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

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**INTRODUCTION**

*Ralstonia pickettii* is a non-fermenting Gram-negative rod that is isolated from water, soil, plants, fruits and vegetables (Giligian, 1995). It is rarely involved in nosocomial septicemia and tissue infections (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-lactamas-

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

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the *bla*$_{OXA-22}$ genetic environment reported in this paper is AF064820.

*Ralstonia pickettii*, an environmental bacterium that may also be responsible for human infections, produces two unrelated, inductible 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 inductive 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.

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
In the present study we investigated the molecular mechanism involved in the expression of the \( \text{bla}_{\text{OXA-22}} \) and \( \text{bla}_{\text{OXA-60}} \) \( \beta \)-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 \( \text{bla}_{\text{OXA-22}} \) and \( \text{bla}_{\text{OXA-60}} \) genes and determined the DNA sequence necessary for induction and expression. Furthermore, as for other systems, we investigated the genetic environment of both \( \beta \)-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 \( \beta \)-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/OXA-22 and PIC-1/OXA-60 iso- genic 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 Ultraspec 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 \( \beta \)-lactamase assay.** Inducibility of the \( \beta \)-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 \( \beta \)-lactam inducers are known to be good inducers of \( \beta \)-lactamase expression (Poirel et al., 1999). For \( \beta \)-lactamase induction kinetics, \( \beta \)-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 (\( OD_{600} \) value of 0.7). Ten millilitres of the culture was collected by centrifugation during the growth and the \( \beta \)-lactamase activity in crude extracts was determined as described by Poirel et al. (1999). The \( \beta \)-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 \( \beta \)-lactamase activity was defined as the amount of enzyme that hydrolysed 1 \( \mu \)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 (Coger). 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\text{OXA-40} 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\text{OXA-22} gene in \textit{E. coli} DH10B, a ligase-mediated PCR (LMPCR) was developed (Prod'hom et al., 1998). Genomic DNA from \textit{R. pickettii} PIC-1 was digested with SacI restriction endonuclease, blunt-ended with \textit{Pfu} polymerase and ligated into pPCR-BluntII-TOPO (Invitrogen), used as a linker for subsequent PCR amplification with internal primers (Table 1), located at each end of the \text{rp3} gene of \textit{R. pickettii} PIC-1 (Girlich et al., 2004b). A PCR product of 1684 bp, including the complete sequence of the \text{rp3} gene, was generated using primers RP3A and RP3B (Table 1), located at each end of the \text{rp3} gene of \textit{R. pickettii} PIC-1 (Girlich et al., 2004b). PCR amplicon of the entire ORF-RP3 was then cloned into plasmid pCRBluntII-TOPO, as recommended by the manufacturer (Invitrogen) and expressed in \textit{E. coli} DH10B. The cloned insert was then removed by EcoRI (Amersham Biosciences) restriction and subcloned into the EcoRI-digested shuttle vector pLAFR-3 (Staskawicz et al., 1987), which replicates in both \textit{R. pickettii} and \textit{E. coli}. The recombinant plasmid, named pLAF-RP3, was introduced into \textit{R. pickettii} PIC-1 by electroporation as previously described (Girlich et al., 2004b).

**Gene inactivation.** Recombinant plasmids p\text{ORF-RP2}, p\text{ORF-RP3}, p\text{ORF-RP4}, p\text{ORF-D} and p\text{ORF-E} were used as suicide vectors for homologous recombination in \textit{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 \text{bla}_{\text{OXA-22}} and \text{bla}_{\text{OXA-40}} genes obtained after a single recombination event were selected onto TSA plates containing kanamycin (Kan, 30 μg mL\textsuperscript{-1}). The disruption of the targeted genes in \textit{R. pickettii} was verified by PCR.

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

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

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<th>Primer</th>
<th>Position</th>
<th>Sequence (5'→3')</th>
<th>GenBank no.</th>
</tr>
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<td>1513–1491</td>
<td>TCTGCAGCTACGGTGCAGCATC</td>
<td>AF064820</td>
</tr>
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<td>694–674</td>
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<tr>
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<td>ΔE-2</td>
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<tr>
<td>Am22mut-1*</td>
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<td>2674–2697</td>
<td>GGCAGCTGCGCCGCGCCGCGAGCCTGTCGATC</td>
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*Primers used for site-directed mutagenesis: deletion of the hyphenated tandem repeat GTTAC-nt-GTTAC.
†\textit{E. coli} σ\textsuperscript{70}–35 promoter consensus sequence TTGACA is underlined.

http://mic.sgmjournals.org 2663
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 pCR-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 TTAGCA (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 an *E. coli* σ^{70} −35 promoter-consensus sequence TTAGCA (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-n_{4}-GTTCAC 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-n_{4}-GTTCAC 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 Su 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 Quiagroup 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.](image-url)
hydrolase) and cefoxitin (5 µg 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 µg ml⁻¹) cultures of *A. hydrophila* CIP76.14 and cefoxitin-induced (10 µg 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).

