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"Distribution and functional identification of complex class 1 integrons"

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ABSTRACT

The emergence of extended-spectrum β-lactamases and plasmid-mediated resistance to quinolones has been previously found to be associated with the dissemination of complex class 1 integrons in Argentina. In this study, we analyzed their distribution through time and evaluated the functionality of the Orf513 protein, which is the putative recombinase of the ISCR1 mobile element. We investigated the presence of the *orf513*, $bla_{CTX-M-2}$, *dfrA3b*, *qnrB10* and bla_{DHA-1} genes by PCR and DNA sequencing as well as their linkage to class 1 integrons in 451 non-epidemiologically related nosocomial strains resistant to at least one expanded-spectrum cephalosporin and to one aminoglycoside, isolated between 1989 and 2010 from 7 hospitals from Buenos Aires City. The epidemiology of complex class 1 integrons was found to be notably different among fermenting (94/171) and non-fermenting clinical bacilli isolates (1/280). The ISCR1::*qnrB10* positive isolates were found since 1993, confirming its presence in clinical isolates more than a decade before its first description. As expected, In35::ISCR1::$bla_{CTX-M-2}$ was the most common complex class 1 integron among *Enterobacteriaceae* isolates, particularly in *Proteus mirabilis*. Experimental analysis corroborated the activity of the Orf513 protein, which was found to bind specific DNA sequences containing the previously suggested *ori*IS region. These findings showed the high dispersion and maintenance of complex class 1 integrons across time in our nosocomial isolates. The contribution of the ISCR1 mobile element to multidrug resistant phenotypes is significant due to its sustained association to class 1 integrons.

1. Introduction

The first complex class 1 integron was described in plasmid pDGO100 (Parsons et al., 1991) from an *Escherichia coli* strain isolated from an outbreak in Sydney in the early 1980s (Groot Obbink et al., 1985). Since then, these elements have been also known as unusual class 1 integrons, complex *sul1*-type integrons or ISCR1 elements because they share a common region (CR) which includes the *orf513* gene and has been identified as the Insertion Sequence Common Region 1 (ISCR1) (Arduino et al., 2002; Toleman et al., 2006a; Verdet et al., 2000). Until now, ISCR1 has been found usually located between two 3′-Conserved Segment (3′-CS) of class 1 integrons (Fig. 1a) (Partridge et al., 2009; Stokes and Hall, 1989).

When several complex class 1 integrons are aligned, the general organization can be identified as a composite of three genetic structures (Fig. 1): (i) the typical class 1 integron harboring the 5′-CS with the variable region 1 (vr-1) and a first copy of the 3′-CS; (ii) the *orf513* gene, which codifies the putative recombinase Orf513 related by sequence homology to the ISCR family proteins and by its genetic organization to the IS*91*-like transposases (Partridge and Hall, 2003; Toleman et al., 2006a), and (iii) the variable region 2 (vr-2) coding for a remarkable variety of antimicrobial resistance genes followed by a duplication of the 3′-CS (Quiroga et al., 2007; Toleman et al., 2006a). The vr-2 has been described with genes encoding resistance to several families of antimicrobial agents such as chloramphenicol (*catA2*), trimethoprim (*dfrA3b*, *dfrA10*, *dfrA23*, *dfrA18*, *dfrA19*), aminoglycosides (*armA*, *nbrB*), β-lactamases ($bla_{CTX-M-2}$, $bla_{CTX-M-4}$, $bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-20}$, bla_{PER-1}, bla_{PER-3}, bla_{VEB-3}, bla_{DHA-1}, bla_{CMY-1}, bla_{CMY-8} to bla_{CMY-11}, bla_{MOX-1}, bla_{FOX-4}) and quinolones (*qnrA1*, *qnrA3*, *qnrA6*, *qnrB2*, *qnrB4*, *qnrB6*,

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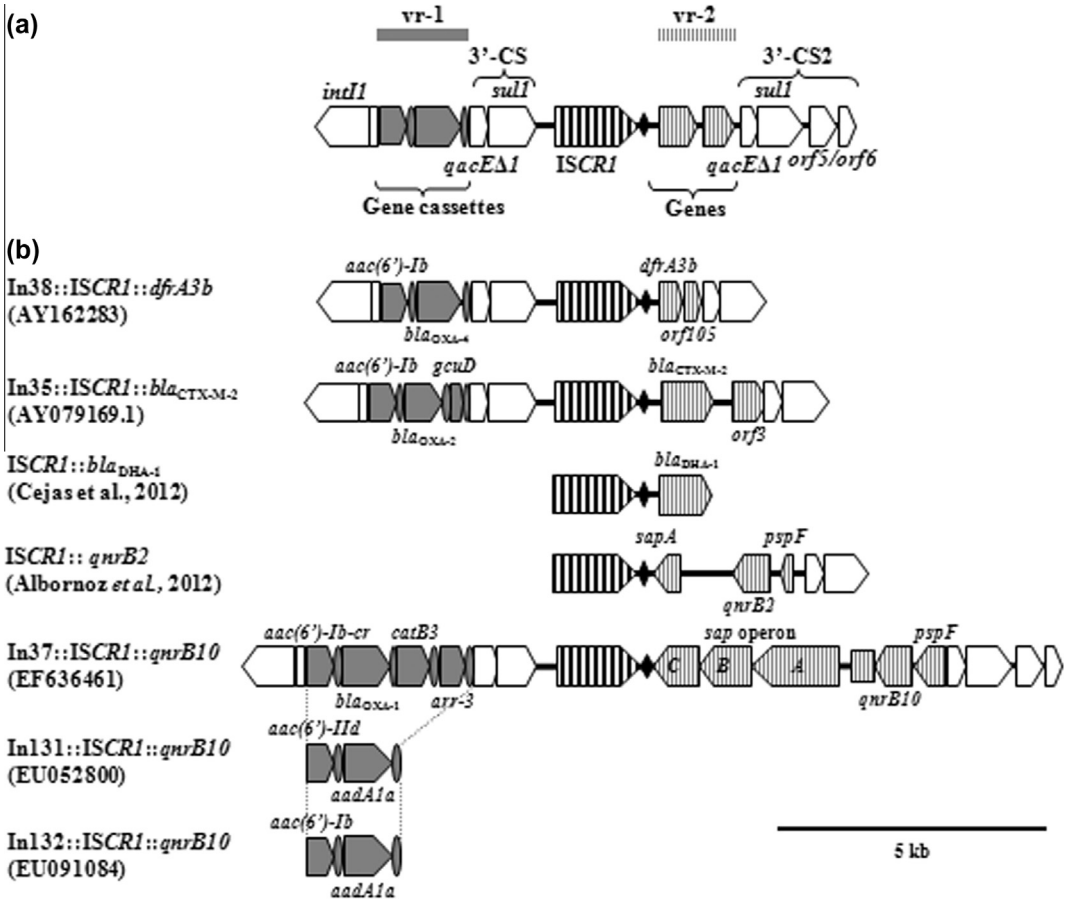

