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Original Article

Evolution of trimethoprim/sulfamethoxazole resistance in *Shewanella algae* from the perspective of comparative genomics and global phylogenic analysis

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Abstract *Objective: Shewanella algae* is a zoonotic marine bacterium that causes a variety of infections in immunocompromised patients or those who have been exposed to seawater. The development of trimethoprim/sulfamethoxazole (TMP/SMX) resistance in *S. algae* are found in human and environment isolates during the past ten years, and thus the treatment options are decreasing.
Methodology: In the study, we conduct a comparative genomic study to identify the resistant mechanism of TMP/SMX-resistance in *S. algae*.
Results: We found the resistance of TMP/SMX in *S. algae* is associated with the existence of *sul1* and *dfrA12* within the class 1 integron. The gene cassette *dfra12-aadA2-qacEΔ1/sul1* within the class 1 integron is highly conserved. In addition, the class 1 integron and encapsulated *sul1* are significantly enriched in Enterobacteriaceae in NCBI and UniProt databases.

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Conclusion: Our study suggests that the horizontal transfer of TMP/SMX resistance via class 1 integron is most frequently occurred within Enterobacteriaceae and has spread to a wide range of sources including soil, poultry, and marine water.

Introduction

Shewanella algae is a gram-negative, rod-shaped, non-fermentative, marine bacterium, that can cause skin and soft tissue infection, otitis, pneumonia, with or without bacteremia. A higher risk of *S. algae* infection is present for people who have had seawater exposure, consume raw seafood, or have an impaired immune system.[1–3] *S. algae* induce hemolysis at 25 °C and 37 °C, which may be an important factor in its pathogenicity.[2] Antimicrobial therapy combined with surgical or drainage treatment is the mainstream approach in the treatment of *Shewanella* infection.[4] In general, *S. algae* are susceptible to aminoglycosides, carbapenems, erythromycin, and quinolones but resistant to penicillin.[3,4] *S. algae* with TMP/SMX resistance have been found in humans and the environment over the past decade.[3,5–7] A similar trend was noted in Taiwan (CL. Shyu, personal communication, July 24, 2020). The resistance of TMP/SMX further limited the treatment choice.

Resistance to sulfonamides develops through intrinsic chromosomal gene mutation or acquired drug-resistant gene.[8] Currently, a number of mechanisms had been reported to be associated with sulfonamide resistance in different bacteria. In Europe and Canada, *Escherichia coli*, which carried *sul1*, *sul2*, or *sul3* genes, produced insensitive forms of dihydropteroate synthase (DHPS).[9] Point mutations in the *folP* gene may lead to structural change in dihydropteroate synthetase. For example, the presence of *folP* gene mutations in *Campylobacter jejuni* results in four amino acid substitutions and allows for the resistance of sulfonamides.[10] F28L/I and P64S mutations in DHPS may affect the P-aminobenzoic acid (PABA) binding site in *E. coli*.[11,12] P-aminobenzoic acid (PABA) hyperproduction, found in *Neisseria* and *Staphylococcus aureus*, reduced the inhibitory action of the sulfonamides.[13] Currently, little is known about the mechanism of TMP/SMX-resistance in *S. algae*.

Comparative genomics is introduced and used to discover antibiotic resistance genes.[8,14] To identify the genetic background of TMP/SMX-resistance in *S. algae*, we conduct a comparative genomics investigation. In addition, to better know the evolution and distribution of genetic determinants of TMP/SMX-resistance, we further performed an integrative computational analysis.

Materials and methods

Isolates and antimicrobial susceptibility testing

A total of 24 *S. algae* isolates collected from various sources were used for the study (Supplementary Table 1). Isolates were frozen with Luria Bertani (LB) broth containing 30% V/V glycerol and stored at −80 °C until required. We used Vitek 2 system (bioMérieux, Inc., Durham, NC, USA) to identify the initial species by the manufacturer's instructions. The minimum inhibitory concentration (MIC) values for TMP/SMX were determined by Vitek 2 (bioMérieux, Inc., Durham, NC, USA). As quality controls, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were utilized. Susceptibilities were determined using a Clinical and Laboratory Standards Institute-established criteria (CLSI M100-S29).

