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A novel genomic island harbouring *lsa*(E), *lnu*(B) genes and a defective prophage in a *Streptococcus pyogenes* isolate resistant to lincosamide, streptogramin A and pleuromutilin antibiotics

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Abstract

The lincosamide-resistant and macrolide-susceptible phenotype has not been described to date in *Streptococcus pyogenes* (group A *Streptococcus*; GAS). The aim of this study was to characterize a GAS isolate susceptible to macrolides but resistant to lincosamide, streptogramin A and pleuromutilin antibiotics (LS_AP phenotype). The antimicrobial susceptibility was tested by the microdilution broth method and the resistance phenotype by D-test. The GAS2887HUB isolate was subjected to whole-genome sequencing. The isolate showed a positive Gots' test (clindamycin inactivation). WGS revealed the strain was ST10 and *emm*93 and had five resistance genes (*lnu*(B)*, ant*(6)-Ia*, aph*(3')-III, *tet*(M)*,* and *dfrG*). The *tet*(M) gene was located into a Tn*916*-like transposon. The *lsa*(E)–*lnu*(B)-containing sequence (inserted downstream of the *rumA* gene) was formed by a 39.6-kb prophage, followed by a gene cluster encoding aminoglycoside-streptothricin resistance [*ant*(6)Ia–*sat4*–*aph*(3')III] and *lsa*(E)–*lnu*(B) genes. This structure was not transferred by conjugation. In conclusion, we have described a new genetic element carrying a determinant of lincosamide resistance in a GAS. Further

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molecular epidemiological surveys are needed to determine the prevalence of this mechanism of resistance in GAS.

Key words: L phenotype, LS_AP phenotype, *Streptococcus pyogenes*, GAS, *lnu*(B), *lsa*(E).

1. Introduction

Streptococcus pyogenes (group A *Streptococcus*; GAS) is a major human pathogen that causes infections such as tonsillitis and skin and soft tissue infection [1]. Penicillins and cephalosporins are widely used to treat GAS infections due to the universal susceptibility of this bacterium to β-lactams. In cases of β-lactam allergy, macrolides or lincosamides are the recommended alternatives. Moreover, the addition of protein synthesis inhibitors, such as clindamycin, may effectively reduce the synthesis of virulence factors and improve patient outcomes [2].

To date, two transferable mechanisms of macrolide and lincosamide resistance have been described in GAS (the ML phenotype). One involves 23S rRNA methylation (*erm* genes), responsible for resistance to macrolides, lincosamides and streptogramins B (the MLS_B phenotype). The other involves active efflux (*mef* genes), conferring resistance to 14- and 15-membered macrolides (the so-called M phenotype) [3,4]. These genes are widespread in streptococci, mostly through the transfer of mobile genetic elements (MGEs) of the Tn*916* family. Two additional phenotypes have been reported sporadically in other

streptococci: lincosamide inactivation by a nucleotidyltransferase (L phenotype, *lnu* genes); and resistance to lincosamide, streptogramin A and pleuromutilin antibiotics due to ribosomal protection (LS_AP phenotype; *lsa* genes) [5,6]. The latter mechanism, also carried by MGEs, has been described in several human and animal streptococcal species [7–12], including one *S. pyogenes* isolate (*erm*(B) together with *lsa*(C); MLS_B phenotype) [13].

In this study we describe, to the best of our knowledge, the first *S. pyogenes* isolate with an LS_AP phenotype due to the presence of *lsa*(E) and *lnu*(B) genes. Moreover, we show that the genetic element carrying these genes was inserted into the *S. pyogenes* chromosome with a defective prophage similar to those recovered from swine pathogens.

