Coexistence of $\text{bla}_{\text{OXA-58}}$ and tet(X) on a Novel Plasmid in Acinetobacter sp. From Pig in Shanghai, China

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The purpose of this study was to characterize the complete sequence of a novel plasmid carrying tigecycline resistance gene tet(X) and carbapenemase gene $\text{bla}_{\text{OXA-58}}$ from a swine Acinetobacter sp. strain SH19PTT10. Minimal inhibitory concentration (MIC) was performed using microbroth dilution method. The isolate SH19PTT10 was highly resistant (16 mg/L) to tigecycline, and also exhibited resistance to ampicillin, streptomycin, tetracycline, chloramphenicol, florfenicol, ciprofloxacin, and sulfamethoxazole/trimethoprim. Although SH19PTT10 harbored $\text{bla}_{\text{OXA-58}}$, it was susceptible to cefotaxime and meropenem. The genome sequence of SH19PTT10 was determined using PacBio single-molecule real-time sequencing. Plasmid pYUSHP10-1 had a size of 174,032 bp and showed partial homology to several plasmids found in Acinetobacter isolates. It contained two repA genes, putative toxin-antitoxin systems (HipA/HipB, RelE/RelB, and BrnT/BrnA), partitioning genes ($\text{parA}$ and $\text{parB}$), and heavy metal resistance-associated genes ($\text{copA}/\text{copB}$, $\text{nrp}$, and $\text{czcA}/\text{czcD}$) but the transfer region or proteins was not found. pYUSHP10-1 carried 16 resistance genes, mainly clustered in two mosaic multiresistance regions (MRRs). The first MRR contained $\text{sul3}$, $\text{qacI-aadA1-clmA1-aadA2-blaCARB-2-drA16}$ cassette, $\text{aac(3)-lld}$, and $\text{bla}_{\text{OXA-58}}$. The $\text{bla}_{\text{OXA-58}}$ gene was associated with ISAba3, as previously described. The second MRR is the tet(X) region (ISAcsp12-aph(3')-la-1S26-ΔxerD-tet(X)-res-ISCR2-sul2) related to the corresponding region in other tet(X)-bearing plasmids. The $\text{pdiff}$ sites, as well as mobile elements, play an important role in mobilization of DNA modules and plasmid evolution. Coexistence of numerous resistance genes on a single plasmid may contribute to the dissemination of these genes under pressure posed by different agents, which may explain the presence of clinically crucial resistance genes tet(X) and $\text{bla}_{\text{OXA-58}}$ in livestock. Thus, rational drug use and continued surveillance of tet(X) and $\text{bla}_{\text{OXA-58}}$ in livestock are warranted.

Keywords: Acinetobacter, $\text{bla}_{\text{OXA-58}}$, plasmid, tet(X), tigecycline resistance
INTRODUCTION

The genus *Acinetobacter* currently includes more than 60 species with valid species names1, and most of these are important nosocomial pathogens. Carbenamens are clinically crucial antimicrobial agents for treating multidrug-resistant Gram-negative pathogens, including *Acinetobacter* isolates (Asif et al., 2018; Rodriguez-Baño et al., 2018). The rapid increase in the prevalence of carbapenem-resistant *Acinetobacter* is mainly attributed to the acquisition of carbapenem-hydrolyzing class D β-lactamases (e.g., OXA-23, -40, -51, -58, and -143; Evans and Ameyes, 2014). OXA-58 has been detected in *Acinetobacter* isolates from patients, animals, and the environment from distinct geographical areas, particularly in clinics (Poirel et al., 2005; Fu et al., 2014; Feng et al., 2016; Klotz et al., 2017; Narciso et al., 2017; Chen et al., 2019; Matos et al., 2019; Suzuki et al., 2019). Though OXA-58 shows weak carbapenem-hydrolyzing activity, the insertion of other insertion sequence (IS) elements into the upstream ISAba3 of blaOXA-58 may provide an alternative promoter that enhances its transcription and the level of carbapenem resistance (Poirel et al., 2005; Narciso et al., 2017; Chen et al., 2019; Matos et al., 2019).

