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

Delphine Girlich, Thierry Naas and Patrice Nordmann

*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 \( \text{bla}_{\text{OXA}} \) 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 \( \text{bla}_{\text{OXA-22}} \) and \( \text{bla}_{\text{OXA-60}} \) genes, respectively. Analysis of the genetic environment of both \( \text{bla}_{\text{OXA}} \) genes revealed several ORFs that were inactivated by homologous recombination. Disruption of ORF-RP3, located 190 bp upstream of \( \text{bla}_{\text{OXA-60}} \) 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 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.

**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 septicemia and tissue infections (Chen 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., 2004). 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
The only known regulation system of chromosomally encoded class \( D \)-\( \beta \)-lactamases is that of \( A.\) \textit{boronae} spp. The \( D \)-\( \beta \)-lactamase induction system in \( A.\) \textit{boronae} spp. depends upon the \( \beta \)-lactamase activity in crude extracts was determined as described by Poirel et al. (1999). The \( D \)-\( \beta \)-lactam inducers are known to be good inducers of \( \beta \)-lactamase expression (Poirel et al., 1999). For \( D \)-\( \beta \)-lactamase induction kinetics, \( D \)-\( \beta \)-lactam inducers were added to 200 ml TS broth cultures of \( R.\) \textit{pickettii} PIC-1, \( A.\) \textit{hydrophila} CIP76.14 and \( C.\) \textit{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 (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 \( R.\) \textit{pickettii} PIC-1 was extracted as previously described (Nordmann et al., 2000) and used as template for PCR amplification. For each PCR experiment, the **METHODS**

**Bacterial strains and plasmids.** \( R.\) \textit{pickettii} clinical isolate PIC-1 has been previously described (Girlich et al., 2004b; Nordmann et al., 2000). \( R.\) \textit{pickettii} PIC-\( \lambda \)-\( \alpha \)-\( \Delta \)-\( \beta \)-lactamase-encoding class \( D \)-\( \beta \)-lactamase genes. We compared the induction kinetics with those of two well-known regulation systems (AmpC from \( C.\) \textit{freundii} and Amp, Cep and Imi from \( A.\) \textit{boronae} spp.). We characterized the promoters of the \( \beta \)-lactamase genes. 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.
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<sub>OXA-40</sub> 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<sub>OXA-22</sub> gene in <i>E. coli</i> DH10B, a ligation-mediated PCR (LMPCR) was developed (Prod’hom et al., 1998). Genomic DNA from <i>R. pickettii</i> PIC-1 was digested with SacII 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<sub>OXA-22</sub> 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 (ARP2-1, ARP2-2, ARP3-1, ARP4-1, ARP4-2, ΔD-1, ΔD-2, ΔE-1, ΔE-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 pAORF-RP2, pAORF-RP3, pAORF-RP4, pAORF-D and pAORF-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 <i>R. pickettii</i> 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 <i>E. coli</i> 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 <i>R. pickettii</i> and <i>E. coli</i>. The recombinant plasmid, named pLAF-RP3, was introduced into <i>R. pickettii</i> PIC-1 by electroporation as previously described (Girlich et al., 2004b).

**Gene inactivation.** Recombinant plasmids pΔORF-RP2, pΔORF-RP3, pΔORF-RP4, pΔORF-D and pΔORF-E were used as suicide vectors for homologous recombination in <i>R. pickettii</i> 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<sub>OXA-22</sub> and bla<sub>OXA-40</sub> genes obtained after a single recombination event were selected onto TSA plates containing kanamycin (Kan, 30 μg ml<sup>−1</sup>). The disruption of the targeted genes in <i>R. pickettii</i> 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>
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<td>1513–1491</td>
<td>TCTGCAGCCTACGGTCCAGACTC</td>
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<td>1034–1015</td>
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<td>TTCGCTTGGCCGAGGAGCT</td>
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<td>2674–2697</td>
<td>GCGCTTGGCAGCAGGCGGACGTC</td>
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*Primers used for site-directed mutagenesis: deletion of the hyphenated tandem repeat GTTAC-n4-GTTAC. 
†E. coli σ<sup>70</sup> −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. picketti* PIC-1 total DNA was used as template in PCR experiments with primers located upstream of the \( \text{bla}_{\text{OXA-22}} \) gene on the one hand (MR1, MR2, MR3, MR4, MR5) and one internal primer, OXA-22C, located in the \( \text{bla}_{\text{OXA-22}} \) 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. picketti* PIC-1(OXA-60). All constructs contained the putative \( \sigma^{30} \text{bla}_{\text{OXA-22}} \) promoter intact or with an *E. coli* \( \sigma^{30} -35 \) promoter-consensus sequence TGGACA (pMR3) (Lisser & Margalit, 1993).

