Identification of a metagenomic gene cluster containing a new class A beta-lactamase and toxin-antitoxin systems

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Abstract

Several reports mention the presence of antibiotic resistance genes in natural and polluted environments; however, many studies are based on their detection via polymerase chain reaction (PCR amplification of known genes and not on an activity screening. We constructed a metagenomic fosmid bank from DNA isolated from a polluted river in Brussels, Belgium, the Zenne. A total of 120,000 clones were pooled and plated directly on solid media containing different antibiotics. Several clones were isolated which could grow in the presence of ampicillin. The DNA from several clones was extracted and subjected to restriction analysis, and based on their restriction pattern, two different clones were found. One of the clones was selected for further study as it showed a higher level of resistance to different beta-lactams antibiotics (ticarcilline and ceftazidime). To find out which gene is responsible for the resistance, an in vitro transposon mutagenesis was performed and clones having lost the resistance phenotype were analyzed via inverse PCR amplification. Several clones had an insert in a gene encoding a new type of beta-lactamase. The amplified fosmid DNA was fully sequenced revealing an insert of 41 kb containing 39 open reading frames (ORFs). Transposon insertions inactivating the resistance to beta-lactams were also found in the ORF upstream of the blaA gene, encoding an aminotransferase, suggesting a polar effect on the transcription of the gene downstream. In addition, other genes were found such as histidine biosynthesis genes, which were found to be scattered on the insert, a relA/spoT gene, and genes belonging to type II toxin–antitoxin system. This predicted system was experimentally validated in Escherichia coli using an inducible expression system.

Introduction

It is now well established that natural environments can be a reservoir of antibiotic resistance genes (Martinez et al. 2009; Allen et al. 2010). The increased use of antibiotics in agriculture and in medicine has also caused a massive release of antibiotics in streams and rivers, allowing the selection of antibiotic resistant bacteria (Baquero et al. 2008; Martinez et al. 2009; Allen et al. 2010). As a result of industrial activity, toxic metals are also released in streams selecting for bacteria able to resist both metal and antibiotic imposed stresses (Calomiris et al. 1984; Baker-Austin et al. 2006). A recent study revealed a positive correlation between the levels of metals and resistance genes (Knapp et al. 2011). For metals, as well as for antibiotics, efflux via efflux pumps is involved in resistance (Grass et al. 2011; Kim et al. 2011). Some metals such as copper or chromium can cause an important production of reactive oxygen species (ROS) via a Fenton reaction (Lloyd and Phillips 1999), and recently, it has been pro-
posed that many antibiotics also generate an oxidative stress (Kohanski et al. 2010; Martinez and Rojo 2011). Metal and antibiotic resistance genes can be moved between bacteria via horizontal transfer of mobile genetic elements (de la Cruz and Davies 2000; Gootz 2010; Andam et al. 2011; Zhang et al. 2011). Two approaches can be used to detect antibiotic resistance genes from metagenomic DNA. The first one is based on the amplification of known antibiotic resistance genes using gene-specific markers, whereas the second, termed functional metagenomics, involves the construction of metagenomic libraries, which are further screened for expression of drug resistance (Riesenfeld et al. 2004; Allen et al. 2010). With the power of new sequencing methods, it is also possible to sequence the inserts of metagenomic clones, hence discovering the genomic context. In this study, we present the construction of a polluted stream metagenomic library, which was functionally screened for beta-lactam resistance, and the identification of a new beta-lactamase gene as well as its genomic context. We also present evidence for the presence and biological activity of toxin–antitoxin genes.

**Materials and Methods**

**Strains and plasmid constructions**

See Table 1 for the list of used strains and plasmids.

**Media and growth conditions**

Yeast were grown on 863 media (1% yeast extract, 1% bactopeptone, and 2% glucose). Luria-Bertani medium (LB) and M9 minimal medium (KH₂PO₄ (22 mmol/L), Na₂HPO₄ (42 mmol/L), NH₄Cl (19 mmol/L), MgSO₄ (1 mmol/L), CaCl₂ (0.1 mmol/L), NaCl (9 mmol/L), vitamin B1 (1 µg/mL)) supplemented with casamino acids and carbon sources were used to grow bacteria. Ampicillin and chloramphenicol were added at respective final concentration of 50 and 12.5 µg/mL. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at 0.1 mmol/L.

