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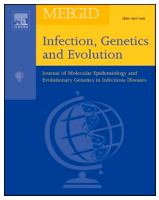

Insights into the genetic contexts of sulfonamide resistance among early clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Since the late 1930s, resistance to sulfonamides has been accumulating across bacterial species including *Acinetobacter baumannii*, an opportunistic pathogen increasingly implicated the spread of antimicrobial resistance worldwide. Our study aimed to explore events involved in the acquisition of sulfonamide resistance genes, particularly *sul2*, among the earliest available isolates of *A. baumannii*. The study utilized the genomic data of 19 strains of *A. baumannii* isolated before 1985. The whole genomes of 5 clinical isolates obtained from the Culture Collection University of Göteborg (CCUG), Sweden, were sequenced using the Illumina MiSeq system. Acquired resistance genes, insertion sequence elements and plasmids were detected using ResFinder, ISfinder and Plasmidseeker, respectively, while sequence types (STs) were assigned using the PubMLST Pasteur scheme. BLASTn was used to verify the occurrence of *sul* genes and to map their genetic surroundings. The *sul1* and *sul2* genes were detected in 4 and 9 isolates, respectively. Interestingly, *sul2* appeared thirty years earlier than *sul1*. The *sul2* gene was first located in the genomic island GI*sul2* located on a plasmid, hereafter called NCTC7364p. With the emergence of international clone 1, the genetic context of *sul2* evolved toward transposon Tn*6172*, which was also plasmid-mediated. Sulfonamide resistance in *A. baumannii* was efficiently acquired and transferred vertically, e.g., among the ST52 and ST1 isolates, as well as horizontally among non-related strains by means of a few efficient transposons and plasmids. Timely acquisition of the *sul* genes has probably contributed to the survival skill of *A. baumannii* under the high antimicrobial stress of hospital settings.

1. Introduction

Antimicrobial resistance (AMR) is an evolutionary response of microbes to withstand the onslaught of antimicrobial agents introduced into their environment. This response has been stocking up in human bacterial pathogens since the beginning of the modern antimicrobial era, marked in 1910 by the introduction of arsphenamine (Salvarsan) into clinical use to treat syphilis cases in humans (Netherton, 1937). Twenty years later, the discovery of sulfonamido-chrysoidine (Prontosil) and development of sulfonamides (1932–1938) was a turning point in the war against bacterial infections (Bickel, 1988). As the only available broad-spectrum antibiotic, sulfonamides were heavily used in the years before penicillin, and this continued into the early years of World War II (Mitchell et al., 2004). Nowadays, the most commonly used sulfonamide for treatment of a variety of human infections is sulfamethoxazole (SMX), mainly in combination with trimethoprim (Nunes et al., 2020). However, several sulfonamide drugs, such as sulfamethazine and sulfadiazine, are extensively used for treatment and prophylaxis of infections in livestock and aquatic animals, as well as for growth promotion purposes in husbandry (Ovung and Bhattacharyya, 2021).

Sulfonamides are chemically synthesized bacteriostatic drugs that function as competitive antagonists of para-aminobenzoic acid, which is an essential compound for the synthesis of folic acid, and thus for bacterial growth (Ovung and Bhattacharyya, 2021). They reversibly inhibit the synthesis of folic acid through binding to dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway. Sulfonamide resistance was first reported around 1938, where particular strains of *Streptococcus* did not respond to the bactericidal effects of

Abbreviations: AMR, Antimicrobial resistance; SMX, Sulfamethoxazole; DHPS, Dihydropteroate synthase; CS, Conserved segment; IS, Insertion Sequence; CCUG, Culture Collection University of Göteborg; ST, Sequence Type; BIGSdb, Bacterial Isolate Genome Sequence Database; MLST, Multilocus sequence typing; NCTC, National Collection of Type Cultures; GI, Genomic island; *orf*, Open reading frame; bp, Base pair; AbaR, *A. baumannii* resistance island; MARR, Multiple antibiotic resistance region; AbGRI, *A. baumannii* genomic resistance island.

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sulfonamide (Buttle, 1939). Vertically acquired resistance to sulfonamides is conferred by mutations in the chromosomal DHPS gene (*folP*), enabling bacteria to produce altered forms of the DHPS enzyme that are resistant to inhibition by the drug (Skold, 2000). However, a majority of the clinically occurring sulfonamides resistance in Gram-negative bacteria is associated with the horizontal acquisition of transferable genes encoding alternative drug-resistant types of the DHPS enzyme, as first reported in 1975 (Wise Jr. and Abou-Donia, 1975).