DNA extraction, sequencing, and assembly of genomes

The manufacturer's instructions for extraction of Genomic DNA were followed using the kit QIAGEN Genomic-tip 100/G and by the DNA Buffer Set Genomic DNA (QIAGEN, Paisley, UK). The production of DNA libraries and genomic sequencing were carried out as previously reported.[15] Ultrasonication was used to fragment the genomic DNA by Covaris S2 (Covaris). The indexed PCR-free library was built using the multiplexed high-throughput sequencing TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). On a MiSeq platform, whole-genome shotgun sequencing was carried out using 2 × 250 bp paired-end sequencing (Illumina, San Diego, CA, USA). To obtain *de novo* assemblies, we used ALLPATHS, version v. R46652,[16] and Velvet, version 1.2.07[17] (See Supplementary Table 2 for summary statistics for assembly). Whole-genome phylogeny was performed by the pairwise comparison of average nucleotide identity.[18]

Identification of trimethoprim/sulfamethoxazole resistance determinants

A multi-database approach with the Comprehensive Antibiotic Resistance Database[19] and ResFinder 3.1[20] was used to identify candidate TMP/SMX resistance genes. Queries with the best hits were validated using the Integrated Microbial Genomes & Microbiomes pipeline.[21] All search results were manually curated to assure the consistency of annotations among different databases. The NCBI nomenclature would be chosen if there were competing nomenclatures.

Assess the prevalence of sul1 genes among organisms

The protein-coding sequence of *sul1* of *S. algae* CLS1 is aligned against 80,410,975 bacterial proteins in UniProt

using BLASTP. By requiring alignment identity 100% and alignment coverage of at least 70%, 403 proteins highly similar to *sul1* in *S. algae* CLS1 were uncovered in different organisms. The isolation source of each organism (e.g., human, seawater) was further extracted from the source feature field in the UniProt database. The remaining organisms without isolation source attributes were manually annotated according to publication or literature search. The isolation distribution of 403 *sul1* genes is compared with that of 80,410,975 UniProt proteins using the Chi-square test ($p < 0.05$).

Distribution of class 1 integrons among bacterial organisms

The entire sequence of class 1 integron from *S. algae* CLS1 is extracted and aligned against NCBI nr database using blastn, requiring alignment identity at least 99% and coverage at least 95%. 211 class 1 integrons were identified and their corresponding organisms were retrieved. The NCBI nucleotide database was retrieved from BLAST ftp (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/). The background distribution of families was constructed by randomly sampling 1,000 sequences from the nucleotide database. The organism distribution of class 1 integrons is compared with background distribution in the NCBI database using the Chi–Square test. The synteny plot of class 1 integrons of multiple organisms is done by multiple sequence alignment using MEGA.

Results

Genome sequencing and assembly

The sequencing used 250 bp paired-end reads and produced results ranging from 6.14 Gbps to 21.77 Gbps for each isolate. The *de novo* assembling of genome sequencing data indicated that there were 27–159 contigs for each genome (>200 bp). The maximum contig size among the genomes was 502 bp aligned to 1,200,621. The GC content ranged from 52.24 for MSW to 53.11 for JFL. A summary of the genomic features is shown in Supplementary Table 3. Whole-genome phylogeny using average nucleotide identity of the 24 isolates further revealed substantial genomic diversity of the species (Supplementary Fig. 1).

Comparative genomics reveals trimethoprim/sulfamethoxazole resistance determinants

Isolate-specific antimicrobial susceptibilities to TMP/SMX are shown in Table 1. *S. algae* CLS1 was resistant to TMP/SMX. All other isolates were susceptible to TMP/SMX. MIC of all TMP/SMX-susceptible isolates was ≤20 μg/mL except 2 isolates, *S. algae* CHL and CLS4, which had a MIC of 40 μg/mL. The TMP/SMX resistance determinant *sul1*, *sul2*, and *dfrA12* were identified in TMP/SMX-resistant *S. algae* CLS1 (Table 1). Among 23 TMP/SMX-susceptible isolates, 4.3% (1/23) carried the *sul2* gene. We were unable to detect *sul1* and *dfrA12* in any of the TMP/SMX-susceptible strains. Besides, no mutation was found in the *folP*, *dhfrIII*, which could play a role in imparting resistance to TMP/SMX. Consequently, *sul1* and *dfrA12* are the major determinants of TMP/SMX resistance in *S. algae* CLS1.