Similar constructs were made with DNA fragments located upstream of \( \text{bla}_{\text{OXA-60}} \). *R. picketti* PIC-1 total DNA was used as template in PCR experiments with primers located upstream of the \( \text{bla}_{\text{OXA-60}} \) gene on the one hand (MR6, MR7, MR8, MR9, MR10) and one internal primer, OXA-60E, located in the \( \text{bla}_{\text{OXA-60}} \) gene on the other hand (Table 1). All constructs contained the putative \( \sigma^{30} \text{bla}_{\text{OXA-60}} \) promoter, intact or with an *E. coli* \( \sigma^{30} -35 \) promoter-consensus sequence TGGACA (pMR10). The constructs with \( \text{bla}_{\text{OXA-60}} \) minimal regions (MRs) were introduced in *R. picketti* PIC-1(OXA-22), an isogenic mutant of *R. picketti* PIC-1 lacking \( \text{bla}_{\text{OXA-22}} \) gene expression. The level of \( \beta \)-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. picketti* PIC-1 (Qiagen) and the OXA-22GSP1 and OXA-22GSP2 antisense \( \text{bla}_{\text{OXA-22}} \) gene-specific primers were used to determine the transcription initiation site of the \( \text{bla}_{\text{OXA-22}} \) gene (Table 1).

**Site-directed mutagenesis.** Since the identified sequence upstream of the \( \text{bla}_{\text{OXA-22}} \) gene contained a tandem repeat GTTAC-n\( _4 \)-GTTAC upstream of the chromosomal copy of the \( \text{bla}_{\text{OXA-22}} \) gene.

**Stress assays.** The susceptibility of wild-type strain *R. picketti* PIC-1 and mutant PIC-1(ORF-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. picketti* PIC-1 and mutant PIC-1(ORF-RP3) to heat shock was determined as described by Sub 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-1(ORF-RP3) grown in TS medium, washed in M\( _9 \) 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 mg 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 Quickpure 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 \( \beta \)-lactamase expression in *R. picketti* 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 \( \mu \)g ml\(^{-1}\)) is the best inducer of OXA-22 (no

**Fig. 1.** \( \beta \)-Lactamase levels produced by cultures of *R. picketti* PIC-1 (○), *A. hydrophila* CIP76.14 (●) and *C. freundii* P478 (■) cultures after induction. Imipenem (1 \( \mu \)g ml\(^{-1}\)) was used as the \( \beta \)-lactam inducer for *R. picketti* PIC-1 and *A. hydrophila*. Cefoxitin (10 \( \mu \)g ml\(^{-1}\)) was used as the \( \beta \)-lactam inducer for *C. freundii* P478 cultures. Benzylpenicillin (100 \( \mu \)M) was used as substrate.
Regulation of blaOXA gene expression in R. pickettii

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 blaOXA-22

Shotgun cloning with Sau3AI-restricted genomic DNA from R. pickettii PIC-1 yielded only a single E. coli DH10B strain containing the blaOXA-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 blaOXA-22-containing inserts. In order to determine the sequence located upstream of the blaOXA-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 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 blaOXA-22

Using 5'RACE PCR experiments, the site of initiation of transcription of the blaOXA-22 gene was mapped in R. pickettii PIC-1 and the deduced promotor region was compared with that of the blaOXA-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)]⁺</th>
<th>PIC-1</th>
<th>PIC-1ΔOXA-22</th>
<th>PIC-1ΔOXA-60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal level</td>
<td>Induced†</td>
<td>Basal level</td>
<td>Induced†</td>
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<tr>
<td>Imipenem (0-1)</td>
<td>1</td>
<td>24</td>
<td>&lt;1</td>
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<td>Cefoxitin (5)</td>
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<td>1200</td>
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</table>

*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 bla\textsubscript{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\textsubscript{OXA-60} gene, the transcription started at the cytosine located 55 bp upstream of the bla\textsubscript{OXA-60} translation start site (Girlich et al., 2004b). A putative σ\textsuperscript{70} promoter was identified at position 64–92 nucleotides (TGGCCG–n\textsubscript{17}–TACGAT) upstream of the promoter was identified at position 64–92 nucleotides (TGGCCG–n\textsubscript{17}–TACGAT) upstream of the bla\textsubscript{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\textsuperscript{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\textsubscript{OXA-22} and bla\textsubscript{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\textsubscript{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\textsuperscript{70} promoter. Since the expression of the bla\textsubscript{OXA-22} and bla\textsubscript{OXA-60} genes was inducible, the constructs with bla\textsubscript{OXA-22} minimal regions (MRs) were introduced into R. pickettii PIC-1 ΔOXA-60, an isogenic mutant of R. pickettii PIC-1 lacking a bla\textsubscript{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\textsubscript{OXA-22} gene expression.

The bla\textsubscript{OXA-60} promoter region was similarly determined. The constructs with bla\textsubscript{OXA-60} MRs were introduced into R. pickettii PIC-1ΔOXA-22, an isogenic mutant of R. pickettii PIC-1 lacking bla\textsubscript{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\textsubscript{OXA-60} gene expression.