To date, four different bacterial genes encoding resistance to sulfonamides have been identified and denoted as *sul1*, *2*, *3*, and *4* (Perreten and Boerlin, 2003; Rådström and Swedberg, 1988; Razavi et al., 2017). The *sul1* gene, for which the nucleotide sequence was first determined in 1981 (Swift et al., 1981), is usually a key component of the 3′ conserved segment (3′-CS) of class I integron (Tn*21* type) and has frequently been plasmid-mediated (Rådström and Swedberg, 1988). The *sul2* gene, first sequenced in 1988, is also commonly carried on plasmids but has not been found to be part of class I integrons (Rådström and Swedberg, 1988). Notably, the Sul1 enzyme (279 amino acids) was heat-labile while Sul2 (271 amino acids) was heat-stable, and their encoding genes shared only 57% nucleotide identity to each other (Rådström and Swedberg, 1988). In 2003, Perreten and Boerlin detected *sul3*, encoded by the 54-kb conjugative plasmid pVP440, in *E. coli* isolated from swine in Switzerland (Perreten and Boerlin, 2003). Sul3 (263 amino acids) showed 40.9% and 40.6% identity to Sul1 and Sul2, respectively. In the pVP440 plasmid, *sul3* was not part of class I integron but was rather located in a 3.6 kb segment flanked by two copies of the insertion sequence (IS) *15Δ/26*. Interestingly, ancient mutations in the chromosomal *folP* genes of *Rhodobiaceae* and *Leptospiraceae* were found to confer resistance to sulfonamides and were proposed as the likely origins of the *sul1–3* genes based on the results of a Bayesian phylogenetic analysis of multiple alignments of their FolP/Sul sequences (Sanchez-Osuna et al., 2018). The fourth sulfonamide resistance gene, *sul4*, was reported in 2017 in association with class I integrons and with 31–33% nucleotide identity to the three other *sul* genes (Razavi et al., 2017). The *sul4* gene was discovered during systematic analysis of DNA obtained from polluted Indian river sediments. *In silico* screening of 6489 publicly available metagenomic datasets revealed the occurrence of *sul4* in different types of environmental samples obtained from seven countries across Asia and Europe (Razavi et al., 2017).

Over the last 3 decades, *Acinetobacter baumannii* has gained lots of interest because of the frequent occurrence of nosocomial outbreaks caused by strains exhibiting exacerbated resistance to several classes of antimicrobials such as carbapenems, extended-spectrum cephalosporins, fluoroquinolones or even to last-resort antimicrobial agents including colistin or tigecycline (Havenga et al., 2022). This bacterium is currently one of the most successful opportunistic pathogens worldwide, causing a broad range of severe infections especially among hospitalized patients, including ventilator-associated pneumonia, bloodstream infections, skin and soft tissue infections, wound infections, catheter-associated urinary tract infections and secondary meningitis. Apart from its innate resistance traits, the genomic plasticity of *A. baumannii* is well-suited for the acquisition of foreign resistance genes by every means of horizontal gene transfer (Sarshar et al., 2021). In addition, a few but extremely relevant virulence attributes have been evolutionarily conserved in the pan-genome of *A. baumannii* providing it an adaptive advantage to thrive in the hospital environment (Sarshar et al., 2021).

High rates of sulfonamide resistance have frequently been reported among clinical isolates of *A. baumannii*. For example, a study from Taiwan showed that 71% (286/403) of the *A. baumannii* clinical isolates collected between 2014 and 2015 were resistant to sulfonamides (Chen et al., 2017). Similarly, non-susceptibility to trimethoprim/sulfamethoxazole was detected in 94.5% (69/73) of non-duplicate *A. baumannii* urinary isolates collected over a period of 12 months from patients with severe urinary tract infection in India (Girija et al., 2019). *sul1* and *sul2* were present in 52.1% (38/73) and 45.2% (33/73) of these isolates, respectively. All the clinical isolates (52/52) that were reported in a recent study from Pakistan were resistant to trimethoprim/sulfamethoxazole (Karah et al., 2020). The *sul1* and *sul2* sulfonamide resistance genes were detected in 11/25 and 14/25 of the investigated isolates, respectively.

The relatively recent appearance of *A. baumannii* as a pathogen of increasing clinical relevance in human health care has raised much interest regarding its adaptability, and how its genome has successfully been able to acquire and retain new traits for AMR. As resistance to sulfonamides was one of the first appearing resistance traits in bacteria, our present study was focused on elucidating genetic events involved in the initial acquisition of the *sul* genes, particularly *sul2*, by the earliest available isolates of *A. baumannii*.

2. Methods

2.1. A. baumannii strains and growth conditions

The study involved genomic data of 19 isolates of *A. baumannii* collected before 1985 (Table 1), including the whole genome sequences of 5 clinical strains obtained from the Culture Collection University of Göteborg (CCUG), Sweden. The CCUG isolates were revived on Luria agar media (Fluka, Millipore Sigma, Sweden) at 37 °C. 20% glycerol was used for storage at −80 °C.

2.2. Antimicrobial susceptibility testing

Susceptibility of the CCUG isolates to ampicillin/sulbactam (10/10 μg), piperacillin/tazobactam (100/10 μg), cefotaxime (30 μg), ceftazidime (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), doxycycline (30 μg), and tetracycline (30 μg) was tested by the Kirby Bauer's disk diffusion method using antimicrobial discs and Mueller Hinton agar from Oxoid™ (Fisher Scientific, Sweden). The minimum inhibitory concentration of sulfamethoxazole (alone without trimethoprim) was tested using the Liofilchem® MIC Test Strips (Liofilchem S.r.l., Italy). The tests were performed, and susceptibility patterns were interpreted following the guidelines as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Version 13.0, 2023; http://www.eucast.org) and/or the Clinical and Laboratory Standards Institute (2022).

2.3. Whole genome sequencing

Genomic DNA of the CCUG isolates was extracted using the DNeasy blood and tissue kit (Qiagen, Germany) and quantified with Qubit 2.0 fluorometer using the Qubit™ dsDNA HS Assay kit (Invitrogen, USA). Using the Nextera XT DNA Library Preparation Kit and Nextera™ DNA CD Indexes (Illumina, San Diego, USA), and the Agencourt AMPure XP system (Beckman Coulter, Life sciences, USA), an indexed paired end library was prepared for each isolate. Followed by manual normalization and pooling, the library was sequenced using the MiSeq Reagent Kit v3 (600-cycle) on Illumina MiSeq system (Illumina, San Diego, USA). All steps of DNA preparation, library construction, clean up, and genome sequencing were done according to the suppliers' instructions. The SPAdes version 3.15.4 genome assembly toolkit was applied for *de novo* assembly of the sequence reads.