Tigecycline, belonging to the novel glycylcycline class, has been a last-resort antibiotic to treat serious infections caused by extensively drug-resistant Gram-negative bacteria, including *Acinetobacter* (Noskin, 2005; Asif et al., 2018). However, novel plasmid-mediated high-level tigecycline resistance genes *tet* (X) [former name *tet*(X3)~(X5)]2 have been identified in *Acinetobacter* isolates from animals and humans in China in 2019 (He et al., 2019; Wang et al., 2019). The emergence and dissemination of *tet*(X) will impair the efficacy of tigecycline in clinical treatment, thus would pose a significant threat to public health. The co-location of *tet*(X) and carbapenem resistance gene *bla*<sub>NDM-1</sub> was previously described in *Acinetobacter* isolates from animals (duck, goose, and cow) and the environment (soil and sewage; Cui et al., 2020; He et al., 2020). In this study, we aimed to determine and analyze the complete sequence of a single plasmid bearing *tet*(X) [formerly designated as *tet*(X3), GenBank accession no. MK134375] and *bla*<sub>OXA-58</sub> obtained from a swine *Acinetobacter* sp. strain in Shanghai, China, providing insights into the genetic structures of the plasmid and these genes.

MATERIALS AND METHODS

Bacterial Strain and *tet*(X) Detection

In September 2019, one strain SH19PTT10 was isolated from the feces sample of a pig by Tryptic Soy Agar plate containing tigecycline (2 mg/L) from a pig farm located in Shanghai, China and was identified using 16S ribosomal RNA (rRNA) gene sequencing (Kim et al., 2010). The presence of *tet*(X) [former name *tet*(X3), *tet*(X4), and *tet*(X5)] was detected by PCR and sequencing (He et al., 2019; Wang et al., 2019).

Antimicrobial Susceptibility Testing

The isolate SH19PTT10 was tested for minimal inhibitory concentrations (MICs) of ampicillin, cefotaxime, meropenem, amikacin, streptomycin, tetracycline, minocycline, tigecycline, chloramphenicol, florfencil, ciprofloxacin, colistin, and sulfamethoxazole/trimethoprim using microbroth dilution method recommended by the guidelines of the Clinical and Laboratory Standards Institute [CLSI, Wayne, PA, United States; Clinical and Laboratory Standards Institute (CLSI), 2012]. The results were interpreted according to CLSI M100, 28th edition [Clinical and Laboratory Standards Institute (CLSI), 2018], Tigecycline (≥1 mg/L), streptomycin (≥32 mg/L), and florfencil (≥32 mg/L) were interpreted according to the clinical breakpoint or epidemiological cutoff values for *Escherichia coli* set by EUCAST3. The *E. coli* strain ATCC 25922 was used for quality control.

Conjugation/Transformation Experiments

Conjugation experiments were performed using streptomycin-resistant *E. coli* C600 as the recipient strain, as previously described (Chen et al., 2007). Transconjugants were selected using 2 mg/L tigecycline and 3,000 mg/L streptomycin. Transformation was carried out by heat-shock and electroporation using *E. coli* DH5α and *Acinetobacter baumannii* ATCC 19606. Transformants were selected by 2 mg/L tigecycline.

Whole Genome Sequencing and Analysis

The whole genome of SH19PTT10 was extracted and sequenced using PacBio single-molecule real-time sequencing (RSI platform, Pacific Biosciences, Menlo Park, CA, United States). Raw sequence data were introduced into the non-hybrid hierarchical genome assembly process (HGAP version 4). The 16S rRNA gene sequences of SH19PTT10 and other representatives across the genus *Acinetobacter* were aligned using ClustalW, and a phylogenetic tree was constructed by the neighbor joining algorithm using MEGA 7.0 (Kumar et al., 2008). The plasmid sequence was analyzed and annotated using the RAST server (Aziz et al., 2008), ResFinder 3.2 (Zankari et al., 2012), ISfinder (Siguer et al., 2006), PlasmidFinder (Carattoli et al., 2014), BLAST4, and Gene Construction Kit 4.5 (Textco BioSoftware, Inc., Raleigh, NC, United States). The replicate genes (rep) of plasmids from SH19PTT10 were assigned to a group according to the typing scheme for plasmids in *A. baumannii* (Bertini et al., 2010). Plasmid pYUSHP10-1 was compared with other plasmids using BLASTn and BRIG (Alikhan et al., 2011).