Fig. 1. (a) General genetic organization of complex class 1 integrons. The type 1 integrase gene, the 3′-CS and the second copy of this element (3′-CS2) are shown by white arrow boxes; the gene cassettes are shown by gray arrow boxes; *att*Cs are represented by vertical ovals; genes of the variable region 2 (vr-2) are shown with striped arrow boxes and the putative origin of replication of IS*CR1* is represented by a black diamond. (b) Complex class 1 integrons previously found in Argentina.

qnrB10) (Andres et al., 2013; Bae et al., 2007; Bou et al., 2000; Cambau et al., 2006; Garnier et al., 2006; Gazouli et al., 1998; Ma et al., 2009; Quiroga et al., 2007; Toleman et al., 2006b; Verdet et al., 2000). As part of the genetic platform of class 1 integrons (Stokes et al., 1993), complex class 1 integrons can be disseminated to Gram-negative clinical isolates around the world (Arduino et al., 2002; Poirel et al., 2008; Stokes et al., 1993; Verdet et al., 2000). However, their distribution has not been well established among species usually isolated from clinical samples. In Argentina, nosocomial isolates harboring complex class 1 integrons with $bla_{\text{CTX-M-2}}$, $bla_{\text{DHA-1}}$, *qnrB2* or *qnrB10* genes have been described since 2002 (Albornoz et al., 2012; Andres et al., 2013; Arduino et al., 2002; Cejas et al., 2012; Di Conza et al., 2002; Quiroga et al., 2007).

The IS*91*-like family and relatives are DNA mobile elements quite different from conventional ISs and transposons (Mahillon and Chandler, 1998). Instead of terminal inverted repeats, their extremities consist of the *ori*IS and the *ter*IS regions, which are associated with rolling circle transposition (Tavakoli et al., 2000). The mechanism of rolling circle transposition has been shown to form free circular entities that may be intermediates in the transposition process, mobilizing sequences flanking the *ter*IS end of the element as result of misreading it in the IS*91*-like element IS*1294* (Tavakoli et al., 2000). In the case of the IS*CR1* element in complex class 1 integrons, it has been suggested that the *ter*IS end has been lost since no homology in this region is found and because it is shorter than other IS*CR*s and IS*91*-like elements (Toleman et al., 2006a). It was also proposed that a deletion occurred in the event of rolling circle transposition, which created these complex class 1 integrons resulting also in the duplication of the 3′-CS (Toleman et al., 2006a). Up to now, there is no experimental evidence on the IS*CR1* mechanism of mobilization; however, bioinformatics studies clearly showed that antimicrobial resistance genes found in the vr-2 of these complex class 1 integrons are taken from chromosomal DNA. This is the paradigmatic case of $bla_{\text{CTX-M-2}}$, which comes from the ubiquitous *kluA* gene from the genome of *Kluyvera ascorbata* showing the ability of this IS to move DNA from a chromosome to a vr-2 of complex class 1 integrons (Arduino et al., 2002; Humeniuk et al., 2002; Rodriguez et al., 2007).

The aim of this study was to know the distribution of complex class 1 integrons among nosocomial isolates from Argentina, and to shed some light on the mechanism of recombination mediated by the IS*CR1* element.

2. Material and methods

2.1. Bacterial strains and growth conditions

The study was carried out with 451 non-epidemiologically related strains isolated from 1989 to 2010 from 7 hospitals from Buenos Aires City. When isolates of a species were collected in the same hospital during a short period of time, only one isolate per antibiotic susceptibility profile was selected to avoid the inclusion of possible outbreaks. The isolates were identified using the standard biochemical tests, and the microbiological test strip (API20NE-Biomerieux). *Acinetobacter* spp. strains were identified at species level by phenotypic method (Gerner-Smidt et al., 1991) and by amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte et al., 1995). All

isolates used in this study were resistant to at least one expanded-spectrum cephalosporin (ceftazidime or cefotaxime) and to one aminoglycoside (amikacin or gentamicin): (i) 26 *E. coli*, 45 *Klebsiella pneumoniae*, 33 *Serratia marcescens*, 35 *Proteus mirabilis*, 7 *Citrobacter freundii*, and 25 *Enterobacter cloacae*, 50 *Burkholderia cepacia* complex, 30 *Stenotrophomonas maltophilia*, 100 *Acinetobacter baumannii* and 100 *Pseudomonas aeruginosa* isolates. The cultures were routinely performed at 37 °C in general Columbia blood agar (Difco Laboratories, Detroit, Mich.) supplemented with cefotaxime (16 μg/ml) for subsequent DNA extraction.

2.2. Antimicrobial susceptibility testing

Tests for susceptibility to cefotaxime, ceftazidime, amikacin, gentamicin, chloramphenicol, trimethoprim–sulfamethoxazole, imipenem, meropenem, ciprofloxacin and cefoxitin were performed by using the agar diffusion method (Clinical and Laboratory Standards Institute, 2012). Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.).