**Freshwater sampling and treatment**

Freshwater was collected in November 2008 and February 2009 from the surface of the Zenne river at the Buda bridge (Haren, Belgium) situated at an industrial site. The Zenne water (2 L) was transferred into sterile bottles at 4°C and immediately filtered using a 1.2-µm glass fiber filter (Whatman, GE Healthcare) to eliminate the sediments and further through a 0.2-µm filter to capture the microorganisms. The 0.2 µm filters with the retained microorganisms were stored at −80°C and thawed at room temperature just before extraction of the DNA.

**DNA extraction**

DNA from the filters was extracted using the Metagenomic DNA Isolation kit for Water from Epicentre®. The final concentration of DNA was 0.27 µg/mL.

**Metagenomic library construction**

Electrocompetent Escherichia coli TransformMax EPI300 T1R cells (Epicentre®, Madison, WI) were used for library construction and grown on LB broth at 37°C containing (LB) and M9 minimal medium (KH₂PO₄ (22 mmol/L), Na₂HPO₄ (42 mmol/L), NH₄Cl (19 mmol/L), MgSO₄ (1 mmol/L), CaCl₂ (0.1 mmol/L), NaCl (9 mmol/L), vitamin B1 (1 µg/mL)) supplemented with casamino acids and carbon sources were used to grow bacteria. Ampicillin and chloramphenicol were added at respective final concentration of 50 and 12.5 µg/mL. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at 0.1 mmol/L.

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chloramphenicol (Cm) (12.5 μg/mL). The metagenomic library was constructed using the CopyControl Fosmid Library Production Kit from Epicentre®. Briefly, the extracted DNA was blunt ended at the 5′-PO4- site, ligated into the pCC1FOS vector and packaged into λ phages using MaxPlax™ Lambda packaging extracts (Epicentre®). E. coli EP1-300 T1R cells were infected with the packaged bacteriophages and the clones inoculated on LB plates containing 12.5 μg/mL Cm. Recombinant clones growing on Cm plates were scraped from the selective agar plates into 10 mL of LB medium plus 20% glycerol and stored in aliquoted 1 mL pools at −80°C. The final titer was 10^11 colony-forming units (CFU) per mL.

### Control of the quality of the metagenomic library

Twenty colonies growing on selective medium plus Cm were selected randomly and grown in 1 mL LB medium plus Cm plus the Copy Control Autoinduction Solution from Epicentre. The DNA was extracted according to the manufacturer protocol (Epicentre) and was restricted using the Fast Digest Restriction enzymes Ndel, PstI, and HindIII (Fermentas, Thermo Scientific).

### Functional screening of the metagenomic library

The E. coli TransformMax EPI300 T1R::pCC1FOS cells were tested for ampicillin susceptibility using the minimum inhibitory concentration assay (MIC, Oxoid, Thermo Scientific) and were found to be sensitive to less than 10 μg/mL Amp. The metagenomic library was therefore plated on LB agar containing 12.5 μg/mL Cm and 50 μg/mL ampicillin. The ampicillin resistant clones (20 clones) were also tested for resistance to other β-lactams, including amoxicillin, aztreonam, cefepime, ceftazidime, ceftriaxone, meropenem, piperacillin, and ticarcillin, using the disk assay (each disk contains 30 μg of one antibiotic). The resistant clones were analyzed by restriction digestion using fast digest PstI and Ndel restriction enzymes (Thermo scientific).

### In vitro transposition mutagenesis

The EZ-Tn5<KAN-2> insertion kit (Epicentre®) was used for the generation of ampicillin-sensitive clones. Briefly, the fosmid carrying the ampicillin resistance gene was purified using the Qiagen miniprep kit. First a 1:1000 diluted overnight culture was induced for 5 h at 37°C at 225 rpm with the CopyControl Autoinduction Solution (Epicentre®) in order to increase the plasmid copy number. The purified recombinant fosmid was then incubated for 2 h with the transposon carrying the kanamycin resistance gene and the transposase under the conditions recommended by the manufacturer. This mixture was used for transformation of the E. coli TransformMax EPI300 T1R cells. The transposon mutants were grown on LB containing Cm and LB containing Cm and Amp (50 μg/mL). The fosmids of the candidates having lost their resistance to ampicillin were extracted, as mentioned above, and sent for sequencing using the KAN-2 FP-1 forward primer (5′ ACCTAACAAGCTCTCATCAACC 3′) and KAN-2 RP-1 reverse primer (5′ GCAATGTAACATCAGAGATTTTGAG 3′).