**Cloning of the upstream sequence of bla*OXA-22***

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’hom 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.

**Mapping of the transcription start site of bla*OXA-22***

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

**Table 2. β-Lactamase activity of *R. pickettii* PIC-1 and isogenic mutants PIC-1ΔOXA-22 and PIC-1ΔOXA-60, deficient in OXA-22 and OXA-60, respectively.**

<table>
<thead>
<tr>
<th>β-Lactam inducer (µg ml⁻¹)</th>
<th>Specific activity [mU (mg protein)⁻¹]*</th>
<th>PIC-1</th>
<th>PIC-1ΔOXA-22</th>
<th>PIC-1ΔOXA-60</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Basal level</td>
<td>Induced†</td>
<td>Basal level</td>
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<tr>
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<td>&lt;1</td>
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*Nitrocefin (100 µM) was used as substrate. Data are means of three independent experiments. Standard deviations were within 10% of the geometric means.
†Induction was carried out for 3 h.
PCR product showed that transcription started at the A located 34 bp upstream of the blaOXA-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 blaOXA-60 gene, the transcription started at the cytosine located 55 bp upstream of the blaOXA-22 translation start site (Girlich et al., 2004b). A putative σ70 promoter was identified at position 64–92 nucleotides (TGGCGG-n17-TACGAT) upstream of the blaOXA-60 translation start site (Fig. 2). Promoter sequence analysis revealed in both cases a −35 promoter sequence that diverged from the E. coli σ70 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 blaOXA-22 and blaOXA-60 promoter (Fig. 3a). Different sizes of DNA regions containing the promoter sequence were constructed. The constructs carried either the largest portion of the blaOXA-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 σ70 blaOXA-22 Promoter. Since the expression of the blaOXA-22 and blaOXA-60 genes was inducible, the constructs with blaOXA-22 minimal regions (MRs) were introduced into R. pickettii PIC-1 ΔOXA-60, an isogenic mutant of R. pickettii PIC-1 lacking a blaOXA-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 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 blaOXA-60 gene expression.

These results suggested that blaOXA-22 and blaOXA-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 (oxacillinases) 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 blaOXA-22 and blaOXA-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’ (TTAC-n6-TTAC) from Aeromonous sp. (Avison et al., 2004). These tandem repeats were located at position −62 to −49 relative to blaOXA-22 (+1) and at position −80 to −67 relative to blaOXA-60 (+1) (Fig. 2). In addition, a third copy of this GTTAC motif was found further upstream of the blaOXA-22 (+1), at position −188 to −184 (data not shown). However, these repeats were not involved in the regulation of blaOXA-22 and blaOXA-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 blaOXA-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 TGGCGG, respectively, in the expression of the blaOXA-22 and blaOXA-60 genes, this sequence was replaced by the E. coli σ70 −35 promoter-consensus sequence TTGACA (pMR5, pMR10) (Fig. 3). Increased expression of both blaOXA-22 and blaOXA-60 was obtained in, respectively, R. pickettii PIC-1ΔOXA-60 and R. pickettii PIC-1ΔOXA-22 with the E. coli σ70 −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 blaOXA-22 and blaOXA-60 promoter. The open arrow indicates the direction of transcription of the non-modified blaOXA gene. In R. pickettii PIC-1 isogenic mutants, harbouring inserted plasmids pMR1 to 10, which conferred resistance to kanamycin (Kan'), the sequence of pPCR-Blunt TOPO is grey and the cloned fragment is hatched. Upon insertion, recombination yields two copies of the blaOXA 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 blaOXA-22 (b) or blaOXA-60 (c) gene and the corresponding truncated blaOXA gene. β-Lactamase expression was determined with each construction in R. pickettii PIC-1ΔOXA-60 (b) and in R. pickettii PIC-1ΔOXA-22 (c) before and after imipenem induction. Specific activity was measured with nitrocefin (100 μM) as the substrate.](http://mic.sgmjournals.org)
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 \( \text{bla}_{\text{OXA-22}} \) 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 \( \beta \)-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 \( \beta \)-lactamase gene as for \textit{Aeromonas} spp. (Avison et al., 2004).

