The Role of ISCR1-Borne P\textsubscript{OUT} Promoters in the Expression of Antibiotic Resistance Genes

Claire Lallement, Cécile Pasternak, Marie-Cécile Ploy* and Thomas Jové*

INSERM, CHU Limoges, RESINFIT, U1092, University of Limoges, Limoges, France

The ISCR1 (Insertion sequence Common Region) element is the most widespread member of the ISCR family, and is frequently present within γ-proteobacteria that occur in clinical settings. ISCR1 is always associated with the 3′Conserved Segment (3′CS) of class 1 integrons. ISCR1 contains outward-oriented promoters P\textsubscript{OUT}, that may contribute to the expression of downstream genes. In ISCR1, there are two P\textsubscript{OUT} promoters named P\textsubscript{CR1−1} and P\textsubscript{CR1−2}. We performed an in silico analysis of all publically available ISCR1 sequences and identified numerous downstream genes that mainly encode antibiotic resistance genes and that are oriented in the same direction as the P\textsubscript{OUT} promoters. Here, we showed that both P\textsubscript{CR1−1} and P\textsubscript{CR1−2} significantly increase the expression of the downstream genes \textit{bla}\textsubscript{CTX-M−9} and \textit{dfrA19}. Our data highlight the role of ISCR1 in the expression of antibiotic resistance genes, which may explain why ISCR1 is so frequent in clinical settings.

Keywords: antibiotic resistance, promoters, ISCR1, expression, insertion sequence element

INTRODUCTION

Antimicrobial resistance is often mediated by the dissemination of antibiotic resistance genes (ARG) that are carried by mobile genetic elements (MGEs) including plasmids, insertion sequences (IS), transposons (Tn) and integron gene cassettes (Partridge, 2011) which are harbored by bacteria across all phyla and environments (Aminov, 2011). In addition, some MGEs may carry promoters that ensure or increase expression of downstream ARG. Several IS including IS1999, IS\textit{Ecp1}, IS\textit{Kpn23} (reviewed in Vandecraen et al., 2017) display a complete outwardly oriented functional promoter usually referred as P\textsubscript{OUT} that enhances expression of downstream ARGs. Other IS like IS1 or IS257 only contain the −35 element that generates a hybrid functional promoter when associated with a downstream putative −10 element (Goussard et al., 1991; Simpson et al., 2000). Most often, these IS-borne promoters allow sufficient expression of ARGs to confer the antibiotic resistance phenotype. IS from the ISCR family are related to the IS91 family and display a \textit{rcr} gene encoding a putative RCR transposase belonging to the ubiquitous HUH endonuclease superfamily (Chandler et al., 2013). HUH transposases of the IS91 family catalyze the transposition of their cognate IS by the rolling-circle replication of the element from one boundary, named \textit{ori}IS, to the other referred to as the \textit{ter}IS (Tavakoli et al., 2000; del Pilar Garcillán-Barcia et al., 2001; Yassine et al., 2015). However, so far, there is no experimental evidence for transposition of any of the ISCR elements. Four out of the fifteen members of the ISCR family are commonly found in γ-proteobacteria, namely ISCR1, ISCR2, ISCR3 and ISCR5, and ISCR1 predominates in strains isolated in clinical settings (Toleman et al., 2006). ISCR1 was first identified as a conserved region disrupting the 3′ conserved segment (3′CS) of class 1 integrons...
(Figure 1A; Stokes et al., 1993). The region downstream of ISCR1 (oriIS side) is variable and often associated with ARG (Arduino et al., 2002; Toleman et al., 2006; Rodriguez-Martinez et al., 2007; Wachino et al., 2011). Previous studies identified the presence of two putative promoters located on the oriIS side of the ISCR1, namely P_{CR1−1} and P_{CR1−2}, suggesting that ISCR1 could impact the expression of downstream genes (Figure 1B; Mammeri et al., 2005; Rodriguez-Martinez et al., 2006). To assess the potential function of these promoters in the expression of downstream genes, we first performed an extensive in silico analysis of all ISCR1 sequences publicly available (GenBank®) to determine the diversity of putative downstream ARGs. Here, we show experimentally by means of a reporter gene assay that ISCR1 directly contributes to the expression of different ARGs via these two P_{OUT} promoters.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids are listed in (Supplementary Table S1). Bacterial strains were grown in Lysogeny Broth (LB) broth at 37°C. Media were supplemented with kanamycin (25 µg/mL) when required.