### 454 pyrosequencing and gene annotation

The clone containing the multiresistant ampicillin fosmid was sent for sequencing. Briefly, the AmpR clone was grown overnight at 37°C and purified as mentioned above followed by an 1-hour incubation with Riboshredder RnaseA (Epicentre). The 454 pyrosequencing reaction was done by DNAvision (Gosselies, Belgium). The sequences were assembled using CAP3 version 3 (available at Mobyle Pasteur: http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::cap3) and annotated by the free online program Softberry (http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb).

### Phylogenetic analysis

In order to get a broad picture of the diversity of the Class A Beta-lactamase, 29 reference sequences were chosen from the study of Hall and Barlow (2004). In addition, the five best BLAST hits were added with the new sequences identified in this study. Altogether, the 35 protein sequences were aligned with ClustalW2 with default parameters (Larkin et al. 2007) implemented in Seaview 4.2.4 (Gouy et al. 2010). After removing positions with gaps, conserved blocks of the multiple alignments were selected by using Gblocks 0.91b with default parameters (Talavera and Castresana 2007). A neighbor-joining tree was inferred using MEGA v5.0 (Tamura et al. 2011) with Poisson correction. The phylogeny was rooted with the group that includes CGA1, CME2, and CFXA2 because those genes are in the chromosomes of species within the Cytophaga–Flexibacter–Bacteroides (CFB) group, the group that is closest to the root of the Eubacterial tree among the groups represented in this phylogeny (Hall and Barlow 2004). The node supports (on the branches) were evaluated based on 1000 bootstrap replicates and the bar indicates sequence divergence.

### Cloning of toxin and antitoxin genes

Primers used to amplify genes were flanked by 40 bp homologous to the cloning sites of the pBAD33-lev or the
pKK223-3-lev vectors. These pBAD33 (Guzman et al. 1995) and pKK223-3 vectors contain the URA3 selection gene and a yeast replication origin obtained from the pFL44 vector (Brosius and Holy 1984; Bonneaud et al. 1991) (see Table 2). Polymerase chain reaction (PCR) products were cotransformed with the linearized pBAD33-lev or pKK223-3-lev vectors into yeast by Gietz and Schiestl (2007) method. Plasmids were constructed by in vivo recombination between PCR products and linearized plasmids in yeast. Candidate vectors were sequenced and transformed into the E. coli MC 1061 strain and then into the DJ624 Δara.

Toxicity and antitoxicity assays

Strains carrying the pBAD33-lev or its derivatives with the toxic genes and the pKK223-3-lev or its derivatives with the hypothetical antitoxin genes were diluted 10 times up to a dilution of 10^-6 CFUs, and 10 μL was plated on minimum media supplied with casamino acids 0.2%, glycerol 1%, ampicillin, and Cm, and either glucose 1% or arabinose 1% and IPTG 0.01 mmol/L.

Results

Isolation of metagenomic DNA and construction of a library

Metagenomic DNA was extracted from 2 L of filtered river water and was sheared before cloning in the fosmid vector pCC1fos (Epicenter®) as described in Material and Methods. Twenty colonies were picked and fosmid DNA extracted after a copy number amplification step. Fosmid DNAs were submitted to a restriction analysis and each extracted after a copy number amplification step. Fosmid DNAs were submitted to a restriction analysis and each clone provided a different pattern, which confirmed that the library is representative (results not shown). A total of 120,000 clones, each containing an insert of ~35 kb, were pooled and screened for antibiotic resistance. A first screening of 12,500 clones was done on ampicillin-containing media (50 μg/mL), resulting in 20 resistant colonies (1/625 clones). DNA was isolated from these clones and analyzed by restriction, resulting in two different restriction patterns, suggesting the existence in the library of two types of AmpR clones. One representative of each clone was selected and plated on media containing ceftazidime and piperacillin. One clone showed resistance to different classes of β-lactams (ampicillin, ceftazidime, and piperacillin) and shows a high MIC against ampicillin with many colonies growing in the inhibition zone, even at the highest concentration of antibiotic (Fig. 1).