2.4. Sequence analysis

All the isolates were assigned sequence types (ST) using the Bacterial Isolate Genome Sequence Database (BIGSdb) software available at https://pubmlst.org/ according to the multilocus sequence typing (MLST) method, Pasteur and Oxford schemes (Diancourt et al., 2010). The presence of acquired antimicrobial resistance genes was detected by ResFinder 4.0, available on the website of the Center for Genomic

Table 1
List of the *Acinetobacter baumannii* isolates included in this study.

Name	Year of isolation	Country of isolation	Source of isolation	Sequence type (ST)[1]	Acquired antibiotic resistance genes[2]	*sul* genes
DSM 30011	≤ 1944	Not available	Environmental (guayule shrubs)	738^{P} (3, 3, 105, 6, 4, 2, 5) / 1113^{Ox} (96, 150, 12, 12, 1, 103, 4)	—	—
NCIB 8209	≤ 1944	Not available	Environmental (guayule shrubs)	1197^{P} (1, 4, 2, 2, 7, 58, 2) / Not available (the *gdhB* gene is missing)	—	—
NCTC 7364	≤ 1947	United Kingdom	Not available	494^{P} (3, 3, 2, 5, 4, 1, 4) / 155^{Ox} (1, 57, 78, 12, 1, 54, 51)	*sul2*	*sul2*
NCTC 7412	≤ 1948	United Kingdom	Urine	241^{P} (40, 3, 15, 2, 40, 4, 4) / 613^{Ox} (15, 48, 58, 42, 36, 54, 41)	—	—
ATCC 19606^{T}	≤ 1948	United States of America	Urine	52^{P} (3, 2, 2, 7, 9, 1, 5) / 931^{Ox} (1, 10, 8, 6, 1, 110, 14)	*sul2*	*sul2*
CCUG 33549	≤ 1948	Not available	Not available	52^{P} (3, 2, 2, 7, 9, 1, 5) / 931^{Ox} (1, 10, 8, 6, 1, 110, 14)	*sul2*	*sul2*
ATCC 17945	≤ 1949	Not available	Not available	1168^{P} (1, 62, 156, 2, 40, 4, 4) / New^{Ox} (18 with 51C → T, 48, 58, 42, 4, 54, 26)	*sul2*	*sul2*
CIP 70.10	≤ 1950	Not available	Not available	126^{P} (3, 2, 7, 2, 7, 1, 3) / 819^{Ox} (21, 15, 2, 28, 1, 107, 4)	—	—
ATCC 17978	≤ 1951	Not available	Cerebrospinal fluid	77^{P} (3, 2, 2, 2, 3, 4, 28) or 437^{P} (3, 2, 2, 2, 30, 4, 28) / 959^{Ox} (1, 12, 56, 1, 1, 61, 3) or 112^{Ox} (1, 12, 56, 36, 1, 61, 26)	*sul2*	*sul2*
CCUG 2488	≤ 1959	Not available	Not available	138^{P} (3, 3, 7, 26, 7, 1, 4) / 877^{Ox} (21, 12, 2, 28, 1, 107, 4)	*sul2*	*sul2*
CCUG 890	≤ 1962	Germany	Urine	54^{P} (12, 3, 18, 2, 17, 4, 5) / 956^{Ox} (23, 35, 3, 27, 23, 144, 7)	*sul2*	*sul2*
ATCC 17961	≤ 1964	Not available	Blood	438^{P} (3, 2, 2, 7, 9, 4, 5) / 931^{Ox} (1, 10, 8, 6, 1, 110, 14)	*sul2*	*sul2'* [3]
CCUG 6644	≤ 1977	Sweden	Osteitis, fibia	New^{P} (3, 2, 40, 2, 7, 2 with 310 T → G, 6) / Not available (the *gdhB* gene is missing)	—	—
HK302	1977	Switzerland	Respiratory tract	1^{P} (1,1,1,1,5,1,1) / Not available	*tet(A)*, *catA1*, *bla*$_{TEM-1}$, *aphA1*, *aacC1*, *aadA1*, *sul1*[4]	*sul1*
CCUG 26383	1980–81	Sweden	Sputum	53^{P} (1, 1, 2, 2, 3, 4, 2) / Not available (the *gpi* gene is missing)	—	—
A1	1982	England	Not available	1^{P} (1, 1, 1, 1, 5, 1, 1) / 231^{Ox} (10, 12, 4, 11, 4, 98, 5) or 1604^{Ox} (10, 12, 182, 11, 4, 98, 5)	*sul1*, *aacC1*, *qacE*, *aadA1*, *tet(A)*, *catA1*	*sul1*
RUH 134	1982	Netherlands	Urine	2^{P} (2, 2, 2, 2, 2, 2, 2) / 6^{Ox} (1, 4, 3, 2, 2, 3, 3) or 350^{Ox} (1, 12, 3/189, 2, 2, 102, 3)	*bla*$_{TEM-1D}$, *aacC1*, *aphA1*, *strA*, *strB*, *sul1*, *qacE*, *tet(B)*, *catA1*	*sul1*
RUH 875	1984	Netherlands	Urine	1^{P} (1,1, 1, 1, 5, 1, 1) / 231^{Ox} (10, 12, 4, 11, 4, 98, 5)	*bla*$_{TEM-1D}$, *aphA1*, *aadB*, *strA*, *strB*, *sul1*, *sul2*, *dfrA5*, *qacE*, *tet(A)*, *catA1*	*sul2* *sul1* *sul1'* [5]
RUH 1486	1985	Netherlands	Umbilicus	25^{P} (3, 3, 2, 4, 7, 2, 4) / 229^{Ox} (1, 15, 2, 28, 1, 107, 32)	—	—