Plasmid Constructions

We used the reporter plasmid pSU38ΔtotlacZ (Jové et al., 2010) and three derived plasmids in which ISCR1 and/or regions adjacent to ISCR1 were inserted in transcriptional fusion with the reporter gene lacZ. Fragments of ISCR1 and/or regions adjacent to ISCR1 were amplified from two Salmonella enterica subsp. enterica strains carrying ISCR1 followed by either blaCTX−M−9, or dfrA19 genes (Espéli et al., 2001) as they belong to the most prevalent antibiotic resistance gene families found in the variable region downstream ISCR1. Primers (Sigma-Aldrich®) used for cloning are listed in Supplementary Table S2. For each construction, amplifications were performed using the Phusion® Polymerase (Thermo Fisher Scientific). PCR products were loaded and visualized by means of a 0.8% agarose gel, extracted and purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). PCR products were cloned into the EcoRI and BamHI unique restriction sites of pSU38ΔtotlacZ. Transformants were selected on LB medium supplemented with kanamycin. Recombinant plasmids were verified by PCR with primers targeting the insert and by sequencing.

β-Galactosidase Assays

β-galactosidase assays were performed in the E. coli MGI656 strain (Supplementary Table S1; Espéli et al., 2001) as previously described (Miller, 1993) for nine independent assays for each construct.

Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentrations were performed by the microdilution method in Mueller-Hinton broth in three independent experiments as recommended by the French Antiibiogram committee guidelines1.

Statistical Analysis

Statistical analyses were performed using the Mann-Whitney test with two paired groups.

GenBank® ISCR1 Element Sequence Analysis

The amino acid sequence of the RCR1 transposase encoded by ISCR1 (accession number CAJ84008) was blasted with BLASTp (NCBI). The matching sequences were filtered to retain RCR1 peptide sequences with an amino acid identity level (equal or) higher than 98%. Corresponding nucleotide sequences in which the oriIS region was partial or truncated were discarded. The remaining nucleotide sequences were sorted out in 93 groups according to the nature of the first gene adjacent to ISCR1: non-annotated nucleotide sequences with identified open reading frame (ORF) longer than 150 bp were included into the analysis. To define a novel gene group, we used a cut-off of 95% amino acid identity of the encoded protein, except for antimicrobial resistance genes (ARGs) for which a single amino-acid variation was used as threshold for inclusion into a group. Finally, one nucleotide sequence representing each gene group was submitted to blastN to identify previously non-annotated nucleotide sequences (only 100% identical sequences were kept). This data extraction was performed on 2017-01-19.

Quantification of bla_{CTX−M−9} and dfrA19 Transcripts

Total RNA was extracted with the NucleoSpin® RNA Extraction Kit (Macherey-Nagel Inc.). Contaminating DNA was removed from RNA samples by using the Turbo DNA-free Kit (Ambion). cDNAs were synthesized from 1 µg of DNase-treated total RNA by using PrimeScript® RT Reagent kit (TaKaRa Clontech). cDNA was quantified by PerfeCTa SYBR Green FastMix® Kit (Quanta BioSciences™) with adequate oligonucleotides (Supplementary Table S2). Three independent experiments were performed, each in triplicate. Relative expressions of the bla_{CTX−M−9} (Primers 16 and 17) and dfrA19 (Primers 18 and 19) genes were estimated by normalizing transcript copy number to those of the housekeeping gene rpoB (Primer 20 and 21). The impact of ISCR1 oriIS has been calculated as ratio between the relative expression of each gene in presence and in absence of the ISCR1 oriIS.