Identification of the gene(s) conferring a β-lactam resistance

To determine which gene(s) encode(s) the β-lactams resistance, in vitro transposon mutagenesis of the AmpR clone was performed as described in Material and Methods. After transformation of E. coli cells, the clones were selected on plates containing Cm and kanamycin (to select for the fosmid- and transposon-containing bacteria). After replication on plates containing ampicillin, several Amp-sensitive clones were selected and further analyzed. The DNA sequence flanking the transposon was obtained using transposon-specific primers as described in the Material and Methods section. Three of the insertions causing the loss of resistance to ampicillin occurred in a gene open reading frames (ORF5), which encodes an aminotransferase, and the remaining six insertions were detected in ORF6, which codes for a β-lactamase (Fig. 2). The transposon insertions in ORF5 could have a polar effect.

| Primer                    | Sequence (5′-3′)                         |
|---------------------------|------------------------------------------|
| ORF24-for-Bad-lev-xba1    | TTTTGGGCTAGCAATTGAGCTCGTACCCGGATTCGAGCTCAGAACGGAGGgagtaATGAAAAATGAAATAATCGAC |
| ORF24-rev-Bad-lev-psy1    | AAATCTTCTCTACCGCAAACACGCAAGCTGACGTCGAGCTGCGATTTATATCTCGATACGAG |
| ORF25-for-psy3-lev-ecoR1  | TGTGGAAATTTGTAGCCGATGAAACAATTTCCACACAGGAGAACAGAATTCGAGAAACACACTATTGTGTTT |
| ORF25-rev-psy3-lev-psy1   | CTTGGAACATCTCTACGCGAATACACAGCAGACGCAGATGAAAAATGAAATAATCGAC |
| ORF26-for-Bad-lev-xba1    | TTTTGGGCTAGCAATTGAGCTCGTACCCGGATTCGAGCTCAGAACGGAGGgagtaATGAAAAATGAAATAATCGAC |
| ORF26-rev-Bad-lev-psy1    | AAATCTTCTCTACCGCAAACACGCAAGCTGACGTCGAGCTGCGATTTATATCTCGATACGAG |
| ORF27-for-psy3-lev-ecoR1  | TGTGGAAATTTGTAGCCGATGAAACAATTTCCACACAGGAGAACAGAATTCGAGAAACACACTATTGTGTTT |
| ORF27-rev-psy3-lev-psy1   | CTTGGAACATCTCTACGCGAATACACAGCAGACGCAGATGAAAAATGAAATAATCGAC |
| relB93-for-lev-ecoR1      | CTTGGAACATCTCTACGCGAATACACAGCAGACGCAGATGAAAAATGAAATAATCGAC |
| relB93-rev-lev-psy1       | CTTGGAACATCTCTACGCGAATACACAGCAGACGCAGATGAAAAATGAAATAATCGAC |
| relE93-for-lev-xba1       | TTTTGGGCTAGCAATTGAGCTCGTACCCGGATTCGAGCTCAGAACGGAGGgagtaATGAAAAATGAAATAATCGAC |
| relE93-rev-lev-psy1       | AAATCTTCTCTACGCGAATACACAGCAGACGCAGATGAAAAATGAAATAATCGAC |

Table 2. Oligonucleotides used in this study, 40 bp regions homologous to the pBAD33lev and pKK223-3lev required for in vivo recombination yeast are underlined.
effect on the expression of the \(\beta\)-lactamase gene (ORF6) downstream, explaining why they result in ampicillin sensitivity. Figure 2 shows the gene cluster comprising ORFs 1–6, whereas all ORFs present in the insert are described in Table S1 and presented in Figure S1. ORF6 is predicted to encode a periplasmic protein containing the Pfam 13,354 domain, which corresponds to the serine \(\beta\)-lactamase-like superfamily. The closest homolog is a \(\beta\)-lactamase found in Herminiimonas arsenoxydans, a \(\beta\)-proteobacterium (58% identity [169/291]) (Fig. 3).