[1] According to the Pasteur (^{P}) and Oxford (^{Ox}) schemes for multilocus sequence typing.
[2] According to ResFinder except for HK302.
[3] *sul2'* has one synonymous single base pair substitution (C309A) compared to *sul2* (WP_001043260.1).
[4] According to reference paper (Krizova and Nemec, 2010).
[5] *sul1'* (WP_000259018.1) has one nonsynonymous single base pair substitution (T115G) compared to *sul1* (WP_000259031.1).

Epidemiology (http://www.genomicepidemiology.org/). ISfinder (https://www-is.biotoul.fr/) and Plasmidseeker (https://github.com/bioinfo-ut/PlasmidSeeker) were used to detect standard insertion sequence (IS) elements and plasmids, respectively.

2.5. Genetic context analysis of sul genes

The occurrence of *sul* genes was verified and their genetic surroundings were manually annotated based on the nucleotide similarities detected by the BLASTn suite search tool (https://blast.ncbi.nlm.nih.gov/). Searches were made against the "Nucleotide collection (nr/nt)", "Whole-genome shotgun contigs (wgs)", and/or "RefSeq Genomes Database (refseq_genomes)" databases. Schematic maps of genetic contexts of *sul* genes were drawn using Snapgene® 6.0.5 (https://www.snapgene.com/) and modified in Inkscape version 1.0.2.

3. Results and discussion

In order to identify elements associated with early acquisitions of the *sul* genes by *A. baumannii*, our study included all isolates ($n = 19$) that were obtained from ≥1944 up to 1985 and for which we had available whole genome or *sul*-related sequence records. Overall, *sul2* and *sul1* were detected in 9 (47%) and 4 (21%) isolates, respectively. Interestingly, the earliest appearance of *sul1* in *A. baumannii* was in 1977, while *sul2* first appeared in ≥1947 (thirty years earlier than *sul1*).

A dominant variant of *sul2* was detected in 8/9 of the *sul2*-positive isolates. This variant was identical to hundreds of the records available in GenBank including some of the earliest sequences of *sul2* (e.g. GenBank: M36657.1, CP077211.1, AE014073.1, and AY823412.1). A synonymous single base pair substitution (C309A) was detected in the *sul2* gene of only one isolate (ATCC 17961), resulting in a new variant designated as *sul2′* (GenBank: CP065432.1, locus_tag: I5593_17975). However, the nucleotide sequence of *sul2′* was not identical to any of the other records available in GenBank (as of 22nd July 2022). Both variants encoded the same 271-long amino acid sequence (Fig. S1a), corresponding to the Sul2 sulfonamide-resistant dihydropteroate synthase (RefSeq: WP_001043260.1).

Similarly, two variants of *sul1* were identified, designated as *sul1* and *sul1′* (Fig. S1b). *sul1′* had a single base pair substitution (T115G) compared to *sul1*, leading to the occurrence of a single amino acid change (Ser39Ala) in the encoded protein. Searching for nucleotide similarity to *sul1* in the GenBank databases yielded hundreds of identical

hits, including several old deposits (such as GenBank: D43625.1, M73819.1, U04277.1 and X12869.1), while far fewer identical hits were detected for *sul1′*. The 279 amino acids encoded by *sul1* and *sul1′* corresponded to the Sul1 (RefSeq: WP_000259031.1) and Sul1′ (RefSeq: WP_000259018.1) sulfonamide-resistant dihydropteroate synthases, respectively.

As expected, the *sul-2*-positive CCUG isolates (CCUG 33549, CCUG 2488, and CCUG 890) were all resistant to sulfamethoxazole, both alone and in combination with trimethoprim, while those that were lacking *sul1* or *sul2* (CCUG 6644 and CCUG 26383) were susceptible to sulfamethoxazole (Table S1). Intermediate resistance to ciprofloxacin was noted for all the isolates. One isolate (CCUG 2488) was also intermediate resistant to tetracycline. Intermediate or full resistance to cefotaxime was detected in 4/5 of the isolates. Otherwise, the isolates were all susceptible to ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, imipenem, meropenem gentamicin, amikacin, tobramycin, levofloxacin, and doxycycline.

3.1. Revisiting the genetic contexts of sul2 in ATCC 19606 and ATCC 17978

A. baumannii ATCC 19606, isolated in the USA from a urine sample ≤ 1948 (Schaub and Hauber, 1948), and ATCC 17978, collected from a cerebrospinal fluid sample in France ≤1951 (Smith et al., 2007) were both resistant to sulfonamides, while remained susceptible to a range of other antibiotics (Hamidian et al., 2020; Smith et al., 2007). *sul2* was the only acquired antimicrobial resistance gene detected in the genomic records of these two isolates. The *sul2* gene was located in a genomic resistance island, called GI*sul2* (15,460 bp), in ATCC 19606 (Hamidian and Hall, 2017a), while a similar version of GI*sul2* interrupted by the insertion sequence (IS) element IS*Aba1*, which will hereafter be called IS*Aba1*::GI*sul2* (16,649 bp), was detected in ATCC 17978 (Nigro and Hall, 2011).