RESULTS AND DISCUSSION

Diversity of the ISCR1 Downstream Genes

In this study, 1127 distinct sequences containing the ISCR1 element extracted from GenBank® were analyzed in silico. The

1http://www.sfmb-microbiologie.org/UserFiles/files/casfm/CASFMV1_0_MARS_2017.pdf
majority of these sequences was recovered from γ-proteobacteria (99.9%) while others were found to be present in uncultured bacteria (n = 4) (Supplementary Table S3). All ISCR1 sequences in this study were associated with the 3′ CS region of class 1 integrons (left-hand side, Figure 1). In contrast, the downstream region of ISCR1 was identified to be very variable (right-hand side, Figure 1). Interestingly, a large percentage of the analyzed ISCR1 elements (n = 946, 84%) carried an adjacent gene oriented in the same direction as the rcr1 encoding transposase gene (top strand). This suggests that these genes might be expressed from the ISCR1 P_{OUT} promoters (Figure 1). The functions of these top strand genes adjacent to ISCR1 fell into three categories (Supplementary Table S4). The most represented genes (n = 429) encoded truncated insertion sequence transposases, most often ISEc28 (n = 418), more rarely ISEc29, ISAba125 or ISEcp1 (Supplementary Table S4). The second most important group (n = 379) was identified as known or putative ARGs encoding resistance to five families of antibiotics: trimethoprim (n = 125), β-lactams (including extended-spectrum β-lactamase, ESBL, genes) (n = 121), quinolones (n = 113), chloramphenicol (n = 12), and aminoglycosides (n = 8). For each antibiotic family, different genes or alleles were identified, that may lead to different resistance phenotypes (Supplementary Table S4). The last group (n = 138) includes genes involved in other cellular process or genes of unknown function (Supplementary Table S4).
ISCR1 Contributes to the Downstream Expression of ARGs via Its P\textsubscript{OUT} Promoters

To investigate the impact of P\textsubscript{OUT} promoters on the expression of downstream genes, we focused on the two following ARGs: bla\textsuperscript{CTX−M−9} (conferring an Extended-Spectrum Beta-Lactamase resistance phenotype) (accession number: AM234698) and dfrA19 (resistance to trimethoprim) (accession number: AM234698). Derivatives in which one or both P\textsubscript{CR1} promoters were mutated were also tested for bla\textsuperscript{CTX−M−9} (A) and dfrA19 (B). Derivatives in which one P\textsubscript{CR1} promoter was mutated or both promoters were mutated were also tested for bla\textsuperscript{CTX−M−9} (A). Constructions are described in (Supplementary Table S1). The results are the average of at least three independent experiments. *p < 0.001.

**FIGURE 3** | Activities and characterization of the ISCR1 P\textsubscript{CR1} promoters. β-galactosidase activities were measured from lacZ-transcriptional fusions with the intergenic region (IGR) cloned either in absence (oriIS−) or in presence (oriIS+) of the 232 bp long ISCR1 oriIS region. The genes tested were bla\textsuperscript{CTX−M−9} (A) and dfrA19 (B). Derivatives in which one or both P\textsubscript{CR1} promoters were mutated were also tested for bla\textsuperscript{CTX−M−9} (A). Constructions are described in (Supplementary Table S1). The results are the average of at least three independent experiments. *p < 0.001.

---

downstream genes and confirm that ISCR1 harbors a functional P\textsubscript{OUT} promoter. We thus showed that both ISCR1 oriIS and IGR are involved in gene expression of dfrA19 and bla\textsuperscript{CTX−M−9}. However, as we observed in our in silico analysis (Supplementary Table S4), the sequence and length of the IGR vary from 0 to 1211 bp. Further analysis with other ISCR1 elements with different downstream genes are needed to elucidate to which extent the sequence of the IGR contribute to downstream gene expression.