Nucleotide Sequence Accession Number

The complete sequence of pYUSHP10-1 was deposited in GenBank under the accession number MT107270.

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1https://apps.xzu.cz/anemec/Classification.pdf
2According to the standards of the nomenclature center (http://faculty.washington.edu/marilynr/), all *tet* (X) genes [former name *tet*(X1)-*tet*(X5)] at present can only be designated as *tet*(X).
3https://www.eucast.org
4http://blast.ncbi.nlm.nih.gov/Blast.cgi
RESULTS AND DISCUSSION

tet(X)-Encoding Acinetobacter sp. Strain SH19PTT10

The strain SH19PTT10 was positive for tet(X) [formerly designated as tet(X3), MK134375] and was highly resistant (16 mg/L) to tigecycline. SH19PTT10 also showed resistance to ampicillin, streptomycin, tetracycline, chloramphenicol, florfenicol, ciprofloxacin, and sulfamethoxazole/trimethoprim but was susceptible to cefotaxime, meropenem, amikacin, minocycline, and colistin (Table 1). The 16S rRNA gene sequencing showed SH19PTT10 belonging to Acinetobacter but not to any described species, thus whole genome sequencing (WGS) was further performed.

SH19PTT10 consisted of a 3,440,498-bp chromosome and five plasmids designated as pYUSHP10-1 to pYUSHP10-5, ranging from 5.7 to 174 kb (Supplementary Table S1). Among them, pYUSHP10-1, the largest plasmid found in SH19PTT10, carried numerous resistance genes, particularly tigecycline resistance gene tet(X) and carbapenemase gene blaOXA-58; pYUSHP10-3 also carried resistance gene tet(39) (Supplementary Table S1). The tet(X)-carrying plasmid pYUSHP10-1 was failed to transfer to E. coli C600/DH5α by conjugation/transformation or A. baumannii ATCC 19606 by transformation. The complete sequence of the 16S rRNA gene exhibited 98.76% identity (1515/1534) with A. cumulans strain WCHAc060092 (CP035934) and 98.37% identity (1509/1534) with Acinetobacter haemolyticus strains AN54 (CP041224). The neighbor-joining tree based on partial 16S rRNA gene sequences within Acinetobacter suggested that the isolate SH19PTT10 may take a distinct position within the genus; however, the branch support for SH19PTT10 was low (bootstrap value 36%; Supplementary Figure S1).

The Backbone of tet(X)- and blaOXA-58-Bearing Plasmid pYUSHP10-1

The plasmid pYUSHP10-1 had a size of 174,032 bp with a 41.32% GC content; it could not be assigned to any previously known incompatibility group. pYUSHP10-1 showed partial homology to several plasmids obtained from Acinetobacter isolates, such as OXA-58-encoding plasmids p225n_1 (KT852971, A. baumannii, patient, Vietnam), pC54_001 (CP042365, Acinetobacter pittii, patient, Australia), and p19110F47-2 (CP046044, Acinetobacter towncri, pig, China), as well as tet(X)- carrying plasmids p34AB (MK134375, A. baumannii, swine, China) and pA101 (CP044019, Acinetobacter indicus, feces, China; 29–41% coverage, 97.3–99.9% nucleotide identity; Figure 1).

The plasmid pYUSHP10-1 contained two repA genes, both of them belonged to the Rep_3 type family (pfam01051). The first repA (positions 1–1068) shared highly similarity (>99.9%) with those of tigecycline-resistant plasmids p18TQ-X (CP045132, 1067/1068) and pAB17H194-1 (CP040912, 1067/1068) obtained from Acinetobacter strains in China. The second repA (positions 92451–93302) was identical to those of plasmids pAHTJRI (CP0388010, A. haemolyticus, patient) and pBXI1-9 (CP010351, Acinetobacter johnsonii, hospital sewage; Feng et al., 2016) in China. In addition, pYUSHP10-1 harbored multiple putative toxin-antitoxin systems, such as Hpa/HipB, RelE/RelB, and BrnT/BrnA, and the partitioning genes (parA and parB), which may ensure plasmid maintenance at cell division (Zielenkiewicz and Cegłowski, 2001). However, the transfer region or proteins for plasmid mobilization was not found in pYUSHP10-1. Putative part of the BREX system (brxC-pglX-pglZ-brxL) was identified in plasmid pYUSHP10-1 (Figure 1), which has been reported as a novel plage defense system which confers resistance to a broad range of phages (Goldfarb et al., 2015). Similar structure was also found in plasmids from Acinetobacter, such as pOXA58_010062 (CP033131, Acinetobacter wuhouensis, sewage, China) with 91% coverage and 96.21% identity and pA101 shared 93% coverage and 93.93% identity. Additionally, heavy metal resistance-associated genes were also detected in pYUSHP10-1, such as copA/copB (copper resistance), nrr (nickel resistance), and czaA/czcD (cobalt-zinc-cadmium resistance; Figure 1).