Interestingly, GI*sul2* was also found in the chromosomes of the type strain of *Enterobacter cloacae* subspecies *cloacae* ATCC 13047^{T} and of *Shigella flexneri* ATCC 700930 (also called serotype 2a strain 2457 T), collected in 1890 and 1954, respectively (Nigro and Hall, 2011). The annotations of GI*sul2* and IS*Aba1*::GI*sul2* have been reported in detail by a number of previous studies (Hamidian and Hall, 2017a; Harmer et al., 2017; Nigro and Hall, 2011). As previously stated, the GI*sul2* in ATCC 13047^{T} was interrupted by a mercuric ion resistance transposon related to Tn*5393* (GenBank: CP001918.1). Notably, we detected direct repeats of 14 bp on the boundaries of GI*sul2* in ATCC 13047^{T} and ATCC 700930, of which 5 bp could be a result of target site duplication during the insertion of GI*sul2*. We also detected GI*sul2* in the genomic record of *Pseudomonas aeruginosa* NCTC11445 (GenBank: LR134308.1), isolated in 1957. However, the segment carrying *sul2* together with Δ*glmM* and IS*CR2* was missing in *P. aeruginosa* NCTC11445 (Data not shown).

The GI*sul2* island in ATCC 19606 was part of a larger genomic region, called GI*19606* (36,157 bp), which was inserted in the chromosome between open reading frames $orf_{\text{ribonuclease-PH}}$ and $orf_{\text{phospholipase-C}}$ (Hamidian et al., 2020). GI*19606* included 28 *orfs*, most of which encoded for hypothetical proteins with no putative conserved domains. It had IS*Aba11* on one end and was flanked by a TSD of 5-bp. The origin of GI*19606* was most likely acquired, with the help of IS*Aba11*, from a plasmid related to *Acinetobacter johnsonii* pXBB1–9 or *Acinetobacter pittii* p2014N21–145-1 (GenBank: CP010351.1 and CP033569.1, respectively), as proposed previously (Hamidian and Hall, 2017a).

Among the isolates that were sequenced in this study, *sul2* was detected in the same genetic context (GI*19606*) in *A. baumannii* CCUG 33549, isolated ≤1948 (Ferguson and Roberts, 1950). Interestingly, both ATCC 19606 and CCUG 33549 belonged to ST52 (3,2,2,7,9,1,5). Searching for nucleotide similarity in the GenBank databases enabled us to detect five other isolates belonging to ST52 and carrying GI*19606*, namely MSP4–16 (GenBank: AODW00000000.1) isolated from a mangrove soil in India in 2010 (Singh et al., 2013), ab736 (GenBank: CP015121.1) obtained from a clinical sample in USA in 2015 (Krishnamurthy et al., 2019), BA20352 and BA20475 (GenBank: JAAOQN000000000.1 and JAAOQM000000000.1, respectively) obtained from clinical blood samples in India in 2019, and A.bA112 (GenBank: JAAQWA000000000.1) obtained in Hong Kong. GI*19606* was most likely inserted in the chromosome of these relatively recent ST52 isolates, as shown for the old ST52 ones, although this could not be confirmed since the sequences were located on more than one contig. Deep analysis of the relevant contigs in isolate MSP4–16 enabled us to detect two identical AT-rich direct repeats of 268 bp in GI*19606* in close proximity (around 20 kb) to each other (Fig. S2), raising the possibility that the genetic architecture of GI*19606* could be subject to rearrangement by direct-repeat-mediated intramolecular recombinational events.

In ATCC 17978, Nigro & Hall reported that IS*Aba1*::GI*sul2* was integrated into the *tniB* gene of a transposon related to Tn*6021* (Nigro and Hall, 2011). Later, this *tniB* gene was re-designated as *tniBb* gene, representing the third *orf* of a transposon tentatively called Tn*6022b* (30,331 bp) and officially re-called Tn*6174* (Hamidian and Hall, 2017b; Saule et al., 2013). The inter-gene region between *tniEb* and *tniDb* of Tn*6174* was interrupted by IS*Aba18* (1309 bp). The insertion of Tn*6174* was associated with a characteristic 5-bp target site duplication (TSD), as reported for all other Tn*6019*-related transposons (Post et al., 2010). Tn*6174* was found next to a segment of 6205 bp, called zone 1 (Saule et al., 2013), and another transposon also related to Tn*6019*, called Tn*6021* (13,104 bp) (Post et al., 2010). The *tniC* gene of Tn*6021* was interrupted by IS*Aba11* (1101 bp). The whole Tn*6174*-zone-1-Tn*6021* assembly was carried on plasmid pAB3 (148,955 bp or 148,956 bp) as for GenBank records CP012005.1, CP079932.1, and CP074709.1, or was dispersed into the chromosome of ATCC 17978 according to CP000521.1, CP053098.1, CP059041.1, and CP018664.1.