2.3. DNA techniques

Total DNAs were prepared as described by Sambrook et al. (1989). PCR amplifications were carried out using the Taq DNA polymerase according to the manufacturer's recommendations (Invitrogen) followed by DNA sequencing when required (Orman et al., 2002). The presence of the *orf513* and *intI1* genes, as well as several antimicrobial resistance determinants was assessed in each isolate by PCR using internal primers (Table A) (Orman et al., 2002; Quiroga et al., 2007). Since the primer pairs ctx-M2F and ctx-M2R, and qnrB-for and qnrB-rev could amplify different alleles of the $bla_{\text{CTX-M-2}}$ and *qnrB* genes, respectively, the PCR products were sequenced to confirm the allele. PCR cartography to identify the vr-2 of the complex class 1 integrons was performed as previously described using the primer pair orf513F3′ and qacEΔ1B listed in Table A and subsequent sequencing of the PCR products (Arduino et al., 2003; Quiroga et al., 2007). To confirm the genetic linkage between vr-1 and vr-2, PCRs were performed with primers targeting the last gene cassette of a vr-1 and a gene in the vr-2, respectively (Orman et al., 2002; Quiroga et al., 2007).

2.4. DNA sequencing

Several PCR products were sequenced after purifying the DNA using the Wizard SV Gel and PCR clean-up System kit according to the manufacturer's directions (Promega, USA). Sequencing was performed on both DNA strands using the ABIPrism 3100 BioAnalyzer equipment. Nucleotide sequences were analyzed using the Blast V2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. Bioinformatics analysis

To evaluate the dispersion of complex class 1 integrons in the bacterial genomes, we used the BlastN algorithm of the Basic Local Alignment Search Tool (BLAST) program from the PubMed website (http://www.ncbi.nlm.nih.gov/pubmed/). We performed different searches using the nucleotide collection database, which consists of 17,040,725 DNA sequences published in the GenBank+EMBL+DDBJ+PDB+RefSeq databases by November 15th, 2012. To investigate the presence of In35::IS*CR1*::$bla_{\text{CTX-M-2}}$, we used Accession Number (AN) AJ621187.1 as query (7 hits found for the complete sequence); for IS*CR1*::$bla_{\text{CTX-M-2}}$ in *P. aeruginosa*, we used AN AY079169.1 as query, adjusting the search to this species (no hits); for $bla_{\text{CTX-M-2}}$ associated with different vr-1, we used AN AY079169.1 (1068 hits), and when possible we analyzed the vr-1 of each integron. To investigate the presence of complex class 1 integrons containing *qnrB10*, we searched for IS*CR1* plus *qnrB10* from AN EU091084.1 as query (857 hits). Finally, to investigate if the IS*CR1* is always associated with complex class 1 integrons and the amount of these integrons in the database, we searched for IS*CR1* plus sul1 from AN EU091084.1 (1057 hits). The redundant hits were excluded. The integron numbers for each vr-1 were given taking into account those recorded in the integron database Integrall (http://integrall.bio.ua.pt).

2.6. Orf513 protein expression

The *orf513* gene, obtained with primers 513-Nco 5-GGA GGA TAC CCA TGG CGC TGG CAA-3 and 523-BamHI 5-CAG TGT TGG ATC CGG GGT CGT CAG-3 from DNA of Pmar12 isolate (Arduino et al., 2002), was digested with *Pci*I and *Bam*HI and cloned into vector pet-29b (ampR; Novagen, Madison, WI, USA) digested with *Nco*I and *Bam*HI, creating an S-tag-orf513 fusion of 6908 bp. The plasmid pET-orf513 was introduced in *E. coli* BL21-CodonPlus (DE3) RP. Overexpression of Orf513 was done by inducing the pT7 promoter with 0.5 mM IPTG at 16 °C for 16–18 h. The Orf513 protein was purified using the S-tag thrombin system (Novagen, Madison, WI, USA). The purified protein was quantified and sent for MALDI-Tof for identification at Centre Proteomique de I Est du Quebec, Qc CA.

2.7. Mobility shift assay

DNA fragments of different size were obtained by PCR from pMAR-12 plasmid DNA as template and labeled using α-[^{32}P]-dCTP primers described in Table A (Arduino et al., 2002). Fragments were purified by Sephadex G-50 columns, quantified and diluted at 20,000 cpm/μl. Different concentrations of protein Orf513 were added to the labeled DNA followed by loading to a 5% non-denaturing polyacrylamide gel. The gel was run at 40 V overnight, dried at 80 °C for 1 h and exposed with a BioMax MS film (KODAK), overnight at room temperature.

3. Results and discussion

3.1. Abundance of complex class 1 integrons among nosocomial isolates

In order to detect positive isolates carrying complex class 1 integrons, firstly we performed PCR tests using primers for *orf513*, $bla_{\text{CTX-M-2}}$, $bla_{\text{DHA-1}}$, *dfrA3b* and *qnrB* alleles to 451 clinical isolates, and we sequenced the amplification products to confirm the allele when necessary (Table A). The 21.1% of the isolates showed the presence of the *orf513* gene, while determinants *dfrA3b* and $bla_{\text{DHA-1}}$ were not found in any isolate. The abundance of the *orf513* gene was found to be notably different among glucose fermenting and non-fermenting bacilli (Fig. 2). Only a single *P. aeruginosa* isolate was positive for this gene among 280 non-fermenting bacilli of *A. baumannii*, *B. cepacia* Complex, *S. maltophilia*, and *P. aeruginosa* (Fig. 2). In contrast, 55% of *Enterobacteriaceae* isolates (94/171) gave positive results for the presence of this gene ($P < 0.0001$, Fisher's Exact Test). It is worth mentioning that 94% (33/35) of *P. mirabilis* isolates harbored this element (Fig. 2). Since our initial approach consisted of selecting bacterial isolates resistant to expanded-spectrum cephalosporins (ceftazidime or ceftriaxone), it is not surprising that IS*CR1*::$bla_{\text{CTX-M-2}}$ was more commonly found than IS*CR1*::*qnrB10*. The latter was identified in all species of the *Enterobacteriaceae* family included in this study, having the highest frequency in *E. cloacae* (48%, 12/25) (Fig. 2). Secondly, by PCR using primers targeting the last gene cassette of the vr-1 and the first gene in the vr-2, we found that all IS*CR1*::$bla_{\text{CTX-M-2}}$ and IS*CR1*::*qnrB10* were linked to class 1