To determine whether the positive effect of the ISCR1 oriIS on the expression of the downstream gene relies on the two P\textsubscript{OUT} promoters, P\textsubscript{CR1−1} (TAACG-N\textsuperscript{17}−TAAGAT) and P\textsubscript{CR1−2} (TTCCGA-N\textsuperscript{118}−TTTATA), we constructed derivatives of pULP2 (oriIS-IGR\textsuperscript{CTX−M−9−lacZ}) in which the putative −10 element, of either P\textsubscript{CR1−1} or P\textsubscript{CR1−2} or both promoters was mutated. Mutation of P\textsubscript{CR1−1} (pULP5) or P\textsubscript{CR1−2} (pULP6) reduced by 85 and 40%, respectively, the overall β-galactosidase activity compared to the pULP2 wild-type construction (Figure 2A). Concomitant mutations of both P\textsubscript{CR1−1} and P\textsubscript{CR1−2} dropped the expression to the basal level detected in absence of oriIS (Figure 2A, pULP7 versus pULP1). These results indicated that both P\textsubscript{CR1−1} and P\textsubscript{CR1−2} are functional and, together, are responsible for the contribution of ISCR1 to the expression of downstream top strand genes. Consistently, several earlier reports mapped transcriptional START sites in the oriIS that are compatible with the P\textsubscript{CR1−1} and P\textsubscript{CR1−2} (Mammeri et al., 2005; Rodriguez-Martinez et al., 2006). P\textsubscript{CR1−1} also appears to be stronger than P\textsubscript{CR1−2} in agreement with its higher conservation degree of its −10 hexamer, with respect to the σ\textsuperscript{70} consensus sequence (four bases out of six versus three for P\textsubscript{CR1−2}). These
tandem promoters display a synergistic effect but the exact underlying mechanisms remain to be elucidated.

**The ISCR1 orlS Is Required to Confer the blaCTX-M-9-Mediated Resistance**

To assess whether the increased level of gene expression due to the ISCR1 orlS region has a phenotypic impact, we measured the level of resistance conferred by dfrA19 and blaCTX-M-9 genes in absence or in presence of the orlS region in *Escherichia coli*. For this purpose, each gene was cloned in pSU38 (Supplementary Table S1) with its own IGR preceded or not by the orlS region. Subsequently, we determined the MIC of the respective clones in presence of the corresponding antibiotic (cefotaxime or trimethoprim) (see below). The *Escherichia coli* MG1655 lac-strain harboring the empty plasmid (pSU38 ΔtotlacZ) was susceptible to both cefotaxime (MIC < 0.5 µg/mL) and trimethoprim (MIC < 4 µg/mL). When MG1655 lac- is transformed with a pSU38 ΔtotlacZ derivative that harbors the blaCTX-M-9 coding sequence alone (pULP13) or preceded by its own IGR (pULP12), the MIC for cefotaxime was also <0.5 µg/mL. The MIC for cefotaxime significantly increased in pULP11 which contains both IGR and orlS region (MIC > 512 µg/mL). These findings demonstrated that the ISCR1 orlS region is required for blaCTX-M-9 to confer a cefotaxime resistant phenotype, most likely mediated by the activity of PCR1-1 and PCR1-2. We performed quantification of transcripts and showed that the blaCTX-M-9 transcript number increased by 99-fold (98.82 ± 8.29) in presence of IGR. These results correlate with MIC findings.

In contrast, when preceded by its IGR, the level of trimethoprim resistance conferred by dfrA19 was similar in absence or in presence of the orlS region (pULP08 MIC = > 2048 µg/mL, pULP09 MIC = > 2048 µg/mL), while the dfrA19 coding sequence alone did not confer any resistance (pULP10, MIC < 4 µg/mL). Susceptibility to higher concentrations of trimethoprim could not be determined since they exceed its solubility in DMSO. The dfrA19 resistance gene confers a higher level of resistance compared to other dfrA alleles, such as dfrA10 for example (MIC: 500 µg/mL) (Parsons et al., 1991). We observed a similar resistance phenotype (trimethoprim MIC = > 2048 µg/mL) in absence or in presence of the orlS region. These results were surprising according to β-galactosidase results obtained with or without the IGR sequence (Figure 3). Quantification of transcripts of dfrA19 confirmed that the IGR plays a role in the expression of the gene. Indeed, we obtained a 20-fold change of transcript number (20.03 ± 4.1, dfrA19) in presence of IGR. Such dissociation between the resistance phenotype and the level of gene expression has been previously described (Barraud and Ploy, 2015). Furthermore, this might also be explained by the nature of the enzyme encoded by dfr genes. Indeed, DFR enzymes are insensitive toward trimethoprim, so neither a high concentration of antibiotic nor the quantity of DFR enzymes will affect the level of resistance. Little is known about the dfrA19 gene and noticeably, it seems to only occur associated with ISCR1 element.