The pdfI Sites of pYUSHP10-1

Recently, numerous plasmids from various Acinetobacter species have been identified to contain 28-bp pdfI site, consisting of 11-bp inversely-oriented binding sites for the XerC and XerD recombinases separated by a spacer of 6 bp (Supplementary Table S2; D’Andrea et al., 2009; Merino et al., 2010; Blackwell and Hall, 2017; Cameranesi et al., 2018). We also found 17 pdfI sites on pYUSHP10-1 (Figure 1; Supplementary Tables S2 and S3). The modules flanked by inversely-oriented pdfI sites either with the XerC sites internal (D/C and C/D) or the XerD sites internal (C/D and D/C) are identified as pdfI module and may be able to mobilize mediated by pdfI sites using XerC-XerD recombination system (D’Andrea et al., 2009; Merino et al., 2010; Blackwell and Hall, 2017; Cameranesi et al., 2018). For example, one module in pYUSHP10-1 consisting of IS40换届-like and putative toxin-antitoxin system BrnT/BrnA, was surrounded by inversely-oriented pdfI sites (XerD/C and XerC/D; Supplementary Figure S2). This pdfI module was adjacent to a hypothetical protein and a further dif module carrying another putative toxin-antitoxin system encoding ParE toxin and helix-turn-helix family protein (Supplementary Figure S2). Interestingly, the pdfI sites flanking two dif modules were also

| Antimicrobial agents | MIC (mg/L) | Interpretation |
|---------------------|-----------|---------------|
| ampicillin          | >128      | R             |
| cefotaxime          | 4         | S             |
| meropenem           | 0.5       | S             |
| amikacin            | 1         | S             |
| streptomycin        | >128      | R             |
| tetracycline        | >128      | R             |
| minocycline         | ≤2        | S             |
| tigecycline         | 16        | R             |
| chloramphenicol     | 128       | R             |
| florfenicol         | 128       | R             |
| ciprofloxacin       | >8        | S             |
| colistin            | 0.25      | R             |
| sulfamethoxazole/trimethoprim | >32 | R |
surrounding the hypothetical protein in the opposite orientation, making it being a putative dif module that may be mobile (Supplementary Figure S2). Different dif module combinations were found in some Acinetobacter plasmids such as pABIR (EU294228), pJ9-3 (CP041590), and pM131-2 (JX101647), indicating that the presence of the dif sites facilitated the mobilization of discrete DNA segment through multiple events (Supplementary Figure S2). Additional dif modules identified in pYUSHP10-1 mostly include toxin-antitoxin systems, and copA/ copB were also found in dif module (Figure 1).

**pYUSHP10-1 Carrying Resistance Genes and dif Modules**

pYUSHP10-1 contained 16 resistance genes, which were mainly clustered in two mosaic multiresistance regions (MRRs; Figure 1). The first MRR carried an approximately 32.2-kb segment consisting of three parts. The first of these (~11.6 kb) was bounded at both ends by IS26 and comprised a truncated mefB (encoding macrolide efflux protein), sul3 (sulfonamide resistance), and an incomplete class 1 integron with ΔintI1 and the gacI-aadA1-cmaA1-aadA2-blaCARB-2-dfrA16 cassette array (Figure 2). This fragment showed 99.9% identity to those of plasmids from E. coli such as pMB5876 (MK070495) and pMRSN346355_67.9 (CP018123), suggesting that pYUSHP10-1 may capture this segment from E. coli plasmids by IS26-mediated transposition or homologous recombination (Figure 2).