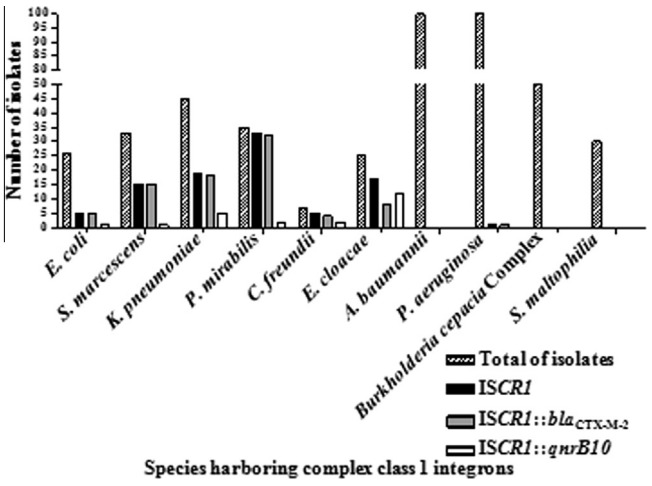

Fig. 2. Abundance of complex class 1 integrons among the species of the survey. Total of isolates analyzed for each species (black columns), *orf513* positive isolates (white columns), IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integrons (solid grey columns), IS*CR1*::*qnrB10* (oblique black lines columns). Note that the total of isolates analyzed for *A. baumannii* and *P. aeruginosa* species are out of scale.

integrons (see below), resulting in the architecture of complex class 1 integrons. In addition, by PCR using primers that target the $bla_{CTX-M-2}$ or the *qnrB10* gene and the *qacEΔ1* gene (Table A), we identified that all vr-2 were located between two 3′-CS as it has been previously reported (Arduino et al., 2002; Quiroga et al., 2007). As shown in Table 1, 11 isolates possessed both complex class 1 integrons, IS*CR1*::$bla_{CTX-M-2}$ and IS*CR1*::*qnrB10* in the same strain.

From a bioinformatics analysis performed on 17,040,725 DNA sequences of the nucleotide collection database, we identified a total of 158 complex class 1 integrons in GenBank, 20 of them with $bla_{CTX-M-2}$ and 3 of them harboring the *qnrB10* allele. These complex class 1 integrons belonged to 6 bacterial families: 85.4% to *Enterobacteriaceae* ($n = 135/158$), 7.6% to *Moraxellaceae* ($n = 12/158$), 3.2% to *Aeromonadaceae* ($n = 5/158$), 1.9% to *Pseudomonadaceae* ($n = 3/158$), 1.3% to *Vibrionaceae* ($n = 2/158$), and only 0.6% belonged to *Xanthomonadaceae* ($n = 1/158$). Regarding to the presence of *orf513* in environmental samples, recent work from our laboratory showed that this gene was mostly encoded in *Pseudomonadaceae* than in other environmental γ-proteobacteria from Patagonia, Argentina (Nardelli et al., 2012). The *orf513* gene found in these isolates was not associated with complex class 1 integrons, showing not only a different epidemiology but also different flanking sequences and therefore alternative genetic platforms for this recombinase in samples recovered from the open environment.

3.2. Different gene cassette arrays among ISCR1::$bla_{CTX-M-2}$ positive species

The *Enterobacteriaceae* family showed a high frequency of the class 1 integron In35 (*aac(6')-Ib-*bla_{OXA-2}*-gcuD*) among *K. pneumoniae* and *P. mirabilis*. Since In35 is usually associated with $bla_{CTX-M-2}$, it is not surprising that the complex integron In35::IS*CR1*::$bla_{CTX-M-2}$ was the most frequent in this study as well as it is in South America (Arduino et al., 2003, 2002; Bado et al., 2010; Brizio et al., 2006; Climaco et al., 2010; Di Conza et al., 2002; Eckert et al., 2006; Hopkins et al., 2006; Orman et al., 2002; Petroni et al., 2002; Power et al., 2005; Soler Bistue et al., 2006; Sompolinsky et al., 2005; Vignoli et al., 2006) (AN: EU597467.1, EU117158.1 and AJ311891.3). In the present study, In35 was detected in *E. coli*, *K. pneumoniae* and *P. mirabilis* (Table 1). Despite its prevalence in the region, it showed a reduced incidence in the rest of the world, where it has been found only in *E. coli* strains from Israel, France, and Portugal (Brizio et al., 2006; Eckert et al., 2006; Hopkins et al., 2006; Sompolinsky et al., 2005). The wide dissemination of In35::IS*CR1*::$bla_{CTX-M-2}$ in South America may be the result of horizontal genetic transfer events mediated by conjugative plasmids as previously found (Arduino et al., 2002; Bado et al., 2010; Climaco et al., 2010; Di Conza et al., 2002; Vignoli et al., 2006).

Besides In35, we identified other gene cassettes arrays in the vr-1 of the $bla_{CTX-M-2}$-containing complex class 1 integrons (Table 1). In2 (*aadA1a*), In37 (*aac(6')-Ib-cr-*bla_{OXA-1}*-catB3-arr-3*), In46 (*aac(6')-Ib*), In131 (*aac(6')-IId-aadA1a*), In132 (*aac(6́)-Ib-aadA1a*), In290 (*aadB-aadA1a*), In293 (*aadB-aadA2*) and In415 (*gcuFΔ3-aadA2*) were identified in different isolates. While In2, In131 and In132 were found in more than 3 species, the remaining integrons were found in a single species, i.e., In46::IS*CR1*::$bla_{CTX-M-2}$ in *E. coli*, In290::IS*CR1*::$bla_{CTX-M-2}$ in *P. mirabilis* (Table 1) suggesting that these complex class 1 integrons may be associated with elements with a limited spectrum of transfer.