**CONCLUSION**

Our data highlight the functionality of the two POUT promoters carried by the ISCR1 element. The fact that those two functional POUT promoters contribute to the expression of various downstream genes, including ARG may explain why ISCR1 is so frequent in clinical settings. Indeed, ISCR1 gives an advantage to the bacteria for antimicrobial resistance expression and one can hypothesize that antibiotic selective pressure has promoted the selection of ISCR1-carrying bacteria.

**AUTHOR CONTRIBUTIONS**

M-CP and TJ conceived the study. TJ coordinated the study. CL, TJ, and CP performed the experiments. All authors analyzed the data and wrote the manuscript.

**FUNDING**

CL gratefully acknowledges the Ministère de l’Enseignement Supérieur, de la Recherche et de l’Innovation (MESRI), and the Fonds Européen de Développement Régional (FEDER) for her doctoral training grant. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**ACKNOWLEDGMENTS**

We thank Amy Gassama for providing us with the *S. enterica* subsp. *enterica* serovar Keurmassar strain and Manon Mellier for her assistance. We also thank Elena Buelow for critical reading of the manuscript.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02579/full#supplementary-material.

**REFERENCES**

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* 2:158. doi: 10.3389/fmicb.2011.00158

Arduino, S. M., Roy, P. H., Jacoby, G. A., Orman, B. E., Pineiro, S. A., and Centron, D. (2002). *blaCTX-M-2* is located in an unusual class 1 integron (In35) which includes Orf513. *Antimicrob. Agents Chemother.* 46, 2303–2306. doi: 10.1128/AAC.46.7.2303-2306.2002

Barraud, O., and Ploy, M.-C. (2015). Diversity of class 1 integron gene cassette rearrangements selected under antibiotic pressure. *J. Bacteriol.* 197, 2171–2178. doi: 10.1128/JB.02455-14
Chandler, M., de la Cruz, F., Dyda, F., Hickman, A. B., Moncalian, G., and Ton-Hoang, B. (2013). Breaking and joining single-stranded DNA: the HUH endonuclease superfamily. *Nat. Rev. Microbiol.* 11, 525–538. doi: 10.1038/nrmicro3067

del Pilar Garcillán-Barcia, M., Bernales, I., Mendiola, M. V., and de la Cruz, F. (2001). Single-stranded DNA intermediates in IS91 rolling-circle transposition. *Mol. Microbiol.* 39, 494–501. doi: 10.1046/j.1365-2958.2001.0261.x

Espéli, O., Moulin, L., and Boccard, F. (2001). Transcription attenuation associated with bacterial repetitive extragenic BIME elements. *J. Mol. Biol.* 314, 375–386. doi: 10.1006/jmbi.2001.5150

Garnier, F., Raked, N., Gassama, A., Denis, F., and Ploy, M.-C. (2006). Genetic environment of quinolone resistance gene *qnrB2* in a complex sul1-type integron in the newly described *Salmonella enterica* serovar Kueurnassar. *Antimicrob. Agents Chemother.* 50, 3200–3202. doi: 10.1128/AAC.00293-06

Goussard, S., Sougakoff, W., Mahlat, C., Bauernfeind, A., and Courvalin, P. (1991). An IS1-like element is responsible for high-level synthesis of extended-spectrum β-lactamase TEM-6 in enterobacteriaceae. *Microbiology* 137, 2681–2687.

