

Global population structure of the *Serratia marcescens* complex and identification of hospital-adapted lineages in the complex

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Abstract

Serratia marcescens is an important nosocomial pathogen causing various opportunistic infections, such as urinary tract infections, bacteremia and sometimes even hospital outbreaks. The recent emergence and spread of multidrug-resistant (MDR) strains further pose serious threats to global public health. This bacterium is also ubiquitously found in natural environments, but the genomic differences between clinical and environmental isolates are not clear, including those between *S. marcescens* and its close relatives. In this study, we performed a large-scale genome analysis of *S. marcescens* and closely related species (referred to as the '*S. marcescens* complex'), including more than 200 clinical and environmental strains newly sequenced here. Our analysis revealed their phylogenetic relationships and complex global population structure, comprising 14 clades, which were defined based on whole-genome average nucleotide identity. Clades 10, 11, 12 and 13 corresponded to *S. nematodiphila*, *S. marcescens sensu stricto*, *S. ureilytica* and *S. surfactantfaciens*, respectively. Several clades exhibited distinct genome sizes and GC contents and a negative correlation of these genomic parameters was observed in each clade, which was associated with the acquisition of mobile genetic elements (MGEs), but different types of MGEs, plasmids or prophages (and other integrative elements), were found to contribute to the generation of these genomic variations. Importantly, clades 1 and 2 mostly comprised clinical or hospital environment isolates and accumulated a wide range of antimicrobial resistance genes, including various extended-spectrum β -lactamase and carbapenemase genes, and fluoroquinolone target site mutations, leading to a high proportion of MDR strains. This finding suggests that clades 1 and 2 represent hospital-adapted lineages in the *S. marcescens* complex although their potential virulence is currently unknown. These data provide an important genomic basis for reconsidering the classification of this group of bacteria and reveal novel insights into their evolution, biology and differential importance in clinical settings.

Received 14 October 2021; Accepted 04 February 2022; Published 22 March 2022

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Keywords: *Serratia marcescens* complex; genomics; population structure; hospital-adapted lineage; antimicrobial resistance.

Abbreviations: AMR, antimicrobial resistance; ANI, average nucleotide identity; ANOVA, analysis of variance; CDI, contact-dependent inhibition; DFAST, DNA Data Bank of Japan Fast Annotation and Submission Tool; DTC, deoxyribonuclease-toluidine blue-cephalothin; ESBL, extended-spectrum β -lactamase; FQR, fluoroquinolone resistance; MDR, multidrug-resistant; ML, maximum-likelihood; QRDR, quinolone-resistance-determining region; Sma, *Serratia marcescens*; Sma_sak, *Serratia marcescens* subspecies *sakuensis*; Sne, *Serratia nematodiphila*; Ssu, *Serratia surfactantfaciens*; Sur, *Serratia ureilytica*; wgMLST, whole-genome multilocus strain typing; WGS, whole-genome sequence.

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The raw sequences obtained in this study have been deposited in GenBank/EMBL/DBJ under the BioProject accession number PRJDB10568 and Sequence Read Archive (SRA) accession numbers DRR253307 to DRR253531.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Nine supplementary figures and twelve supplementary tables are available with the online version of this article.

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DATA SUMMARY

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INTRODUCTION

Serratia marcescens (Sma) is a Gram-negative bacterium that is now recognized as an important opportunistic pathogen. It causes a range of nosocomial infections, such as pneumonia, urinary tract infections, bacteremia, meningitis and endocarditis, particularly in immunocompromised patients [1]. Many hospital outbreaks of this opportunistic pathogen, which sometimes occur recurrently, have also been reported [2–8]. While Sma is known to be naturally resistant to penicillins and first- and second-generation cephalosporins, strains showing higher levels of antimicrobial resistance (AMR) have also emerged and spread widely [9, 10]. In particular, strains producing extended-spectrum β -lactamases (ESBLs) and carbapenemases are highly problematic, as they are resistant to a wide range of β -lactams, thus posing serious problems in treating infections caused by this bacterium [11, 12].

Sma is ubiquitous in nature, being found in both water and soil, and it is associated with plant surfaces, insects and animals [13, 14]. Starting from the first whole-genome sequence (WGS) determination and detailed genomic comparison of two Sma strains (Db11, an isolate from an insect source; and SM39, a multidrug-resistant clinical isolate) [15], both of which were used as representative strains of Sma in this study, several comparative genome analyses have been reported. They included a large-scale genomic analysis of multidrug-resistant (MDR) clones in the UK and Ireland [9] and more recently published studies that analysed many publicly available WGSs [16–18]. The results of three recent studies indicated the presence of clinical isolate-enriched lineages, but WGSs of environmental isolates were limited in the database. Thus, it appears that genomic differences between clinical isolates and those inhabiting natural environments have not yet been fully understood.

Taxonomically, the genus *Serratia* has recently been moved from the family *Enterobacteriaceae* to the newly proposed family *Yersiniaceae* [19], but there are also various debates regarding the species/subspecies classification in the genus, in which more than 21 species/subspecies have been proposed. For example, although *S. marcescens* subspecies *sakuensis* (Sma_sak) has been proposed, recent studies indicated that there is no merit in its separation to subspecies status [20–22]. Although average nucleotide identity (ANI) analysis using WGSs is now widely used in bacterial and archaeal taxonomy [23, 24], the relationship of Sma and its close relatives, such as *S. nematodiphila* (Sne) and *S. ureilytica* (Sur), has not yet been well elucidated even in the above-mentioned comparative genome analyses [16–18, 22].