The earliest occurrence of *sul2* was found in the genome of *A. baumannii* NCTC 7364, isolated ≤1947, representing one of the oldest *A. baumannii* isolates preserved until today (Baumann et al., 1968). The type of sample and country of isolation for NCTC 7364 were unknown. NCTC 7364 was resistant to sulfonamide, trimethoprim, cephalexin, penicillin, and ampicillin (Alexander et al., 1984). Similarly to ATCC 17978, the *sul2* gene was located in IS*Aba1*::GI*sul2*, which was inserted in the *tniBb* gene of Tn*6174*. However, the original version of Tn*6021*, called Tn*6022* (13,104 bp including IS*Aba18*), was present in this isolate (Hamidian and Hall, 2011). The whole Tn*6174*-zone-1-Tn*6022* assembly was carried on a plasmid of 148,956 bp, hereafter called NCTC7364p (Fig. S3a). Particularly, the only difference between Tn*6021* and Tn*6022* is related to a region of 531 bp of Tn*6022* been replaced by an analogous region from Tn*6174* (Fig. S3b; Hamidian and Hall, 2011).

The *sul2* gene in *A. baumannii* ATCC 17945, isolated ≤1949 (Stuart et al., 1949), had the same genetic environment as of NCTC 7364, except that the *tniE* gene of Tn*6022* was interrupted by a second copy of IS*Aba1* in ATCC 17945 (Fig. S4). The whole assembly was most likely carried on a plasmid of 151,457 bp showing >99% nucleotide identity to the NCTC7364p. However, the exact composition of the genetic context of *sul2* in ATCC 17945 and its occurrence on plasmid were tentatively proposed since the corresponding sequences were distributed on 6 contigs of the genomic record of ATCC 17945. Similarly, the whole genome sequences we obtained for *A. baumannii* CCUG 2488, isolated ≤1959 (https://www.ccug.se/strain?id=2488), and CCUG 890, isolated ≤1962 from a clinical urine sample in Germany (Stenzel and Mannheim, 1963) revealed the occurrence of *sul2* on the same Tn*6174*-zone-1-Tn*6022* genetic context. The type of sample and country of isolation for ATCC 17945 and CCUG 2488 were unknown.

Based on our BLASTn search for nucleotide similarities, as of July 2022, the complete Tn*6174*-zone-1-Tn*6022* assembly was found in the genome of only one relatively new *A. baumannii* isolate, namely A758, isolated in Chile in 2017. The Tn*6174*-zone-1-Tn*6022* assembly in A758 was most likely mediated on plasmid related to NCTC7364p (Fig. S5). However, this could not be confirmed since the corresponding sequences were distributed on several contigs (GenBank: JAGTAJ000000000.1). In

addition, our search enabled us to detect *sul2* in three old isolates from other *Acinetobacter* species, which were *Acinetobacter* sp. CIP 56.2 isolated from France in 1955, *Acinetobacter lwoffii* TG19636 isolated from a urine sample in 1962, and *Acinetobacter* sp. CIP 64.7 (ATCC 17910) isolated from Germany in 1964 (Bouvet and Grimont, 1986; Perichon et al., 2014; Sahl et al., 2013). The *sul2* gene was also detected in two relatively new *A. baumannii* isolates, namely Ac23 and Ac246, isolated in Portugal in 2001 and 2004, respectively (Silva et al., 2021). In these five isolates, *sul2* was located in IS*Aba1*::GI*Sul2* (16,649 bp) and Tn*6174* (30,331 bp), showing 100% nucleotide identities to the corresponding regions of NCTC7364p (data not shown). However, Tn*6174* was inserted by itself, without zone 1 or Tn*6022*, in the chromosomes of CIP 56.2, TG19636, CIP 64.7, Ac23, and Ac246 (GenBank: APPH00000000.1, AMJG00000000.1, APRY00000000.1, JABKAR010000000.1, and JABKAS010000000.1, respectively). In accordance, the latter five isolates were lacking sequences related to NCTC7364p.

NCTC 7364 belonged to ST494 (3,3,2,5,4,1,4), while ATCC 17945 to ST1168 (1,62,156,2,40,4,4), CCUG 2488 to ST138 (3,3,7,26,7,1,4), CCUG 890 to ST54 (12,3,18,2,17,4,5), A758 to ST318 (6,6,8,2,3,5,5), and both Ac23 and Ac246 to ST238 (6,3,8,2,3,5,4). ATCC 17978 belonged to ST77 (3,2,2,2,3,4,28) according to Diancourt et al. (2010) or to ST437 (3,2,2,2,30,4,28) according to several publicly available GenBank genomic records (e.g., CP000521.1, CP012004.1, CP018664.1, and CP039028.1). Only ST318 and ST238 shared 5/7 alleles and were double locus variants to each other, while the STs of other isolates shared $\leq$4/7 alleles and were considered to be not related to each other. The scattered distribution of Tn*6174*-zone-1-Tn*6022*, or versions of it, in isolates belonging to non-related STs and among different *Acinetobacter* species could be related to dynamic intra- and inter-species horizontal transferability of the carrier plasmid. Further intra-genomic movement of Tn*6022b* from plasmid into the chromosome was also indicated.