Trimethoprim/sulfamethoxazole resistance genes are linked with a mobile genetic element

Upstream and downstream analysis of the sequences surrounding the *sul1* and *dfrA*12 genes in *S. algae* CLS1 revealed the presence of class 1 integron, indicating the possibility of horizontal transfer of this gene (Fig. 1). Apart from these, the aminoglycoside adenyltransferase gene *aadA2* was also found. We could not find Class 1 integron in other *sul1* and *dfrA12* negative *Shewanella* genomes. All 23 *sul1* and *dfrA12* negative isolates were also *intl1* negatives. Comparative analysis of the gene casette indicates that the integron structure is highly conserved among *Acinetobacter baumannii* WCHAB005078, *Aeromonas* sp. ASNIH5, *Edwardsiella tarda* FL95-01, and *E. coli* QD 1-5-9 (Fig. 1). In addition, the sequence identity of these integrons are extremely high (>99%), implying they are recently horizontally transferred and/or highly conservated.

Significant enrichment of sul1 in Enterobacteriaceae

To investigate the distribution of Sul1 in bacterial organisms, we align the protein sequence of *S. algae* CLS1 Sul1 against 120,801,841 proteins in UniProt (see Method). 403 proteins identical to *S. algae* CLS1 Sul1 were identified and their isolated organisms were retrieved. We found Sul1 is largely isolated from Enterobacteriaceae (46%), followed by Pseudomonadaceae (9%) and *Moraxellaceae* (8%) (Fig. 2a). In comparison with the background distribution (i.e., isolation sources of all proteins in UniProt) (Fig. 2b), Sul1 is significantly enriched in Enterobacteriaceae (46% vs 4%, $P < 0.001$). Statistically significant differences were observed when comparing the distribution of *sul1* genes among isolates of different origins (Fig. 2c). Isolates of human origin are more often contained *sul1* compared to other origins.

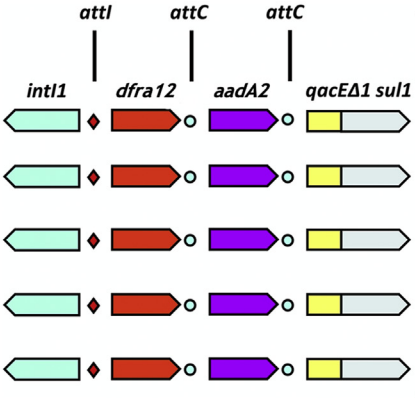
Distribution of class 1 integrons

As *sul1* is contained within and transferred by the class 1 integron in *S. algae* CLS1, we further investigate the distribution of organisms containing the same class 1 integrons. We observed structure of *S. algae* CLS1 class 1 integron is highly conserved (in terms of both structure and identity) across a variety of species. The entire class 1 integron sequence from *S. algae* CLS1 is extracted and blasted against all bacterial sequences in NCBI (see Method). 211 highly-conserved class 1 integrons (>99% identity) were identified and their organism distribution is plotted (Fig. 3). Similar to Sul1, 93.83% of class 1 integron fall within Enterobacteriaceae, which is followed by *Aeromonadaceae* (2.36%). Compared with the background distribution in the NCBI nucleotide database, class 1 integron is indeed significantly enriched in Enterobacteriaceae.

Table 1 Genes associated with TMP/SMX resistance identified in the study.