Jové, T., Da, Re, S., Denis, F., Mazel, D., and Ploy, M.-C. (2010). Inverse correlation between promoter strength and excision activity in class 1 integrons. *PLoS Genet.* 6:e1000793. doi: 10.1371/journal.pgen.1000793

Mammeri, H., Loo, M. V. D., Poirel, L., Martinez-Martinez, L., and Nordmann, P. (2005). Emergence of plasmid-mediated quinolone resistance in *Escherichia coli* in Europe. *Antimicrob. Agents Chemother.* 49, 71–76. doi: 10.1128/AAC.49.1.71–76.2005

Miller, J. H. (1993). *A Short Course In Bacterial Genetics: A Laboratory Manual And Handbook For Escherichia coli And Related Bacteria.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Parsons, Y., Hall, R. M., and Stokes, H. W. (1991). A new trimethoprim resistance gene, *dhfrX*, in the *In7* integron of plasmid *pDGO100*. *Antimicrob. Agents Chemother.* 35, 2436–2439. doi: 10.1128/AAC.35.11.2436

Partridge, S. R. (2011). Analysis of antibiotic resistance regions in gram-negative bacteria. *FEMS Microbiol. Rev.* 35, 820–855. doi: 10.1111/j.1574-6976.2011.00277.x

Rodriguez-Martinez, J.-M., Poirel, L., Canton, R., and Nordmann, P. (2006). Common region *CR1* for expression of antibiotic resistance genes. *Antimicrob. Agents Chemother.* 50, 2544–2546. doi: 10.1128/AAC.00609-05

Rodriguez-Martinez, J. M., Velasco, C., García, I., Cano, M. E., Martinez-Martinez, L., and Pascual, A. (2007). Characterisation of integrons containing the plasmid-mediated quinolone resistance gene *qnrA1* in *Klebsiella pneumoniae*. *Int. J. Antimicrob. Agents* 29, 705–709. doi: 10.1016/j.ijantimicag.2007.02.003

Simpson, A. E., Skurray, R. A., and Firth, N. (2000). An IS257-derived hybrid promoter directs transcription of a tetA(K) tetracycline resistance gene in the *Staphylococcus aureus* chromosomal mcregion. *J. Bacteriol.* 182, 3345–3352. doi: 10.1128/JB.182.12.3345-3352.2000

Stokes, H. W., Tomaras, C., Parsons, Y., and Hall, R. M. (1993). The partial 3′-conserved segment duplications in the integrons In6 from pSa and In7 from pDGO100 have a common origin. *Plasmid* 30, 39–50. doi: 10.1006/plas.1993.1032

Tavakoli, N., Comanducci, A., Dodd, H. M., Lett, M.-C., Albiger, B., and Bennett, P. (2000). IS1294, a DNA element that transposes by RC transposition. *Plasmid* 44, 66–84. doi: 10.1006/plas.1999.1460

Toleman, M. A., Bennett, P. M., and Walsh, T. R. (2006). ISCR elements: novel gene-capturing systems of the 21st century? *Microbiol. Mol. Biol. Rev.* 70, 296–316. doi: 10.1128/MMBR.00048-05

Vandecraen, J., Chandler, M., Aertsen, A., and Van Houdt, R. (2017). The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit. Rev. Microbiol.* 43, 709–730. doi: 10.1080/1040841X.2017.1303661

Wachino, J.-I., Yoshida, H., Yamane, K., Suzuki, S., Matsui, M., Yamagishi, T., et al. (2011). SMB-1, a novel subclass B3 metallo-β-lactamase, associated with ISCR1 and a class I integron, from a carbapenem-resistant *Serratia marcescens* clinical isolate. *Antimicrob. Agents Chemother.* 55, 5143–5149. doi: 10.1128/AAC.00545-11

Yassine, H., Bientz, L., Cros, J., Goret, J., Bebear, C., Quentin, C., et al. (2015). Experimental evidence for IS1294β-mediated transposition of the blaCMY-2 cephalosporinase gene in enterobacteriaceae. *J. Antimicrob. Chemother.* 70, 697–700. doi: 10.1093/jac/dku472

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Lallement, Pasternak, Ploy and Jové. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.