Worldwide, different vr-1 arrays in complex class 1 integrons carrying IS*CR1*::$bla_{CTX-M-2}$ from nosocomial isolates have been reported (Fig. 3a and Table B) (Arduino et al., 2003; Cendejas et al., 2010; Climaco et al., 2010; Chagas et al., 2011; Do Carmo Filho et al., 2008; Eckert et al., 2006; Fursova et al., 2010; Garcia Fernandez et al., 2007; Hammad and Shimamoto, 2011; Karczmarczyk et al., 2011; Lopes et al., 2010; Marquez et al., 2008; Melano et al., 2003; Orman et al., 2002; Pallecchi et al., 2004; Peirano et al., 2009; Priamchuk et al., 2010; Song et al., 2011; Toleman et al., 2006b; Valverde et al., 2006). Several of these complex class 1 integrons were isolated either from an outbreak, or from sporadic or epidemic clones (Arduino et al., 2012, 2002; Canton and Coque, 2006). In the recent years, complex class 1 integrons In2 and In127 were also found in *Enterobacteriaceae* strains isolated from animals in Ireland (2007) and Japan (2009) (Hammad and Shimamoto, 2011; Karczmarczyk et al., 2011), suggesting possible transfer of resistance determinants among environmental and nosocomial niches.

Among the 280 non-fermenting bacilli isolates of our survey, *P. aeruginosa* 2002 strain was found to be the only one which contained a complex class 1 integron (Table 1). This integron harbored a novel array of cassettes (*aadB-aadA1-aacA7*) associated with $bla_{CTX-M-2}$, the In547::IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integron (Table 1, AN: KC879156). To date, there are only 4 reports of complex class 1 integrons in this species, all of them from South America harboring the $bla_{CTX-M-2}$ gene in the vr-2, including In35::IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integron from Argentina (Arduino et al., 2003; Ingold et al., 2011; Picao et al., 2009; Radice et al., 2007). Although infrequent, these data show a regional spread of complex class 1 integrons from *Enterobacteriaceae* to *P. aeruginosa*.

When analyzing the emergence of $bla_{CTX-M-2}$ over time, we realized that the first reports were from Argentina (Fig. 3a). Therefore, we propose that a potential path to the widespread of the IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integrons began in 1989 in our country, with successive dispersion to neighboring countries in the 1990s and 2000s, to finally disseminate to the rest of the world, including Asia and Europe, in the early 2000's (Fig. 3a).

3.3. Distribution of ISCR1::qnrB10 among clinical isolates

The primers and subsequent sequencing of the amplicons used for the identification of different *qnrB* alleles included detection of *qnrB* genes that have been described in the vr-2 of complex class 1 integrons (Andres et al., 2013; Cattoir et al., 2007; Garnier et al., 2006; Quiroga et al., 2007). Sequence analysis confirmed the sole presence of *qnrB10* in all the positive samples. In Argentina, 4 other alleles of *qnrB* have been described: (i) *qnrB1* in an *Enterobacteriaceae* strain (94% of nucleotide identity to *qnrB10*) (AN: DQ351241), (ii) *qnrB2* in strains of *Klebsiella* spp., *E. coli* and *Salmonella* spp.

Table 1
Description of the vr-1 and the respective vr-2 found in complex class 1 integron-harboring isolates.

Isolates[a]	Variable region of class 1 integrons (vr-1)	Variable region of complex class 1 integrons (vr-2)	Complex class 1 integron name[b]
Cf201, Cf203, Cf301, Ecl30, Ecl302, Ecl401[c], Ec205, Sm101	*aadA1a*	$bla_{CTX-M-2}$	In2::IS*CR1*::$bla_{CTX-M-2}$
Ec57[c], Ec511, Kpn130, Kpn133[c], Kpn201, Kpn202, Kpn205, Kpn300, Kpn301, Kpn307, Kpn311, Kpn314[c], Kpn402, Kpn404, Kpn502[c], Kpn505, Kpn508, Pm4[c], Pm5, Pm8–12, Pm202–207, Pm212, Pm213, Pm300, Pm302–306, Pm401–403, Pm412–414	*aac(6')-Ib-bla*$_{OXA-2}$-*gcuD*	$bla_{CTX-M-2}$	In35::IS*CR1*:: :: *blabla*$_{CTX-M-2CTX-M-2}$
Kpn3	*aac(6')-Ib-cr-bla*$_{OXA-1}$-*catB3-arr-3*	$bla_{CTX-M-2}$	In37::IS*CR1*::::*blabla*$_{CTX-M-2CTX-M-2}$
Ec512	*aac(6')-Ib*	$bla_{CTX-M-2}$	In46::IS*CR1*::$bla_{CTX-M-2}$
Cf701[c], Ecl202, Ecl303[c],Ecl304[c], Pm19, Pm408, Pm416, Sm120–123, Sm203–206, Sm310–312, Sm407, Sm13003[c]	*aac(6')-IId -aadA1a*	$bla_{CTX-M-2}$	In131::IS*CR1*::$bla_{CTX-M-2}$
Ecl5, Ecl203, Ec304, Kpn9[c], Pm22	*aac(6')-Ib-aadA1a*	$bla_{CTX-M-2}$	In132::IS*CR1*::$bla_{CTX-M-2}$
Pm25	*aadB-aadA1a*	$bla_{CTX-M-2}$	In290::IS*CR1*::$bla_{CTX-M-2}$
Sm100	*aadB-aadA2*	$bla_{CTX-M-2}$	In293::IS*CR1*::::*blabla*$_{CTX-CTX-M-2}$
Kpn12	*gcuFΔ3-aadA2*	$bla_{CTX-M-2}$	In415::IS*CR1*::::*blabla*$_{CTX-M-2CTX-M-2}$
Pae2002	*aadB-aadA1-aacA7*	$bla_{CTX-M-2}$	In547::IS*CR1*::::*blabla*$_{CTX-M-2}$
Kpn314[c], Kpn411, Kpn502[c]	*aac(6')-Ib-cr-bla*$_{OXA-1}$-*catB3-arr-3*	*qnrB10*	In37::IS*CR1*::*qnrB10*
Ecl9, Ecl702	*aac(6')-Ib*	*qnrB10*	In46::IS*CR1*::*qnrB10*
Ecl92, Ecl94, Ecl98, Ecl401[c], Ecl705, Kpn9[c]	*aac(6')-IId -aadA1a*	*qnrB10*	In131::IS*CR1*::*qnrB10*
Cf1, Cf701[c], Ecl21, Ecl303[c], Ecl304[c], Ecl703, Ecl704, Ec57[c], Kpn133[c], Pm4[c], Pm26, Sm13003[c]	*aac(6')-Ib -aadA1a*	*qnrB10*	In132::IS*CR1*::*qnrB10*