These results suggested that bla\textsubscript{OXA-22} and bla\textsubscript{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 (oxacillinases) and ImiH (carbenemase), 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\textsubscript{OXA-22} and bla\textsubscript{OXA-60} upstream sequences revealed a highly homologous region of 17 bp including a tandem repeat of (GTTAC-n\textsubscript{4}–GTTAC) similar to the ‘cre/blr-tag’ (TTAC-n\textsubscript{4}–TTAC) from Aeromonas sp. (Avison et al., 2004). These tandem repeats were located at position −62 to −49 relative to bla\textsubscript{OXA-22} (+1) and at position −80 to −67 relative to bla\textsubscript{OXA-60} (+1) (Fig. 2). In addition, a third copy of this GTTAC motif was found further upstream of the bla\textsubscript{OXA-22} (+1), at position −188 to −184 (data not shown). However, these repeats were not involved in the regulation of bla\textsubscript{OXA-22} and bla\textsubscript{OXA-60} since: (i) the region essential for induction lies between −240 and −159, while the GTTAC-n\textsubscript{4}–GTTAC repeat lies at −80 bp, and (ii) deletion of the tandem repeat (GTAC-n\textsubscript{4}–GTTAC upstream of the chromosomal copy of the bla\textsubscript{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\textsubscript{OXA-22} and bla\textsubscript{OXA-60} genes, this sequence was replaced by the E. coli σ\textsuperscript{70} −35 promoter-consensus sequence TTGACA (pMR5, pMR10) (Fig. 3). Increased expression of both bla\textsubscript{OXA-22} and bla\textsubscript{OXA-60} was obtained in, respectively, R. pickettii PIC-1ΔOXA-60 and R. pickettii PIC-1ΔOXA-22 with the E. coli σ\textsuperscript{70} −35 promoter sequence.
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 blaOXA 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 blaOXA-22 and blaOXA-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 blaOXA-22 and blaOXA-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 blaOXA-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. picketti. Several ORFs were identified in the DNA sequences surrounding the blaOXA-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. picketti. In order to test whether any of the proteins encoded in these ORFs might be involved in the regulation of expression of the oxacillins in R. picketti, these ORFs were knocked out. R. picketti PIC-1ΔORF-D, PIC-1ΔORF-E, PIC-1ΔORF-RP2, PIC-1ΔORF-RP3 and PIC-1ΔORF-RP4 were analysed for β-lactamase expression and induction (Table 3). Among these R. picketti PIC-1 isogenic mutants, PIC-1ΔORF-RP3 was the only one for which a modification of β-lactamase expression was observed (Table 3). The R. picketti PIC-1ΔORF-RP3 strain presented a single copy of pΔORF-RP3 integrated into ORF-RP3, thus disrupting this ORF. The R. picketti PIC-1ΔORF-RP3 strain was more susceptible to all β-lactams than wild-type R. picketti PIC-1 although it expressed a sixfold higher constitutive β-lactamase level than that of the parental strain (Table 3). Furthermore, R. picketti PIC-1ΔORF-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. picketti PIC-1ΔORF-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. picketti, 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. picketti PIC-1 and isogenic strains, deficient in ORFs surrounding both blaOXA genes and that of R. picketti PIC-1ΔORF-RP3, deficient in RP3, before and after transformation with a plasmid carrying ORF-RP3

<table>
<thead>
<tr>
<th>R. picketti</th>
<th>Specific activity [mU (mg protein)⁻¹]†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal level</td>
</tr>
<tr>
<td>PIC-1</td>
<td>1</td>
</tr>
<tr>
<td>PIC-1ΔORF-D</td>
<td>1</td>
</tr>
<tr>
<td>PIC-1ΔORF-E</td>
<td>1</td>
</tr>
<tr>
<td>PIC-1ΔORF-RP2</td>
<td>1</td>
</tr>
<tr>
<td>PIC-1ΔORF-RP4</td>
<td>1</td>
</tr>
<tr>
<td>PIC-1ΔORF-RP3</td>
<td>6</td>
</tr>
<tr>
<td>PIC-1ΔORF-RP3 + pLAF-RP3§</td>
<td>11</td>
</tr>
<tr>
<td>PIC-1 + pLAF-RP3</td>
<td>7</td>
</tr>
</tbody>
</table>

*Nitrocefin (100 μM) was used as substrate. Data are means of three independent experiments. Standard deviations were within 10% of the geometric means. †Cefoxitin (0.5 μg 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 blaOXA-22 gene, ORF-RP2 and ORF-RP3 are located respectively 1876 bp and 192 bp upstream of the ATG codon of blaOXA-60 and ORF-RP4 is located 657 bp downstream of the blaOXA-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 E. coli.
et al. (Salanoubat et al., 2004b), these two genes seem to have integrated together 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-negative 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 adenyl cyclases, the bacterial transcription factor FhlA, and hundreds of other signalling and sensory proteins (Ho et al., 2000). 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

**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

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**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.
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