‡ORF-D is located 229 bp upstream, and the 3' end of ORF-E10 bp downstream, of the *bla*_{OXA-22} gene, ORF-RP2 and ORF-RP3 are located respectively 1876 bp and 192 bp upstream of the ATG codon of *bla*_{OXA-60} and ORF-RP4 is located 657 bp downstream of the *bla*_{OXA-60} gene (Girlich *et al.*, 2004b).

§The entire ORF-RP3 was amplified by PCR, subcloned into the *Eco*RI-digested shuttle vector pLAFR-3 (Staskawicz *et al.*, 1987) that replicates in *R. pickettii* and in *E. coli*.

(data not shown). These data confirmed that the observed phenotype of mutant *R. pickettii* PIC-1ΔORF-RP3 strain was linked to inactivation of the ORF-RP3. Partial complementation probably resulted from non-optimal expression of ORF-RP3 from the plasmid pLAF-RP3. Interestingly, ORF-RP3 and *bla*_{OXA-60} shared no sequence identity with any other ORFs on the chromosome of *R. solanacearum*, which is the species most related to *R. pickettii*. Furthermore, as indicated previously (Girlich *et al.*, 2004b), these two genes seem to have integrated together between two genes sharing high nucleotide identity with two contiguous genes located in the *R. solanacearum* genome (Salanoubat *et al.*, 2002). *R. solanacearum* contains a single oxacillinase gene that shared 63 % nucleotide identity with *bla*_{OXA-22}. The expression of this oxacillinase gene is not inducible (data not shown) and it is tempting to argue that the absence of an ORF-RP3 in *R. solanacearum* might explain the absence of β-lactamase induction in that species.

Sequence alignment of protein RP3

Homology searches on fragments of the RP3 protein using the BLAST algorithm revealed weak sequence identities with several protein domains. The NH₂-terminus (260 aa) of the RP3 protein shared 27 % amino acid identity with a fragment of an ATPase domain-containing response regulator of the LuxR family from the Gram-positive bacterium *Kineococcus radiotolerans* (GenBank accession no. EAM73200) (Phillips *et al.*, 2002). The central region of RP3 shared 28 % amino acid identity with a tetratricopeptide repeat (TPR)-containing protein from the Gram-negative plant pathogen *Xylella fastidiosa* (GenBank accession no. EAO13786). TPR domains are identified in a variety of organisms including bacteria, cyanobacteria, yeast, fungi, plants and humans and are involved in chaperone, cell-cycle, transcription and protein transport complexes, in particular. They are believed to be ancient modules promoting protein–protein interactions in *Bacillus subtilis* (Core & Perego, 2003). The central region of RP3 also shared 25 % amino acid identity with a signal transduction GAF domain from a sensory transducer from *K. radiotolerans* (GenBank accession no. EAM72954). GAF domains are ubiquitous motifs present in cyclic GMP-regulated cyclic nucleotide phosphodiesterases, certain adenylyl cyclases, the

bacterial transcription factor FhlA, and hundreds of other signalling and sensory proteins (Ho *et al.*, 2000). Finally, another fragment shared 27 % amino acid identity with a periplasmic protease domain of a nisin-resistance protein from *Xanthomonas campestris* (GenBank accession no. AAM42679). Site-directed mutagenesis experiments may elucidate further the precise role of these motifs in DNA and protein binding during the regulatory process.