[a] Cf, *Citrobacter freundii*; Ecl, *Enterobacter cloacae*; Ec, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pm, Proteus *mirabilis*; Pae, *Pseudomonas aeruginosa;* Sm, *Serratia marcescens*.
[b] The name of the corresponding complex class 1 integron was determined as previously described (Quiroga et al., 2007).
[c] Isolates harboring two complex class 1 integrons.

associated with IS*CR1* (93% of nucleotide identity to *qnrB10*), both alleles isolated between 2005 and 2008, (AN: DQ351242), (iii) *qnrB9*-like in a *K. pneumoniae* strain isolated in 1988 (93% of nucleotide identity to *qnrB10*), and (iv) *qnrB19* in small plasmids of *Salmonella* spp. and *E. coli* strains isolated in 2007 (98% of nucleotide identity to *qnrB10*) (AN: EU432277) (Andres et al., 2013; Jacoby et al., 2009; Tran et al., 2012). At present there are more than 70 alleles for the *qnrB* gene with 95.13% amino acid identity among them (http://www.lahey.org/qnrstudies/). Two-thirds of these alleles are usually located in chromosomal DNA of different *Citrobacter* species (Jacoby et al., 2011). Although not all isolates of *Citrobacter* spp. possessed a *qnrB* allele, whole genome sequences indicate that this genus is likely the natural source of these genes (Jacoby et al., 2011). Here, we found by bioinformatics studies, that the vr-2 containing the *qnrB10* has an identity ranging from 92% to 96% with the chromosomal genes of *C. freundii* (NZ_JH414886.1), suggesting the capture of this determinant by IS*CR1* from a donor genome close to this species.

Similar to *qnrB10*, other *qnrB* alleles have been associated with transposons, such as: (i) *qnrB1* in IS*26*/IS*3000* (Andres et al., 2013; Teo et al., 2009), (ii) *qnrB19* in IS*26*/IS*26* (Dolejska et al., 2013; Schink et al., 2012) or in genetic structures that resulted from *ISEcp1* gene capture (Tran et al., 2012); and (iii) *qnrB2*, *qnrB4* and *qnrB6* in complex class 1 integrons (Andres et al., 2013; Cattoir et al., 2007; Garnier et al., 2006). Since the first descriptions of complex class 1 integrons carrying *qnrB* alleles in the vr-2 in the 90's (Fig. 3b), several other isolates were reported from Brazil and the United States and other countries of Oceania, Africa, Asia and Europe in the 2000s, followed by the recent report in Spain in the 2010s (Fig. 3b) (Ahmed and Shimamoto, 2011; Chen et al., 2009; Folster et al., 2011; Garnier et al., 2006; Jacoby et al., 2011; Ma et al., 2009; Meradi et al., 2011; Minarini et al., 2008; Ogbolu et al., 2011; Park et al., 2007; Peirano et al., 2011; Quiroga et al., 2007; Ruiz et al., 2012; Teo et al., 2009; Thomas et al., 2005; Verdet et al., 2006; Wang et al., 2012; Wu et al., 2008; Zong et al., 2008) (AN: JX101693, FJ628167, FJ943244 and EU855788.1). As found for the worldwide dissemination of complex class 1 integrons possessing $bla_{CTX-M-2}$ in the vr-2, the starting point for the spread of IS*CR1*::*qnrB10* genetic elements associated to class 1 integrons in clinical samples was likely to be Argentina in the 80's and 90's, respectively (Fig. 3).

Although the array of gene cassettes in the vr-1 can be easily exchanged, the evaluation of the distribution of IS*CR1*::*qnrB10* in our survey revealed the presence of In37, In46, In131 and In132 (Table 1). While In37::IS*CR1*::*qnrB10* and In46::IS*CR1*::*qnrB10* were only found in *K. pneumoniae* and *E. cloacae*, respectively; In131::IS*CR1*::*qnrB10* and In132::IS*CR1*::*qnrB10* were mainly found in *E. cloacae* (Table 1). We performed PCR fingerprinting by a PCR assay employing degenerate oligonucleotide primers (DO-PCR) (Limansky and Viale, 2002) on all *E. cloacae* strains. We found that all of them had unique DO-PCR patterns, suggesting non-clonal relationships among the isolates (Data not shown). The high frequency found in *E. cloacae* (48%) evidences that this species could be considered as a reservoir for complex class 1 integrons harboring IS*CR1*::*qnrB10*. Moreover, positive strains for IS*CR1*::*qnrB10* were isolated in 1993 (*P. mirabilis*, $n = 1$) and in 1998 (*E. cloacae*, $n = 1$) showing that the emergence of this allele in Argentina occurred at least a decade before its first description (Quiroga et al., 2007). In addition, *qnrB10* has also been detected in an *E. coli* strain from healthy animals from Nigeria isolated in 2006 suggesting a potential transfer of this determinant to other niches (Fortini et al., 2011).

These results clearly showed the maintenance and dissemination across time of the *qnrB10* allele and its consistent localization at the vr-2 of different complex class 1 integrons in the *Enterobacteriaceae* family.