3.2. Novel genetic context for sul2 in ATCC 17961

The *sul2* gene of ATCC 17961, isolated from blood sample $\leq$ 1968 (Baumann et al., 1968) was also located in GI*sul2*. However, GI*sul2* (15,181 bp) in ATCC 17961 was lacking IS*Aba1*, had a boundary deletion of 279 bp, and was inserted in a distinctive large transposon (43,752 bp) belonging to the Tn*6019* family (Fig. 1). In addition to GI*sul2*, this transposon, hereafter called Tn*7523* (number was allocated by The Transposon Registry, https://transposon.lstmed.ac.uk/), had 22 *orfs* and was bounded by imperfect inverted repeats of 26 bp. The first 5 *orfs* corresponded to *tniC*, *tniA*, *tniB*, *tniD*, and *tniE* similarly to other members of the Tn*6019* family (Post et al., 2010). The rest of Tn*7523*, *orf6* to *orf22*, encoded a variety of proteins such as transporters, regulatory protein RecX (PRK14135), carboxymuconolactone decarboxylase, ferredoxin-NADP(+) reductase, NAD(*P*)H-dependent oxidoreductase, redox-sensitive transcriptional activator SoxR, ThiF family adenylyltransferase, nucleotidyltransferase, ImmA/IrrE family metallo-endopeptidase, and metallophosphoesterase, which was completely dissimilar to the previously reported Tn*6019*-related elements. Tn*7523* also had a novel IS element between *orf15* and *orf16*. This IS (1243 bp) had 1021/1243 (82%) nucleotide identity to IS*Acsp3* (https://isfinder.biotoul.fr/scripts/ficheIS.php?name=ISAcsp3).

Tn*7523* was inserted in the chromosomal gene *comM*, known to be the site of insertion of a variety of *A. baumannii* resistance islands (AbaRs) derived from or built on Tn*6019* (Post et al., 2010). Interestingly, ATCC 17961 belonged to ST438 (3,2,2,7,9,4,5), which is a single locus variant of ST52, implying probable intra-chromosomal horizontal transfer of GI*sul2* from GI*19606* to Tn*7523* during the vertical reproduction of lineage ST52 from ATCC 19606 toward ATCC 17961. A related structure was detected in the genome of 3 *A. baumannii* isolates (3207, 7835, and 9201) obtained between 2007 and 2013 in Mexico (Castro-Jaimes et al., 2016; Salgado-Camargo et al., 2020) The backbone of Tn*7523* in these isolates was 100% identical to the corresponding sequences of ATCC 17961. However, the GI*sul2* was 22,781 bp, due to the insertion of IS*Aba1* upstream *sul2* and acquisition of an extra segment of 6130 bp carrying the *floR*, *tetR(G)*, *tetA(G)*, *lysR*, and *fabB* genes, encoding for chloramphenicol resistance, tetracycline resistance transcriptional repressor, tetracycline resistance, LysR-family transcriptional regulator, and 3-oxoacyl-ACP synthase along with a truncated copy of IS*CR2* (Fig. S6). The new version of Tn*7523* (52,257 bp) was inserted into a gene encoding for a HEAT repeat domain-containing protein (Sequence ID: WP_024437283.1) in the chromosomes of these isolates (GenBank: CP015364.1, CP033243.1, and CP023020.1), representing a different location compared to ATCC 17961 or ATCC 19606. Segments related to Tn*7523* were also detected in *A. baumannii* TG27343 and 1583–8, isolated in 2005 and 2006 in the USA (Sahl et al., 2013). Interestingly, all the latter five isolates (3207, 7835, 9201, TG27343, and 1583–8) belonged to ST422 (26,72,2,2,29,4,5).

3.3. Revisiting the genetic context of sul2 in RUH 875

In *A. baumannii* RUH 875 (also known as NIPH 527 or A297), isolated from urine sample in the Netherlands in 1984 (Dijkshoorn et al., 1987), *sul2* was part of the configuration IS*Aba1-sul2*-Δ*glmM*-IS*CR2-strB-strA* (Hamidian et al., 2016). This configuration (6567 bp) arose by the merge of IS*Aba1-sul2*-Δ*glmM*-IS*CR2* (4811 bp), coming from GI*sul2*, with the *strB-strA* operon (1756 bp), derived from Tn*5393*. They were all part of a transposon called Tn*6172* (Hamidian et al., 2016), which was carried on plasmid pA297–3 (220,063 bp; GenBank: KU744946.1). Tn*6172* (11,719 bp) had two regions with 100% nucleotide identity to the corresponding regions of Tn*6174* (Fig. S7). The site of insertion of Tn*6172* in pA297–3 was similar to that of the whole Tn*6174*-zone1-Tn*6022* assembly in NCTC7364p. Furthermore, large parts of pA297–3 and NCTC7364p shared >99% nucleotide identity to each other, as shown in Fig. S8. Among the differences between the two plasmids, we noted that Tn*6022*, part of zone 1, and part of *orf97* were deleted in