Strain	Classification	MIC of TMP/SMX (μg/mL) (interpretation)	Genes associated with TMP/SMX resistance (NCBI locus tags)						
			sul1	*sul2*	*dfrA12*	*folP*	*dhfrIII*	*intI1*	*aadA2*
S. algae CLS1	Clinical	≥320, R	AYI72_20315	AYI72_11570	AYI72_20330	AYI72_09440	AYI72_14685	AYI72_20335	AYI72_20325
S. algae CHL	Clinical	40, S	—	AYI82_21890	—	AYI82_10710	AYI82_19110	—	—
S. algae AC	Non-Clinical	≤20, S	—	—	—	AYI85_12335	AYI85_05225	—	—
S. algae ACCC	Clinical	≤20, S	—	—	—	AYI77_15160	AYI77_06240	—	—
S. algae CLS2	Clinical	≤20, S	—	—	—	AYI98_04335	AYI98_15920	—	—
S. algae CLS3	Clinical	≤20, S	—	—	—	AYJ00_16515	AYJ00_14540	—	—
S. algae CLS4	Clinical	40, S	—	—	—	AYJ01_17535	AYJ01_16305	—	—
S. algae CLS5	Clinical	≤20, S	—	—	—	AYJ02_18415	AYJ02_00485	—	—
S. algae JFC1	Non-Clinical	≤20, S	—	—	—	AYI74_16335	AYI74_13580	—	—
S. algae JFC2	Non-Clinical	≤20, S	—	—	—	AYI75_09040	AYI75_14000	—	—
S. algae JFC3	Non-Clinical	≤20, S	—	—	—	AYI76_05840	AYI76_19435	—	—
S. algae JFL	Clinical	≤20, S	—	—	—	AYI86_06815	AYI86_17685	—	—
S. algae melkephyllucas	Clinical	≤20, S	—	—	—	AYI84_13595	AYI84_18195	—	—
S. algae MSW	Non-Clinical	≤20, S	—	—	—	AYI96_13845	AYI96_10810	—	—
S. algae RC	Clinical	≤20, S	—	—	—	AYI78_14260	AYI78_18865	—	—
S. algae SYT1	Non-Clinical	≤20, S	—	—	—	AYI83_12235	AYI83_14210	—	—
S. algae SYT2	Non-Clinical	≤20, S	—	—	—	AYI94_16465	AYI94_14890	—	—
S. algae SYT3	Non-Clinical	≤20, S	—	—	—	AYI99_07305	AYI99_14950	—	—
S. algae SYT4	Non-Clinical	≤20, S	—	—	—	AYI88_16560	AYI88_15185	—	—
S. algae TYL	Clinical	≤20, S	—	—	—	AYI73_02230	AYI73_14745	—	—
S. algae YHL	Clinical	≤20, S	—	—	—	AYI97_07815	AYI97_01460	—	—
S. algae SYC	Clinical	≤20, S	—	—	—	AYI81_13625	AYI81_11870	—	—
S. algae YTH	Clinical	≤20, S	—	—	—	AYI79_13550	AYI79_18860	—	—
S. algae YTL	Clinical	≤20, S	—	—	—	AYI80_10465	AYI80_18910	—	—

TMP/SMX, trimethoprim/sulfamethoxazole; R, resistant; S, susceptible.



Source	Species	Strain	Accession Number
Human	*Shewanella algae*	CLS1	LTBI00000000
Human	*Acinetobacter baumannii*	WCHAB005078	NZ_CP027246
Waste water	*Aeromonas sp.*	ASNIH5	NZ_CP026122
Fresh water	*Edwardsiella tarda*	FL95-01	NZ_CP011359
Bos taurus	*Escherichia coli*	QD1-5-9	LC318057

Figure 1. Comparison of class 1 integrons of five bacterial genomes isolated from human, cattle, and environment: *S. algae* CLS1, *A. baumannii* WCHAB005078, *Aeromonas sp.* ASHIN5, *E. tarda* FL95-01, and *E. coli* QD1-5-9. The structure of these integrons are not only highly conserved but also embed with the same genes, including *dfrA12*, *aadA2*, and *sul1*.