3.4. The Orf513 protein binds to the oriIS region

Bioinformatics analysis of all IS*CR1* sequences submitted to the nucleotide collection [database], revealed that this element is always

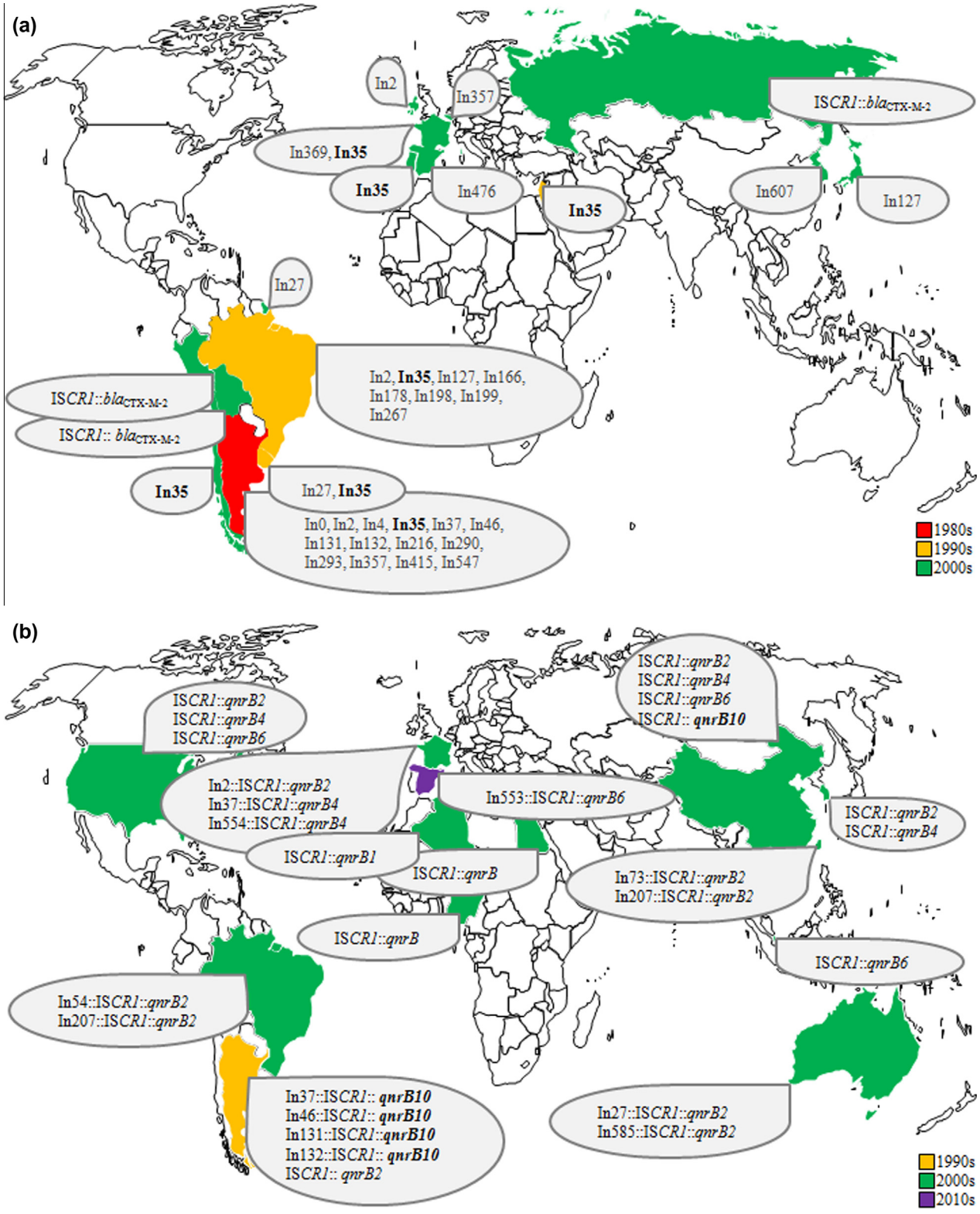

Fig. 3. (a) World map showing the distribution of the IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integrons found in this study, in GenBank and in the literature. All vr-1s are depicted with word balloons indicating the country of origin; In35 is in bold letters. (b) World map showing the distribution of the IS*CR1*::*qnrB* complex class 1 integrons found in this study, in GenBank and in the literature. All vr-1s are depicted with word balloons indicating the country of origin when they were defined, as well as each particular *qnrB* allele; the *qnrB10* allele is in bold letters.

inserted at 24 nt downstream of the stop codon of the *sul1* gene generating the end of the first 3′-CS (nt 3165 of the AN EU091084.1) (Fig. 1). One hundred and three out of 158 complex class 1 integrons found in GenBank were located in plasmids (103/108). In addition, we found that complex class 1 integrons with different vr-2 ($bla_{CTX-M-2}$, bla_{DHA-1}, *qnrA1*, *qnrB2*, *qnrB4*,