Environmental stress response of mutant *R. pickettii* PIC-1ΔORF-RP3

Nishino *et al.* (2003) demonstrated that the response regulator EvgA controlled the expression of multiple genes conferring antibiotic resistance in *E. coli* by regulating the expression of drug transporters. Ramos-Aires *et al.* (2004) showed that inactivation of the GlmR transcriptional regulator, involved in amino sugar metabolism, dramatically sensitized *P. aeruginosa* to a large variety of antibiotics, suggesting interaction between several regulatory pathways. Similarly, *R. pickettii* PIC-1ΔORF-RP3, deficient in RP3, was more susceptible to all β-lactams than wild-type *R. pickettii* PIC-1 (data not shown) and the wild phenotype could be restored when ORF-RP3 was expressed *in trans* from plasmid pLAF-RP3. In order to understand the physiological role of the RP3 protein, we attempted to further characterize the *R. pickettii* PIC-1ΔORF-RP3 strain. Changes in the morphology of the bacteria could be observed on Gram staining: ORF-RP3 deletion mutants were thinner, longer and remained in chains. The growth rate and the ability to resist drastic changes in temperature, pH and osmolarity were also examined. The growth rates of *R. pickettii* PIC-1 and of *R. pickettii* PIC-1ΔORF-RP3 strains at 37 °C in TS broth were similar except for a longer lag phase for the mutant strain (Fig. 4). Stationary-phase cultures of both strains were exposed to a sudden temperature shift from 37 to 50 °C. After 15 min at 50 °C, the mortality of the *R. pickettii* PIC-1ΔORF-RP3 culture was about 30 times higher than that observed for the parental culture (Fig. 4). Exposure of mutant *R. pickettii* PIC-1ΔORF-RP3 to 2 M NaCl resulted in a considerable decrease in the number of viable cells (6 % survival) relative to that in the wild-type (100 % survival). Thus, these results suggested that inactivation of ORF-RP3 seriously impairs

Fig. 4. Growth at 37 °C and resistance to heat shock of the *R. pickettii* PIC-1 parental strain (○) and isogenic strain PIC-1ΔORF-RP3 (●). (a) Growth curve in TS medium at 37 °C. (b) Cell survival assay after exposure to heat shock at 50 °C.

the capacity of *R. pickettii* PIC-1ΔORF-RP3 to adapt rapidly to both temperature and osmotic variations, similarly to what was observed by Ramos-Aires *et al.* (2004) upon inactivation of the *glmR* gene in *P. aeruginosa*. Our results were also in agreement with those of Nishino *et al.* (2003), who showed that *evgA*-overexpressing *E. coli* strains had an increased resistance to high ionic strength. Indeed, overexpression of this gene resulted in a better ability to survive at low pH and high osmolarity. By contrast with this system, exposure to an acidic pH of *R. pickettii* PIC-1 and of its isogenic mutant did not reveal any modifications in their antibiotic resistance patterns. In both cases no cells survived upon 1 h exposure to pH 2.0 and 100 % survived upon 2 h exposure to pH 5.2 (data not shown).

The role of RP3 in the regulation of *bla*_{OXA-22} and *bla*_{OXA-60} remains unclear but inactivation of this protein affects expression and induction of expression of both oxacillinase genes in *R. pickettii*. Interestingly, as for the other systems, the type of organization for β -lactamase genes and their regulators seems to be similar. Indeed, the gene involved in the regulation of β -lactamase expression is located immediately upstream of one of the regulated genes and divergently transcribed. In *ampC-ampR* (Bartowsky & Normark, 1993), as in the *nmcA-nmcR* (Naas & Nordmann, 1994) or *ampH-blrAB* (Niumsup *et al.*, 2003) systems, the putative binding site is located between the regulator and the regulated gene. Although the protein RP3 sequence contained residues suspected to be involved in DNA or protein binding, we were unable to confirm this. Preliminary analyses with inactivation of the promoter upstream regions showed that the weak β -lactamase expression in *R. pickettii* was not the consequence of a repression system, but depends upon the weak activity of both promoters. Further studies are needed to clarify the physiological role and mechanism of action of ORF-RP3 at the 240 bp region of *Pbla*_{OXA-22} and 270 bp region of *Pbla*_{OXA-60}.

Alteration of ORF-RP3 in *R. pickettii* results in a complex, pleiotropic phenotype, probably resulting from perturbation of the peptidoglycan structure in *R. pickettii* PIC-1ΔORF-RP3 mutant, as evidenced by its abnormal response to temperature and osmotic stress. Further studies of the cell shape and of the amount of LPS and phospholipids will be needed to confirm this hypothesis. It is reasonable to think that RP3, like BlaRI from *B. licheniformis*, could be involved in a system of signal transduction through the membrane (Kobayashi *et al.*, 1987).

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