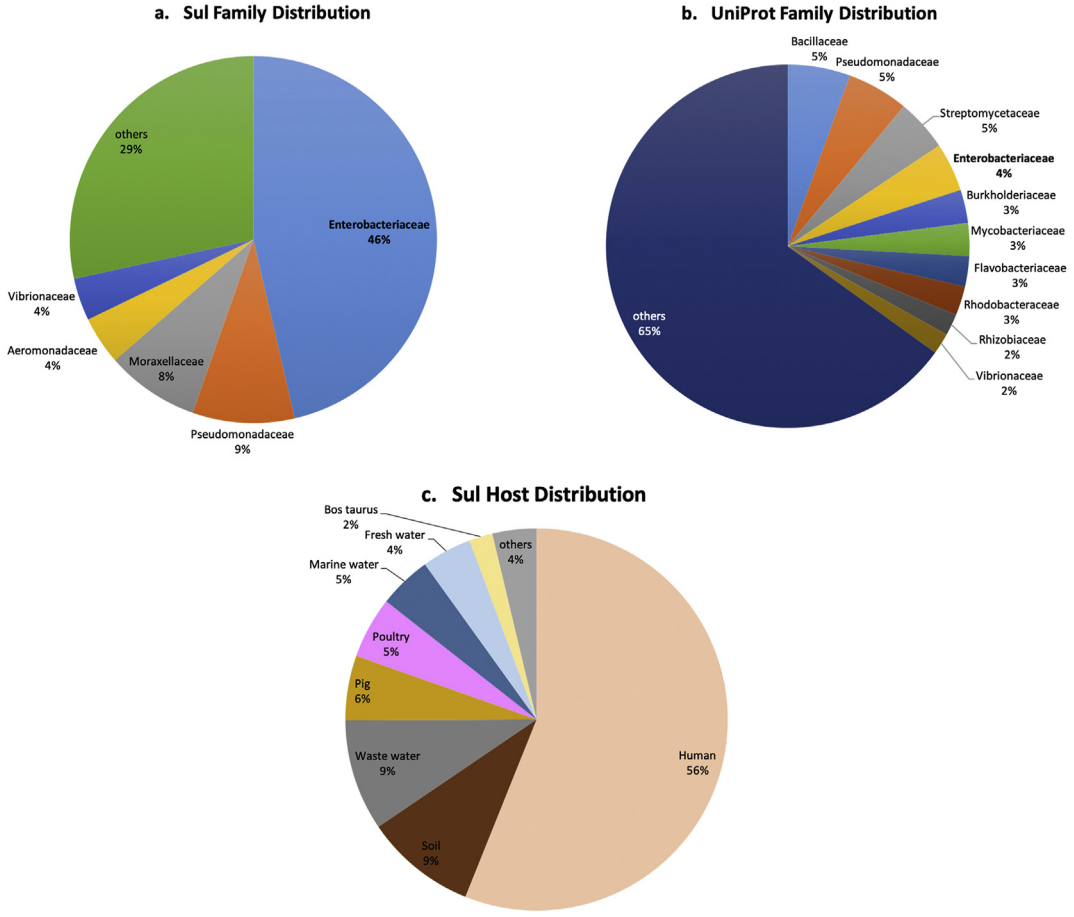


Figure 2. (a) The family distribution of bacterial species containing *sul1* in the UniProt database. Enterobacteriaceae is the most frequent family (46%), which is followed by Pseudomonadaceae (9%); (b) The background family distributions (i.e., with or without *sul1*) in the UniProt database. The Enterobacteriaceae family occupies only 4%, which is significantly lower than that containing *sul1* (46% vs 4%, $P < 0.001$); (c) The frequency of hosts containing *sul1*. Although human are the dominant host of *sul1*, the gene also appears within a variety of sources from marine water, waste water, soil, to poultry.

Discussion

In the study, we identified the association of *sul1*, *sul2*, and *dfrA12* with high-level resistance to TMP/SMX, as evident as *sul1* and *dfrA12* only present in a TMP/SMX-resistant *S. algae* strain as compared to the non-resistant strains. There was no mutation of the *folP* or *dhfrIII* in all isolates. The exist of *sul1* combined with *dfrA12* may contribute to the