A phylogenetic tree shows that the \(\beta\)-lactamase encoded by ORF6 clusters with known class A \(\beta\)-lactamases enzymes, although presenting a different sequence (Fig. 4). The ORF6 encoded \(\beta\)-lactamase clusters with other similar class A enzymes present in the genomes of \(\beta\)-proteobacteria.

**Global analysis of the Amp\(^R\) clone**

To gain information regarding the gene functions associated with the detected \(\beta\)-lactamase, the complete clone sequence was obtained (Genbank accession number KF033132). After aligning the fragments, a single contig of 47,977 nucleotides was obtained. Thirty-eight complete ORFs were detected, plus an incomplete ORF39 (Table S1 and Fig. S1). The first ORF encodes a phosphomannomutase (phosphoglucomutase), ORF2 encodes a peptidase, ORF3 a tryptophanyl-tRNA synthetase, ORF4 a methionine aminopeptidase, ORF5 an aminotransferase, and ORF6 the class A \(\beta\)-lactamase. ORFs 7–10 follow and are transcribed in the same forward orientation and they code for hypothetical proteins (Fig. S1). ORFs 11–14 are transcribed in the reverse orientation and correspond to the \(kdpAFCB\) genes which encode the components of a potassium transporting ATPase (Table S1 and Fig. S1). The ORFs 15 and 16, also transcribed in the reverse orientation, do not correspond to known proteins. Interestingly, ORF17, also transcribed in the reverse orientation, corresponds to the \(hisC\) gene encoding a histidinol phosphate aminotransferase involved in the biosynthesis of histidine. Other histidine biosynthesis genes are also found in the contig, namely, ORF21 (\(hisD\)) and ORFs 30–34 (\(hisGBIF\)), also transcribed in the reverse orientation. Between \(hisC\) and \(hisD\), there are three ORFs (18–20) encoding, respectively, a RelA/SpoT protein (ORF18) and two outer membrane lipoproteins (ORFs 19 and 20). ORF22 encodes a ribosome-associated GTPase. ORFs 23–29 code for hypothetical proteins or proteins involved in toxin activity (ORFs 25 and 28) or in chromosome segregation (ORF27). ORF28 product is predicted to be a RelE toxin and ORF29 a ribbon–helix–helix protein CopG (see also section below). ORFs 34 and 35 are transcribed in the forward orientation and code for a C4-dicarboxylate transporter/tellurite resistance protein and a polyphosphate:nucleotide phosphotransferase, respectively. ORFs 36–39 are transcribed in the reverse orientation and code for a cobalamin S-adenosyl methionine transferase (ORF36) and different subunits of an ethanolamine transporter.

**ORFs 26 and 28 encode proteins that inhibit E. coli growth**

Two of the ORFs were predicted to encode type II toxins (Hayes and Van Melder 2011). ORF25 was predicted to

![Figure 1](image1.png) **Figure 1.** Growth of the Amp\(^R\) clone in the presence of different concentrations of ampicillin (Oxoid strips). Although some growth inhibition could be observed, there is regrowth of colonies inside the inhibition zone.

![Figure 2](image2.png) **Figure 2.** Genomic organization of ORFs 1–6: ORF1 (\(algC\)) encodes a phosphomannomutase, ORF2 a peptidase, ORF3 a tryptophanyl-tRNA synthetase, ORF4 a methionine aminopeptidase, ORF5 an aminotransferase, and ORF6 a periplasmic \(\beta\)-lactamase. The black triangles represent the transposon insertions resulting in the inactivation of the resistance to ampicillin.
Expression of the predicted Doc toxin encoded by ORF25 from the pBAD did not show any toxicity (data not shown). Interestingly, expression of ORF26 from the pBAD vector did inhibit E. coli growth. In an attempt to identify a potential antitoxin for the ORF26 toxin, the flanking ORFs (ORF25 and ORF27) were cloned in the pKK vector and their ability to restore viability was tested. Neither of these ORFs was, however, able to counteract ORF26-mediated toxicity. ORF24 was also tested and was not toxic (data not shown).