**Genetic environment of \( \text{bla}_{\text{OXA-22}} \) and \( \text{bla}_{\text{OXA-60}} \)**

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 \( \text{bla}_{\text{OXA-22}} \) and \( \text{bla}_{\text{OXA-60}} \) 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 \( \text{bla}_{\text{OXA-22}} \) 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 \textit{Streptomyces coelicolor} (Redenbach et al., 1996). Its function remains unknown in \textit{R. pickettii}. Several ORFs were identified in the DNA sequences surrounding the \( \text{bla}_{\text{OXA-60}} \) gene that shared sequence identity with chromosomally encoded genes of \textit{Ralstonia solanacearum} (Salanoubat et al., 2002) and \textit{Chromobacterium violaceum} (Brazilian National Genome Project Consortium, 2003; Girlich et al., 2004b), but their function remains unknown in \textit{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 \textit{R. pickettii}, these ORFs were knocked out. \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-D}, PIC-1\textsuperscript{ΔORF-E}, PIC-1\textsuperscript{ΔORF-RP2}, PIC-1\textsuperscript{ΔORF-RP3} and PIC-1\textsuperscript{ΔORF-RP4} were analysed for \( \beta \)-lactamase expression and induction (Table 3). Among these \textit{R. pickettii} PIC-1 isogenic mutants, PIC-1\textsuperscript{ΔORF-RP3} was the only one for which a modification of \( \beta \)-lactamase expression was observed (Table 3). The \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-RP3} strain presented a single copy of p\textsuperscript{ΔORF-RP3} integrated into ORF-RP3, thus disrupting this ORF. The \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-RP3} strain was more susceptible to all \( \beta \)-lactams than wild-type \textit{R. pickettii} PIC-1 although it expressed a sixfold higher constitutive \( \beta \)-lactamase level than that of the parental strain (Table 3). Furthermore, \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-RP3} lacked inducible \( \beta \)-lactamase expression, thus indicating that ORF-RP3 encoded a protein probably involved in \( \beta \)-lactamase expression (Table 3). 

Trans-complementation of \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-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 \( \beta \)-lactam susceptibility profile and an inducible \( \beta \)-lactamase expression in \textit{R. pickettii}, although at a lower level (Table 3). Both \( \beta \)-lactamases genes were inducible, as revealed by IEF results.

**Table 3.** \( \beta \)-Lactamase activity of \textit{R. pickettii} PIC-1 and isogenic strains, deficient in ORFs surrounding both \( \text{bla}_{\text{OXA}} \) genes and that of \textit{R. pickettii} PIC-1\textsuperscript{ΔORF-RP3}, deficient in RP3, before and after transformation with a plasmid carrying ORF-RP3

<table>
<thead>
<tr>
<th>R. pickettii</th>
<th>Specific activity [mU (mg protein)(^{-1})]*</th>
<th>Basal level</th>
<th>Induced†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIC-1</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-D}‡</td>
<td>1</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-E}</td>
<td>1</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-RP2}</td>
<td>1</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-RP4}</td>
<td>1</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-RP3}</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>PIC-1\textsuperscript{ΔORF-RP3} + pLAF-RP3§</td>
<td>11</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>PIC-1 + pLAF-RP3</td>
<td>7</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

*Nitrocefin (100 \( \mu \)M) was used as substrate. Data are means of three independent experiments. Standard deviations were within 10% of the geometric means. 
†Cefoxitin (0.5 \( \mu \)g ml\(^{-1}\)) was used as \( \beta \)-lactam inducer.
‡ORF-D is located 229 bp upstream, and the 3' end of ORF-E 10 bp downstream, of the \( \text{bla}_{\text{OXA-22}} \) gene, ORF-RP2 and ORF-RP3 are located respectively 1876 bp and 192 bp upstream of the ATG codon of \( \text{bla}_{\text{OXA-60}} \) and ORF-RP4 is located 657 bp downstream of the \( \text{bla}_{\text{OXA-60}} \) gene (Girlich et al., 2004b). §The entire ORF-RP3 was amplified by PCR, subcloned into the EcoRI-digested shuttle vector pLAFR3 (Staskawicz et al., 1987) that replicates in \textit{E. coli}.|

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contiguous genes located in 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 with several protein domains. The NH2-terminus (260 aa) of ORF-RP3 and blaOXA-66 shared no sequence identity with any other ORFs on the chromosome of R. solanacearum. Interestingly, ORF-RP3 and blaOXA-66 shared no sequence identity with any other ORFs on the chromosome of 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 blaOXA-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 GlnR 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
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 *glmS* 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*<sub>OXA-22</sub> and *bla*<sub>OXA-60</sub> 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<sub>OXA-22</sub>* and 270 bp region of *Pbla<sub>OXA-60</sub>*.

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 BlaR1 from *B. licheniformis*, could be involved in a system of signal transduction through the membrane (Kobayashi et al., 1987).

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