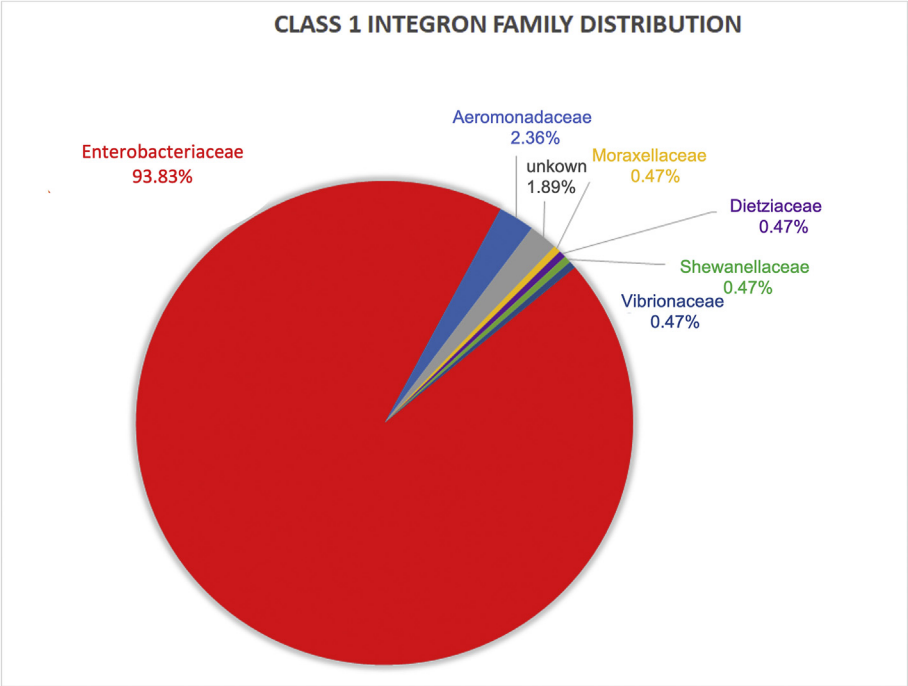


Figure 3. The family distribution of bacterial species encompassing class 1 integrons in the NCBI nr database. The Enterobacteriaceae family is the dominant sources of class 1 integrons in comparison with others (93.83%).

high-level resistance of both TMP/SMX. Apart from this, we also found the association of TMP/SMX resistant determinants and the mobile genetic element, class 1 integron.

Sulfonamides inhibit dihydropteroate synthase, which is a key step to interference the biosynthetic pathway of bacteria. It is often combined with TMP and is effective against a wide variety of aerobic gram-positive and gram-negative bacteria, *P. jirovecii*, and some protozoa. However, an increased TMP/SMX resistance has been noted.[22] Mutation in *folP* gene lead to impaired penetration or insensitive dihydropteroate synthetase, efflux pumps, plasmid-medicated restricted penetration, hyperproduction of p-aminobenzoic acid, or combined more than one mechanism was reported to cause sulfonamide resistance.[13,23] Acquired resistance by drug-resistant target enzymes also plays an important role in sulfonamide resistance. Sulfonamide resistance in gram-negative enteric bacteria is found globally and is largely linked to either of the two genes *sul1* and *sul2*.[24] In Portuguese, the dissemination of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) is found in *Salmonella enterica* strains.[25] The same phenomenon was found in Poland. Among 84 sulfonamide-resistant *Salmonella* spp., which isolated from food, they found an overall prevalence of *sul1* is 44.0% (37/84), and *sul2* is 46.4% (39/84). Besides, a strong association between *intl1* and *sul1* gene had been found. All *intl1*-positive isolates carried the *sul1* gene.[26] Our results provided a further example of the mechanism of SMX resistance involving *sul1* and the genetic linkage with *intl1*.

Our data suggested the underlying connection of *dfrA12* and TMP resistance. TMP is a dihydrofolate reductase (DHFR) inhibitor, which interferes with the synthesis of bacterial folic acid. Impaired permeability, loss of drug-binding capacity, efflux pump, overproduction of or alterations in DHFR have been reported and would cause resistance to TMP.[13,23] In addition, transferable *dfr* genes have been recognized as a significant TMP resistance mechanism. There have been common findings of resistance to TMP due to transferable *dfr* genes in many bacteria, such as *Listeria monocytogenes*[27] and *E. coli*.[28] These genes mediate high-level resistance to TMP with MICs that are greater than normal MIC values by $>$ 1000-fold in gram-negative enteric bacteria.[23] In Korea, 63% (77/122) urinary isolates of *E. coli* were found to be resistant to TMP. Among this TMP resistance *E. coli*, *dfr* genes were detected in 72 isolates. The *dfrA17* and *dfrA12* genes were the most prevalent genes and all were located on class 1 integrons.[29]

We found a strong association between class 1 integron and *sul1*. The gene cassette *dfra12-aadA2-qacEΔ1/sul1* within the integron is identical compared to positions within the genomes of *A. baumannii* WCHAB005078, *Aeromonas* sp. ASNIH5, *E. tarda* FL95-01, and *E. coli* QD 1-5-9. The finding indicates the possibility of horizontal transfer of these genes. The *sul1* gene is normally found with other resistance genes in class 1 integrons.[25] The phenomenon has been observed in other microorganisms, such as *E. coli*, *Salmonella* spp., *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia*.[25,26,30–33] Class 1 integrons are prevalent in antibiotic-resistance bacteria from different ecosystems. In China, they investigated the antimicrobial-resistance genes and integrons in 108 strains of antibiotic-resistant bacteria isolated from eels and aquaculture ponds. Among these 108 strains, class 1 integrons were present in 79.63% of the strains. Among class 1 integron-positive isolates, 73.26% of isolates contained the