The second part (3,296 bp) contained an open reading frame (ORF) encoding AAA family ATPase, aminoglycoside resistance gene aac(3)-IId, and mobile element ISAb3 (Figure 2). It was highly similar (>99%) to the corresponding regions of multiple plasmids found in Acinetobacter and Enterobacteriaceae isolates, such as Acinetobacter plasmids p225n_1 and pC54_001 and E. coli plasmid pEC2-NDM-3 (KC999035). Interestingly, the pI1 site adjacent to ISAb14 and the pI1 site upstream of IS26 were in inverse orientation, suggesting that the acquisition of ~16.5 kb segment including two hypothetical proteins and the first and second parts of MRR I in pYUSHP10-1 was possibly mediated by the pI1 sites via site-specific recombination (Figure 2).

The last part of MRR I (17,301 bp) was highly similar to those of Acinetobacter plasmids p225n_1 (one single nucleotide polymorphism), p19110F47-2, pC54_001, and pAP43-OXA58-NDM1 with additional deletions (Figure 2). The primary component of this fragment is the blaOXA-58 region. As previously described (Fu et al., 2014; Feng et al., 2016; Klotz et al., 2017), blaOXA-58 was flanked by two copies of ISAb3 with opposite orientation, although the upstream ISAb3 was incomplete in
pYUSHP10-1 (Figure 2). OXA-58 shows weak activity against the carbapenems and is unable to hydrolyze some cephalosporins such as ceftazidime and cefotaxime (Poirel et al., 2005). The insertion of other IS elements, such as IS\textsubscript{Aba825}, IS\textsubscript{Ou1}, IS\textsubscript{1008}, and IS\textsubscript{1006} into the upstream IS\textsubscript{Aba3} may provide a promoter to enhance the expression of OXA-58 and leading to the carbapenem resistance (Fu et al., 2014; Narciso et al., 2017; Chen et al., 2019; Matos et al., 2019). In this study, we did not observe insertion of IS element into IS\textsubscript{Aba3}, suggesting the lacking of a putative promoter for \textit{bla}\textsubscript{OXA-58} overexpression, thus the strain SH19PTT10 showed susceptibility to cefotaxime (4 mg/L) and meropenem (0.5 mg/L).

The \textit{bla}\textsubscript{OXA-58} region has been frequently bracketed by two Re27 sequences, recognized as \textit{pdf} sites later (D’Andrea et al., 2009), which have been shown to mediate the mobilization of \textit{bla}\textsubscript{OXA-58} (Poirel and Nordmann, 2006). In this study, two \textit{pdf} sites in inverse orientation (XerD-XerC and XerC-XerD) were identified flanking the 2,257-bp segment containing IS\textsubscript{Aba3}-\textit{bla}\textsubscript{OXA-58}-ΔIS\textsubscript{Aba3} arrangement in pYUSHP10-1, as observed in numerous \textit{Acinetobacter} plasmids (Figure 2). The downstream IS\textsubscript{Aba3} was commonly followed by the putative transcriptional regulator gene \textit{araC1} and threonine efflux protein gene \textit{lysE} in \textit{Acinetobacter} isolates (Figure 2; Fu et al., 2014; Feng et al., 2016; Klotz et al., 2017; Matos et al., 2019). An additional \textit{pdf} site was found adjacent to \textit{lysE} (Figure 2), which may account for the mobilization of this segment together with the \textit{bla}\textsubscript{OXA-58} \textit{pdf} module. However, the conserved structure \textit{araC1}-\textit{lysE} was not found in pYUSHP10-1. A truncated \textit{araC1} was identified in p225n\textsubscript{1}, pAP43-OXA58-NDM1, and pC54\textsubscript{001}, possibly due to homologous recombination between \textit{araC1} and the downstream putative gene encoding site-specific integrase, which shared a sequence of 6 bp (TAAGTT) that might be the site of recombination (Figure 2). The same Δ\textit{araC1} was also observed in p19110F47-2, which truncated by an incomplete IS\textsubscript{Aba20} (Figure 2).