2. Materials and methods

2.1 *Bacterial strain and antibiotic susceptibility testing*

A *S. pyogenes* exhibiting a LS_AP phenotype (GAS2887HUB) was obtained from a wound sample of a 77-year-old woman with cellulitis who was attended in the emergency department in September 2009. She consulted for a fever (39°C), headache, vomits and a confusional state. The physical examination revealed a pretibial leg ulcer was observed with signs of infection and cellulitis. Antipyretic and amoxicillin-clavulanic acid therapy was started after collecting blood cultures and wound samples. The patient presented good evolution after a 15-hour observation period and was discharged. After two weeks of amoxicillin-clavulanic treatment and one month of reepithelialisation therapy the wound resolved.

The antimicrobial susceptibility was tested by the broth microdilution reference method following the EUCAST recommendations. For antibiotics lacking recommendations, the Antibiogram Commitee of the French Society for Microbiology breakpoints were used (CA-SFM: www.sfm-microbiologie.org/). The ML phenotype was assessed by the D-test disk-diffusion method [3]. The following antimicrobials were tested: ampicillin, penicillin, cefotaxime, erythromycin, azithromycin, spiramycin, clindamycin, lincomycin, pristinamycin, quinupristin, dalfopristin, quinupristin-dalfopristin, tiamulin, tetracycline, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole, amikacin, gentamicin, kanamycin, streptomycin and tobramycin. Lincosamide inactivation was screened using Gots' test, as previously described [14].

2.2 *WGS analysis*

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen), quantified with a QuantiFluor dsDNA System (Promega Corporation, US) and was then adjusted to 0.2 ng/uL. Genome was sequenced using a 150 bp paired-end read protocol (NexteraXT kit and MiSeq, Illumina, US) at Macrogen, Inc. (Seoul, Rep. of Korea) .The quality of the sequencing was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Afterwards, reads were trimmed, duplicated reads removed and errors corrected. Finally, they were assembled with Geneious 9.1.7 (Biomatters, New Zealand).

The *emm*-type and MLST were deduced using the *emm* CDC databases for WGS (https://www2a.cdc.gov/ncidod/biotech/strepblast.asp;

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https://pubmlst.org/spyogenes/). Additional sequence analysis was performed using different available online tools (ResFinder 3.0, ICEberg and Phaster) to explore the presence of acquired resistance mechanisms and different MGEs, such as integrative conjugative elements and prophages. The genetic environment of *lsa*(E)–*lnu*(B) was studied through comparison with previously described sequences present in public databases. For the putative prophage sequences predicted by Phaster, further characterisation was achieved using BlastX. Comparisons between GAS2887HUB, sequences found to be similar based on nucleotide sequence alignments, and *lsa*(E)–*lnu*(B) structures previously described in streptococci were displayed using Easyfig program.

2.3 *Mating experiments*

S. pyogenes GAS3493HUB and *S. agalactiae* GBS4777HUB (rifampin-resistant MIC: 32 mg/L and clindamycin susceptible) were used as recipient strains for conjugation experiments performed on membrane filter, as described [15]. Transconjugants were selected using blood agar plates containing 20mg/L of rifampicin and 4mg/L of clindamycin. Mating experiments were repeated three times.

3. Results and Discussion

3.1 *Antibiotic resistance genes, susceptibility testing and molecular typing*.

The GAS2887HUB isolate was resistant to clindamycin, lincomycin, dalfopristin, tiamulin, tetracycline, amikacin, kanamycin, streptomycin and

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trimethoprim/sulfamethoxazole, but it was susceptible to the remaining antibiotics tested (Supplementary material Table S1). Gots' test was positive, proving lincosamide inactivation, whereas the D-test failed to reveal either synergy or induction events. Consistent with this, the analysis of acquired genes for antibiotic resistance (ResFinder 3.0) demonstrated the presence of *lnu*(B), *ant*(6)-Ia, *aph*(3')-III, *tet*(M) and *dfrG*. The *lsa*(E) was not detected because it is not included in the ResFinder database. Besides this, the GAS2887HUB strain harboured a Tn*916* element [*tet*(M) gene], two prophage-like sequences (P1 and P2) and a partly deleted prophage (P3) (Supplementary material Fig. S1).