qacEΔ1/sul1 gene. The gene cassette array *dfrA12-orfF-aadA2* was the most widely distributed and was found in *Citrobacter freundii, Citrobacter werkmanii, Aeromonas veronii, Shewanella seohaensis, Shewanella xiamenensis*.[34] Zhao et al. reported class 1 integron with encapsulates four-gene cassettes (i.e., *aacA3-catB11c-dfrA1z-aadA2az*), in contrast to our integron consists of two-gene cassettes: *aacA3* and *dfrA12*. None of them are equivalent to the two-gene cassettes in our strain, although *dfrA12* and *accA3* are separately found in different arrays in their data. Therefore, all these findings suggest the gene cassettes of class 1 integron is highly diverse and perhaps shaped by local selection pressure.

The integrative computational analysis in our study found Sul1 distributed in Enterobacteriaceae (46%), followed by Pseudomonadaceae (9%) and *Moraxellaceae* (8%). Sul1 is also significantly prevalent in Enterobacteriaceae compared to the background distribution. Besides, the isolates from the human origin are the predominant origin of *sul1* in the global ecosystems. The gene *sul1* is widely spread in many bacterial species with unequal proportions. From 1991 to 1999, the prevalence of *sul1* in *E. coli* increased from 26.7% to 36.5%.[29] In *Salmonella* spp. isolated from the environment, about 44.0%—76% isolates carried the *sul1* gene.[25,26] The resistant strains of sulfonamides were particularly high among *Salmonella* spp. isolated from foods in Asian countries. The proportion of *sul1* is about 45.5%—95% in Malaysia, Vietnam, and China.[35] Of note, *sul1* is not only detected in Enterobacteriaceae but also in other bacterial species. In Korea, *S. maltophilia*, which was collected from 10 hospitals from 2009 to 2010, revealed a high prevalence of *sul1* in TMP/SMX resistant isolates. Among the 32 resistant isolates, the *sul1* gene was accounted for 23 isolates (72%), and all of them had high-level resistance to TMP/SMX.[36] Multidrug-resistant *A. baumannii* isolated from 5 health-care facilities in Algiers reported 36.17% isolates carried *sul1*.[37] In total 143 MRSA collected from two cities in India, they found 39% of these isolates carried *sul1*.[38]

We found the distribution of the class 1 integron family is significantly enriched in Enterobacteriaceae compared to the background distribution in NCBI nr. Similar to Sul1, 93.83% of class 1 integron fall within Enterobacteriaceae, which is followed by *Aeromonadaceae* (2.36%). Multidrug-resistant Enterobacteriaceae carried class 1 integron has been reported in the past decades. For example, multidrug-resistant (MDR) *Vibrio cholerae* were investigated in India. 22 strains (23%) of the total 94 *V. cholerae* strains were found to have class 1 integrons.[39] In Iran, 181 *K. pneumoniae* were isolated from clinical specimens and 150 (82.9%) were identified as MDR isolates. Among this MDR *K. pneumoniae*, 100% carried *intl1* and 33% carried *intl2*.[40] 76 TMP/SMX resistant *K. pneumoniae* were isolated from the four major hospitals in Egypt. Among these isolates, about half of isolates carried both *sul1* and *sul2* genes. Besides, class 1 integrons were found in 37/38 (97.3%) of the *K. pneumoniae* isolates and only one of the isolates harbored the *intl2* gene.[32] The major limitation in the current study is the only one TMP/SMX resistant strain of *S. algae* which hinder our conclusion.

In conclusion, we found an antimicrobial-resistant mechanism of TMP/SMX in *S. algae*, which is associated with *sul1*. The exist of *sul1* combined with *dfrA12* contributes to the high-level resistance of TMP/SMX. These resistant genes are carried by class 1 integron. Besides, we also found a high association between class 1 integron and *sul1*. We found class 1 integron and Sul1 are largely carried by Enterobacteriaceae. In closing, the *sul1* gene is highly associated with the class 1 integron and is mostly found in Enterobacteriaceae.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.09.014.