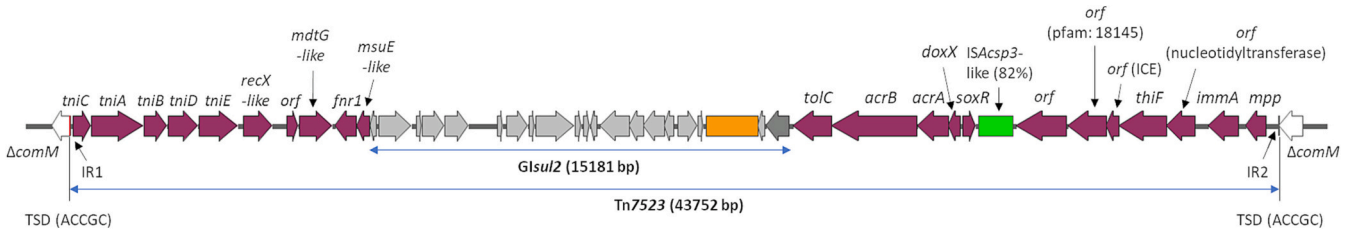

Fig. 1. Genetic context of *sul2* in *Acinetobacter baumannii* ATCC17961. *sul2* was found in GI*sul2*, as part of the genetic configuration *sul2*-Δ*glmM*-IS*CR2-orf*$_{\text{hypothetical protein}}$-*resG*. GI*sul2* was located in Tn*7523* which was inserted in the chromosome of ATCC17961. GI*sul2* was shown as labeled gray box. Genes and open reading frames (*orf*) were shown by labeled white (for the interrupted *comM* gene), gray (for GI*sul2*) or plum (for the rest of Tn*7523*) arrows, with the arrowhead indicating the direction of transcription. The insertion sequence element IS*Acsp3-like* was shown as labeled green box. IS*CR2* was shown as labeled orange box. The inverted repeats (IR) of Tn*7523* were shown as labeled yellow vertical bars. Target site duplications (TSD) were highlighted and shown as labeled red vertical bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pA297–3 compared to NCTC7364p. This isolate, RUH 875, belonged to ST1 (1,1,1,1,5,1,1), representing one of the earliest isolates of International Clone 1 with an available genomic record. Altogether, our analysis indicated that this new genetic context of *sul2* originated from the Tn*6174*-zone1-Tn*6022* assembly by means of few genomic evolutionary events (deletions, insertions, and single nucleotide mutations) combined with inter-strain horizontal transfer of the hosting plasmid.

3.4. Summary of the genetic contexts of sul1

The oldest *A. baumannii* isolate with an identified *sul1* gene was sulfamethoxazole-resistant strain HK302, recovered in 1977 from the respiratory tract of a patient in Zurich University Hospital, Switzerland during an outbreak of nosocomial infections (Devaud et al., 1982) and had the allelic profile of ST1 (International Clone 1). A putative structure of the HK302 resistance island was identified based on polymerase chain reaction-restriction fragment length polymorphism mapping and partial sequencing which revealed the presence of a 63.4-kb AbaR3-like resistance island carrying multiple antibiotic resistance genes, including two copies of *sul1* (GenBank: HM357806.1, Krizova and Nemec, 2010). Although the whole sequence of this AbaR is currently not available, the described genetic structure corresponded to AbaR0 (GenBank: KF483599.2, Hamidian and Hall, 2018). Similarly, two copies of the *sul1* gene were detected in the chromosomes of isolates A1, isolated in the United Kingdom in 1982 (Holt et al., 2015), and RUH 875 which were also members of international clone 1 (ST1). As previously described, the two copies of *sul1* were part of the 3′-conservative segments, either truncated or complete, of a variety of class I integrons, which were integrated, along with other transposon units, into the AbaR3-like resistance islands AbaR24 (GenBank: JN968482.3) and AbaR21 (GenBank: KM921776.1) (Nigro et al., 2011). One isolate, RUH 134 (also known as A320 and NIPH 528), collected in 1982 in the Netherlands and assigned to ST2 (corresponding to international clone 2), carried only one copy of the *sul1* gene, which was located in the 3′-CS of a complete In4-type class I integron of 7994 bp representing one of the main components of the IS*26*-bounded genomic resistance island AbGRI2-0b (GenBank: JN247441.4 and CP032055.1) (Nigro and Hall, 2012).

4. Conclusion

Since their first introduction into clinical use, synthetic sulfonamides have been largely handicapped by the rapid appearance of sulfonamide resistance. Searching for *sul* genes in the genomes of ancient isolates of *A. baumannii* revealed a time gap of 30 years between the first appearance of *sul2* $\geq$ 1947 and *sul1* in 1977. The dynamic ability of *sul2* to change contexts was notable and has most likely contributed to its prolonged existence. Importantly, the occurrence of horizontally acquired sulfonamide resistance elements in *A. baumannii* International Clones 1 and 2 has probably paved the way for the acquisition of additional genes conferring resistance to other classes of antibiotics. In addition, our study added an important clue on the role of plasmids and transposons in the global spread of antimicrobial resistance. One of the limitations of this study was related to the shortfall of available sulfonamide-resistant isolates in the selected timeline (−1985). Accordingly, the identified genetic environments provided only a glimpse into the primitive arrangement of the *sul1* and *sul2* genes in *A. baumannii*. It is appealing to extend the timeline and do further research using a larger sample size, including more recent isolates, although high complexity is anticipated due to the remarkable genomic plasticity of *A. baumannii*.

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Ethical approval

Not required.

CRediT authorship contribution statement

Anju Bala: Conceptualization, Methodology, Data curation, Writing – original draft. **Bernt Eric Uhlin:** Supervision, Writing – review & editing. **Nabil Karah:** Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

The sequence raw reads and draft genome sequences of five CCUG isolates have been deposited in the DDBJ/EMBL/GenBank database under the BioProject accession no: PRJNA836743. Raw sequence data files of the five isolates are deposited in the Sequence Read Archive (SRA) database under the accession numbers: SRR19176915, SRR19153306, SRR19176914, SRR19752888, SRR19752889 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA836743). Individual accession numbers and metadata of all the isolates are described in Table S1. Genome assembly features of the clinical isolates sequenced in this study are given in Table S2. All supporting data and protocols have been provided within the article or through supplementary data files. Two supplementary tables and eight supplementary figures are available with the online version of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2023.105444.

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