The GAS2887HUB strain was *emm93* and the sequence type was ST10, a rare association. In fact, this was the unique isolate with *emm93* among nearly 500 GAS collected over a twenty-year period. Furthermore, the GAS MLST database contains only 14 ST10 isolates, of which only 3 contain *emm93* (2 isolates from India and 1 from Egypt). However, clindamycin susceptibility was not reported for any of these isolates, which meant that we could not clarify the putative clonal association of these resistance mechanisms, as with other resistance mechanisms in GAS [3].

3.2 *WGS analysis: Genetic context of lsa*(E)-*lnu*(B) *genes*

To the best of our knowledge, the genetic environment of *lsa*(E)–*lnu*(B) genes in our strain was different to that previously reported [7,8,13,16,17]**.** The *lsa*(E)–*lnu*(B) genes resided together with the 39.6 kb P2 that was inserted downstream of the *rumA* gene (Fig. 1A). This sequence was formed by a 39.6 kb prophage, followed

by an aminoglycoside-streptothricin-resistance cluster [*ant*(6)Ia–*sat4*–*aph*(3')III] and the *lsa*(E)–*lnu*(B) genes. The extremes of this composite structure were highly similar to the *Streptococcus porci* DSM 23759 prophage (67%–100% identity, average 85.8%, Acc. No. AUIP01000001, positions 1–57586) and to the *lsa*(E)–*lnu*(B) genes (99.7% identity, Acc. No AUIP01000004, positions 2690–7146). Moreover, the GAS2887HUB sequence containing from *ant*(6)Ia–*sat4*–*aph*(3')III to *lsa*(E)–*lnu*(B) was found in a different arrangement in *Erysipelothrix rhusiopathiae* Ery-11 [16] (Acc. No. KP339868, 99.1% identity, positions 11622–17508; 99.9% identity, positions 4868–9307). The *ant*(6)Ia–*sat4*–*aph*(3')III cluster shared 98.1% identity with the *S. suis* TZ080501 genome (Acc. No. KX077897, positions 51296–56201) (Fig. 1B).

Among streptococci (Fig 1C), the MGE harbouring *lsa*(E)–*lnu*(B) genes was first reported in a *S. agalactiae* structure (SGB76) also containing a multidrug resistance cluster (*aadE*–*apt*–*spw*) [8]. This combination was also described with different arrangement in *S.suis* [17,18]. A second *lsa*(E)–*lnu*(B)-containing element was described in a human *S. agalactiae* isolate (Fig 1C; 20162235) [7]. Beside this, the *lnu*(B) gene have been also found in streptococci and staphylococci with L and LS_AP phenotypes isolated from animals and some food products [18], indicating that some streptococci serve as a reservoir for antimicrobial resistance genes [17]. When comparing the characterized structures harbouring *lsa*(E)–*lnu*(B) genes with GAS2887HUB (Fig. 1C), three findings were notable [8,16,18,19]. First, the *orf4*–*orf5*–*lsa*(E)–*lnu*(B) structure was highly conserved (95% identity) among them. Second, in the previously described streptococcal structures, the

lsa(E)–*lnu*(B) genes went together with a multidrug resistance cluster (*aadE*–*apt*–*spw*). Third, the aminoglycoside-streptothricin resistance cluster [*ant*(6)Ia–*sat4*–*aph*(3')III] detected in our strain (GAS2887HUB) was found in a different arrangement in *E. rhusiopathiae.*

The presence of a prophage highly similar to the one previously found in animal isolates of *S. porci* DSM 23759 [19], and *E. rhusiopathiae* [16,20], could suggest a putative animal source for this element. It is likely that the widespread use of lincosamides to treat (or prevent) infections in animals could have favoured the selection and spread of such mechanisms of resistance. In our strain, the genomic island located downstream of the defective prophage, which contains the *ant*(6)Ia–*sat4*–*aph*(3')III–*cluster and lsa*(E)–*lnu*(B) resistance genes, could be a disrupted integrative mobilizable element. Nonetheless, it contains integration- and excision-like genes and one putative insertion sequence, but no genes required for MGE conjugation. However, no transconjugants were obtained after mating experiments.