## Discussion

### Identification of an active class A β-lactamase

Our first aim was to detect, by functional screening, the expression of antibiotic resistance determinants coming from the environment, choosing river water, as it was hypothesized that the Zenne river in Brussels, being heavily polluted, could be a source of drug resistance genes (Baquer et al. 2008; Martinez et al. 2009; Allen et al. 2010; Garcia-Armisen et al. 2011). The Zenne being as well heavily polluted by the presence of heavy metals (Garcia-Armisen et al. 2011), it could also represent a source of both metal and antibiotic resistance genes (Baker-Austin et al. 2006). In a previous study, using the same water samples, we obtained 32 cultivable isolates showing resistance to at least one antibiotic among the nine tested (Cm, erythromycin, gentamicin, meropenem, aztreonam, tetracycline, amoxicillin, ofloxacin, and ceftazidime) (Garcia-Armisen et al. 2011). These cultivable bacteria belonged to different phyla (Bacteroidetes and Proteobacteriales) with a large representation of the Pseudomonas genus. However, a 16S clone library analysis revealed that there was no good correlation between the cultivable and the noncultivable bacteria as no Pseudomonas 16S sequence could be detected (Garcia-Armisen et al. 2011). This prompted us to look for novel resistance determinants by direct functional screening of a metagenomic library. The power of functional metagenomics for the discovery of novel antibiotic resistance genes has been previously established by the analysis of libraries of soil DNA (Allen et al. 2009; Lang et al. 2010; Torres-Cortes et al. 2011) and, more recently, from gulls (Martiny et al. 2011). Using this approach, new β-lactamase genes, different from the clinical enzymes, could be isolated from an Alaskan soil (Allen et al. 2009) and aminoglycoside resistance genes were discovered by screening a soil metagenomic library (Riesenfeld et al. 2004). Although gene expression using E. coli as host is not guaranteed (Uchiyama and Miyazaki 2009; Ekkers et al. 2012), screening nevertheless resulted in the selection of a clone that is able to grow in the presence of high class A β-lactamases as described in the results section.

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concentrations of β-lactam antibiotics. The β-lactamase encoded by ORF6 shows 74% similarity with a class A TEM β-lactamase from the β-proteobacterium *H. arsenoxydans* (Muller et al. 2007). The metagenomic β-lactamase clusters well with A-type enzymes (Fig. 4), suggesting that it belongs to this class of β-lactamase enzymes. Furthermore, our enzyme also contains conserved residues typical of class A enzymes in its active site (Majiduddin...
Identification of a relBE toxin–antitoxin system

In this work we demonstrated that ORFs 28 and 29 constitute a RelBE toxin–antitoxin system, whereas ORF26 is a lone toxin. Serial dilutions of the DJ624ara containing the pBAD33lev control vector or its derivatives with ORFs 24, 26, or relE (ORF28) and the control vector pKK223-3lev or its derivatives with ORF25, ORF27, or relB (ORF29) were plated on M9 minimal media containing arabinose (1%) and IPTG (0.1 mmol/L) and the appropriate antibiotics. Plates were incubated overnight at 37°C.

Histidine biosynthesis genes

Another striking feature of our sequence contig is the presence of different histidine biosynthesis genes, which are either clustered (hisFIBG) or separated by other ORFs (hisC and hisD). However, the gene coding for HisB, which catalyzes the sixth and the eight step of histidine biosynthesis pathway, is not present in our contig, meaning that the probability is high that we did not recover all the histidine biosynthesis genes in our fragment.
concerns ORF35 encoding a polyphosphate:nucleotide phosphotransferase which shows the highest similarity with a protein coming from a methanogenic Archeon (Euryarcheota). The histidine biosynthesis genes appear also to have homologs in quite different bacteria (α-proteobacteria for HisC and HisG, γ-proteobacteria for HisD and HisF, a β-proteobacterium for HisB, and a bacterium belonging to the Bacteroidetes for HisI).

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genomic organization of the 39 ORFs present in the insert of the AmpR clone.

Table S1. The complete list of the 39 ORFs detected in the insert of the AmpR clone.