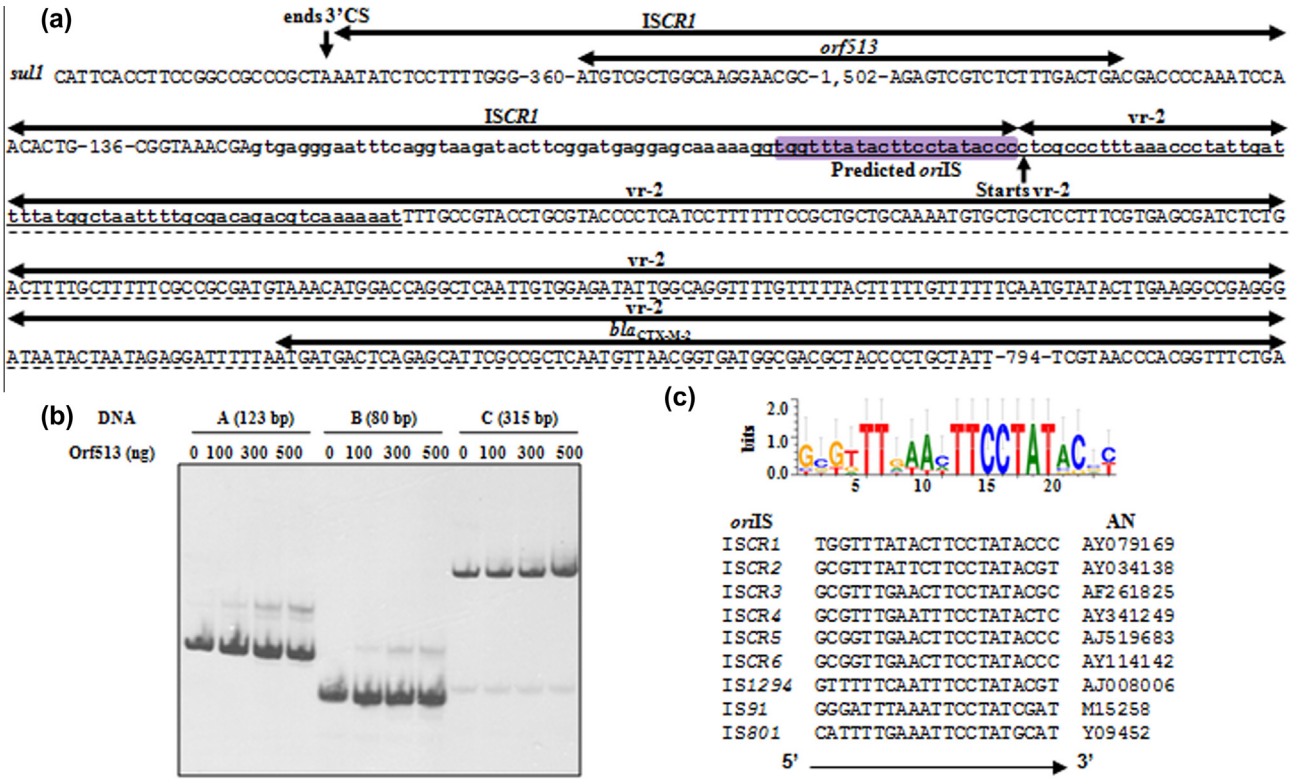

Fig. 4. (a) DNA fragment of In35::IS*CR1*::$bla_{CTX-M-2}$ complex class 1 integron (AN: AY079169 from nt 379 to 3698). Double-headed arrows indicate IS*CR1*, *orf513* and part of the vr-2 with the complete $bla_{CTX-M-2}$ gene. Numbers indicate the amount of nt not showed of each sequence. Vertical arrows indicate the end of the first 3′CS and the beginning of vr-2, respectively. Rectangle indicates the region with identity to the *ori*IS of IS*91*, which is within the IS*CR1* binding region obtained in this study by mobility shift assays with purified Orf513 protein. Lowercase letters represent fragment A, which was obtained using primers 1 and 3 (123 pb). Underlined letters with a solid line represent fragment B, which was obtained using primers 2 and 3 (80 bp). Underlined letters with a dashed line represent fragment C, which was obtained using primers 4 and 5 (315 bp). (b) Mobility shift assay with purified Orf513 protein. Each DNA fragment (A, B, C) was incubated with different concentrations of Orf513 protein. c) Alignment of the last 21 bp of various IS*CR* elements and their counterparts corresponding to the *ori*IS from relevant insertion sequences. Sequence conservation is depicted using sequence logos from WebLogo (http://weblogo.berkeley.edu/logo.cgi).

qnrB10, catA2) were inserted between the IRs of Tn*402* transposons (Marquez et al., 2008) (AN: EU780013, GU943791, EU195449, JX193301, GU943791, JX141473).

In agreement with previous bioinformatics studies, every IS*CR1* element from GenBank possessed the proposed *ori*IS*CR1* 5′-TGGTTTATACTTCCTATACCC-3′, while no homology was found for a putative *ter*IS end (Partridge and Hall, 2003; Toleman et al., 2006a,b). The *ori*IS*CR1* has been identified as the origin of transposition of IS*CR1* and its sequence is highly conserved in the family of IS*CR* elements, e.g. IS*91*-like transposons. Thus, we decided to assess whether this region was involved in the mechanism of recombination. We performed mobility shift assays in order to evaluate the ability of the Orf513 protein to bind to specific DNA sequences containing the *ori*IS. After exposing the Orf513 protein to 2 DNA fragments containing different regions of the IS*CR1* (Fig. 4a), and to a DNA fragment containing the first nucleotides of the vr-2 and of the $bla_{CTX-M-2}$ gene, we noticed a clear shift confirming a DNA–protein interaction between Orf513 and the previously proposed *ori*I*SCR1* (5′-TGGTTTATACTTCCTATACCC-3′) (Fig. 4b and c). The Orf513 protein binds to the region which contains the proposed *ori*IS (Fig. 4b, fragments A and B), but no binding is observed to the region downstream of it (Fig. 4b fragment C). Taken together, this data confirms that Orf513 recognizes the *ori*IS and suggests that the binding activity of Orf513 would be involved in the mobilization of IS*CR1* transposon elements.

Additional experiments are needed in order to discern its complete mechanism which involves the capture of genes from a donor strain, the invasion of IS*CR1* into class 1 integrons, and the subsequently spread among clinical samples under antibiotic pressure.

4. Conclusions

A high dispersion and maintenance along the time (1989–2010) of complex class 1 integrons was found in clinical samples from Argentina, with $bla_{CTX-M-2}$ and *qnrB10* genes always located in the vr-2. The epidemiology of complex class 1 integrons was found to be notably different among fermenting and non-fermenting bacilli. While only one positive complex class 1 integron isolate was found among 280 non-fermenting isolates, the 55% of the fermenting isolates were positive for the presence of this genetic element (94/171). According to our findings, to bioinformatics analysis and to previous studies, complex class 1 integrons possessing $bla_{CTX-M-2}$ or *qnrB10* in the vr-2 began to spread in clinical isolates from Argentina to the rest of the world in the 80's and 90's, respectively.

The identification of the functional properties of the Orf513 protein as well as the maintenance across time of complex class 1 integrons in our nosocomial isolates highlight its active role in the spread of genetic platforms that encode multiple antimicrobial resistance genes linked to different mobile elements that enhance their dissemination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.06.029.

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