Clindamycin is frequently used, combined with a β-lactam, for the treatment of severe skin and soft tissue infections as prevents the ribosomal synthesis of virulence factors. Furthermore, the WHO has included lincosamides and streptogramins in the list of critically important antimicrobials for the human medicine. To date clindamycin-resistance in GAS has been associated to *erm* genes responsible of the MLS_B phenotype. The description of this new genomic island encoding lincosamide-resistance in macrolide-susceptible GAS is a cause of concern and deserves surveillance of this rare resistance mechanism that could be

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underestimated if clindamycin is not routinely tested. Moreover, *lnu*(B)-*lsa*(E) confers resistance to pleuromutilinas. Among them, lefamulin is one of the eleven phase III antimicrobials included in the WHO 2017 priority list for antibacterial agents.

4. Concluding remarks

In conclusion, we have described a genomic island conferring LS_AP phenotype in a GAS similar to those found in *S. porci* and *E. rhusiopahiae*. *In vitro* mobilization of this element by conjugation was not achieved. The presence of a lincosamide inactivation gene precludes the use of lincosamides in the treatment of severe GAS infections from this pathogen. Further studies are needed to understand if this mechanism of resistance is spread among GAS.

Nucleotide sequence accession number

Sequence data were deposited in the European nucleotide archive under the accession number ERS2881644.

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Declarations

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Competing Interests: None

Ethical Approval: Not required

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Figure legends:

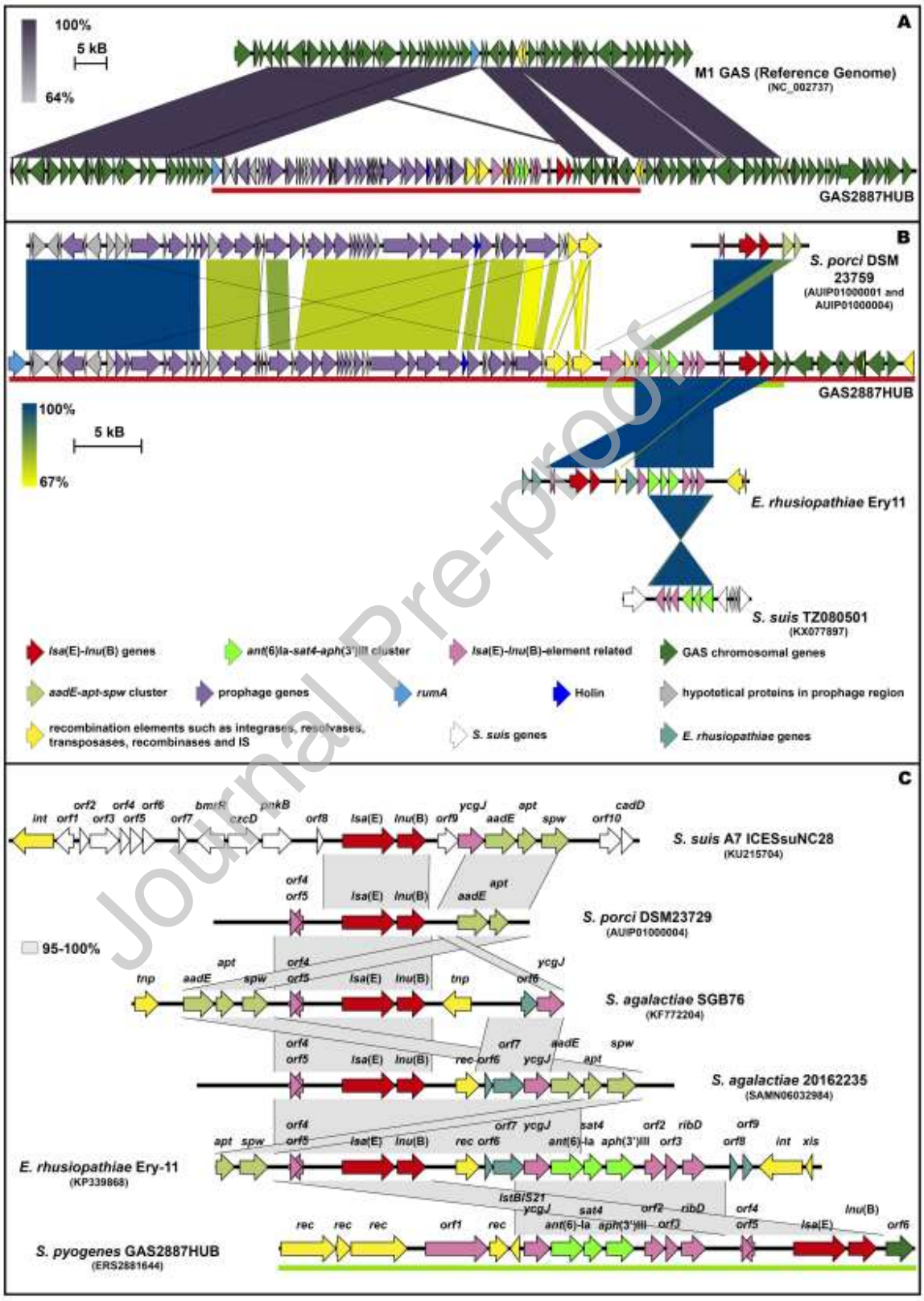

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Fig. 1. Schematic and comparative of the sequence containing *lsa*(E)–*lnu*(B). **A)** Comparison of GAS2887HUB to the M1 GAS strain (reference GAS genome: Acc. No. NC_002737 positions 1080518–1152718) showing the insertion of the sequence containing *lsa*(E)–*lnu*(B) genes together with the prophage 39.6 kb P2 downstream of *rumA* (23S rRNA (uracil-5)-methyltransferase). The purple-gradated regions show 64%–100% sequence identity, and the red line highlights the part of the GAS2887HUB sequence shown in more detail in Fig. 1B. **B)** Representation of the sequence containing *lsa*(E)–*lnu*(B) genes together with the prophage 39.6 kb P2, showing the relatedness of the prophage sequence and *lsa*(E)–*lnu*(B) genes with *S. porci* DSM 23759 [19] genome (Acc. No. AUIP01000001 positions 1–57586 and Acc. No. AUIP01000004 positions 1–8831), the different of arrangement of the *ant*(6)Ia–*sat4*–*aph*(3')III cluster and *lsa*(E)–*lnu*(B) genes in *E. rhusiopathiae* Ery-11 [16] (Acc. No. KP339868, positions 11622–17508 and 4868–9307) and the presence of the *ant*(6)Ia–*sat4*–*aph*(3')III cluster in *S. suis* TZ080501 [17] (Acc. No. KX077897 positions 46803–68299). The yellow-to-blue-shaded regions show 67%–100% sequence identity. The green line emphasises the GAS2887HUB sequence shown in more detail in Fig. 1C. **C)** Distribution of the *lsa*(E)–*lnu*(B) genes, the *ant*(6)Ia–*sat4*–*aph*(3')III cluster and the *aadE*–*apt*–*spw* cluster among the previously described structures in streptococci containing *lsa*(E)–*lnu*(B) genes [ICESsuNC28 of *S. suis* A7 [18] (Acc. No. KU2115704); *S. porci* DSM23759; *S. agalactiae* SGB76 [8] (Acc. No. KF772204); *S. agalactiae* 20162235 (SRA number SAMN06032954) [13] and *E. rhusiopathiae* Ery-11. There is a different

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arrangement to that found in GAS2887HUB. The grey regions indicate that there is up to 95% sequence identity.

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