The genetic contexts downstream of IS\textsubscript{Aba3}-\textit{bla}\textsubscript{OXA-58}-ΔIS\textsubscript{Aba3} is diverse in \textit{Acinetobacter} (Figure 2; Fu et al., 2014; Feng et al., 2016; Klotz et al., 2017; Matos et al., 2019). In pYUSHP10-1, several putative ORFs encoding two-component regulatory system AdeR/AdeS, RND family efflux transporters, LysE family protein, and \textit{araC} family transcriptional regulator were identified (Figure 2). In addition, a ~5.1 kb segment was further present downstream consisting of sulfonamide resistance gene \textit{sul2} and several intact or truncated insertion sequences, including IS\textsubscript{1008}, IS\textsubscript{Aba1}, IS\textsubscript{CR2}, and IS\textsubscript{1006} (Figure 2). Two \textit{pdf} sites in inverse orientation (XerD-XerC and XerC-XerD) were found within this segment to create a \textit{dif} module (Figure 2). The first one disclosed an additional \textit{adeR/adeS} \textit{dif} module with the \textit{pdf} site downstream of ΔIS\textsubscript{Aba3}, which may readily explain its co-transfer with the \textit{bla}\textsubscript{OXA-58} \textit{dif} module in several plasmids, e.g., p19110F47-2 (Figure 2). The structure IS\textsubscript{Aba1}-\textit{sul2}-ΔIS\textsubscript{CR2}-IS\textsubscript{1006} was also observed in many plasmids found in \textit{Acinetobacter} and \textit{Enterobacteriaceae} isolates, such as
The generation of a circular molecule [ISCR2-xerD-tet(X)-res] by recombination between the two copies of ISCR2 in the same orientation could lead to the insertion of the tet(X) module (He et al., 2019). Although two copies of ISCR2 were only intact in p34AB, the formation of different but related tet(X) structures may have resulted from additional molecular events mediated by mobile elements (e.g., IS26 and Tn5393) via transposition or homologous recombination and have the potential to evolve diverse tet(X) genetic contexts.

pYUSHP10-1 also carried other resistance genes, including macrolide resistance genes mph(E)/msr(E), florfenicol resistance gene floR, and streptomycin resistance genes strA/strB (Figure 1). As previously described (Blackwell and Hall, 2017), a 2,950-bp segment including the macrolide resistance genes mph(E)/msr(E) was surrounded by two pdfif sites in inverse orientation (Figure 1), further suggesting that the pdfif sites may mediate mph(E)/msr(E) mobilization in Acinetobacter plasmids. Furthermore, many intact or truncated insertion sequences were present in the backbone of pYUSHP10-1, such as ISaba14 (Figure 1). These IS elements may enable this plasmid to capture more genes and evolve through IS-mediated recombination events.

**CONCLUSION**

We report the isolation and genetic characterization of an Acinetobacter sp. strain exhibited multiresistance phenotype, due to the acquisition of a novel plasmid carrying 16 resistance genes, including tigecycline resistance gene tet(X) and carbapenemase gene blaOXA-58. The Acinetobacter sp. may serve as an important reservoir of antimicrobial resistance genes. Coexistence of numerous resistance genes on a single plasmid may facilitate its dissemination and persistence under different selection pressure, which may explain the presence of clinically crucial antibiotic resistance genes tet(X) and blaOXA-58 in livestock. Additionally, at least two different mechanisms, site-specific
recombination via the pdf sites and transposition of mobile elements (e.g., ISCR2 and IS26) could account for the acquisition of DNA modules containing resistance structures and/or other genes in pYUSHP10-1. It highlights the ability of resistance structures to be captured by multiple events and their capacity to evolve during horizontal transfer.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

Z-MP, XJ, and JW conceived the study. YW, HW, P-CS, JW, Y-QT, and FS carried out the experiments. JW and Z-YW analyzed the data. JW wrote the manuscript. Z-MP and XJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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FUNDING

This work was supported by National Natural Science Foundation of China (grant number 31902319), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and grant supported by Ministry of Agriculture and Rural Affairs, China (no. 14162130110239004).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/article/10.3389/fmicb.2020.578020/full#supplementary-material
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Conflict of Interest: 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.

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