Herein, we performed a large-scale genome analysis of Sma and closely related species, which we referred to as the '*S. marcescens* complex (Sma complex)'; including 108 soil and 117 clinical isolates collected and sequenced in this study. Our analysis revealed their phylogenetic relationships and global population structure, which consisted of 14 clades defined based on the results of whole-genome ANI analysis. The 14 clades showed different genomic properties and distinct trends related to isolation sources. Based on the results, we propose the presence of two hospital-adapted clades in the Sma complex. The marked accumulation of AMR genes and mutations conferring fluoroquinolone resistance (FQR) is also demonstrated in these clades.

METHODS

Analyses of the type strains of genus *Serratia*

We downloaded the genome sequences of the type strains of 16 species and one subspecies belonging to genus *Serratia* from the NCBI Assembly database (last access: 21 January 2021). These sequences and those of two completely sequenced Sma strains, SM39 and Db11 [15], were used for core-gene-based phylogenetic analysis, with the type strain of *Y. enterocolitica* (NCTC 12982^T) as an outgroup (see Table S1, available in the online version of this article, for the list of type strains used). The core genes, which were defined as those present in all 20 strains ($n=801$), were identified using Roary version 3.7.0 [25] with a 60% BLASTp identity cutoff, and a core-gene alignment was generated using MAFFT [26]. Using the 296627 SNPs identified in the alignment, a maximum-likelihood (ML) phylogenetic tree was constructed using RAxML [27] with the GTR-GAMMA model of nucleotide substitution and 500 bootstrap replicates. The tree was displayed and annotated using ggtree version 2.2.4 [28] in the R package. ANI was calculated using PYANI version 0.2.7 [29] in ANIb mode. The 16S rRNA sequences of each strain were identified by BLASTn searches using the consensus 16S rRNA reference sequence of Sma SM39, which was obtained by aligning the sequences of seven copies using Clustal Omega [30] and created with the cons command in the EMBOSS package [31]. The 16S rRNA consensus sequences of other strains were obtained via the same procedure. As the finished genome sequence of the Sma-type strain was not available, we used the sequence of strain SM39 as a reference in this analysis.

Impact Statement

Serratia marcescens causes various opportunistic infections. Although *S. marcescens* and its close relatives are widely distributed in natural environments, their genomic differences have yet to be elucidated. Here, we present the results of a large-scale and comprehensive genome analysis of *S. marcescens* and its closely related species (referred to as the '*S. marcescens* complex' in this study). Our analysis revealed the global population structure of the complex, comprising 14 distinct clades, which will be an important basis for its reclassification in the future, and we identified two hospital-adapted clades. These two clades are clearly distinguished from *S. marcescens sensu stricto* and should be paid special attention because of their marked accumulation of a wide range of antimicrobial resistance genes, including various extended-spectrum β -lactamase and carbapenemase genes and mutations conferring fluoroquinolone resistance.

Isolation and genome sequencing of *Sma* strains from soil samples

We collected soil samples (approximately 10 g for each) in several regions in Japan and in Toulouse, France. Each sample was suspended in 30 ml saline in a 50 ml tube, vortexed for 3 min, and centrifuged at 100 g for 5 min at 4 °C. The supernatant was filtered through several sheets of sterilized gauze, centrifuged at 4000 g for 15 min at 4 °C, and inoculated onto a deoxyribonuclease-toluidine blue-cephalothin (DTC) agar plate [32], followed by a 24 h incubation at 37 °C under aerobic conditions. When colonies with a purple halo formed, one of the colonies was picked and inoculated onto a fresh DTC agar plate. After a 24 h incubation at 37 °C, a single colony was isolated from the plate and used for subsequent analyses. For species identification by 16S rRNA sequence determination, colonies were suspended in 50 μ l of 10 mM Tris-HCl (pH 8.0) buffer in 1.5 ml tubes, boiled at 100 °C for 5 min and rapidly cooled on ice. Samples were centrifuged at 18700 g for 10 min at room temperature to obtain supernatants to be used as PCR templates. PCR was performed using the KAPA Taq EXtra PCR kit (Nippon Genetics, Tokyo, Japan) and the 27F and 1492R universal primers. Each reaction mixture for PCR (15 μ l) contained 1 μ l of template DNA, 0.75 μ l of each primer (10 μ M), 0.45 μ l of dNTPs, 1.5 μ l of MgCl₂, 3 μ l of 5 \times EXtra buffer, and 0.075 μ l of KAPA Taq EXtra DNA polymerase. The PCR conditions employed were as follows: 96 °C for 2 min; 20 cycles of 1 min at 96 °C, 1 min at 55 °C, and 2 min at 72 °C; and 72 °C for 5 min. The PCR products were analysed by 1.5% agarose gel electrophoresis to confirm the generation of ca. 1.5 Kb amplicons. The amplicons were sequenced with capillary sequencers (Applied Biosystems 3130xL or 3500xL Genetic Analyzers, Thermo Fisher Scientific Japan, Tokyo, Japan) and compared to the 16S rRNA sequence of SM39 by using BLASTn. Isolates showing >99% sequence identity were used as *Sma* strains in this study. According to this procedure, we collected 108 soil strains in Japan and France. In addition, we obtained 117 clinical strains from various hospitals in Japan and France.

For genome sequencing, strains were cultured in Lysogeny broth overnight at 37 °C, and genomic DNA was purified from the cultures using a DNeasy Blood and Tissue kit (Qiagen, CA, USA). Sequencing libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, USA) and sequenced using the Illumina MiSeq platform to generate 300 bp paired-end reads.

Collection of publicly available genome sequences

We searched the NCBI database (<https://www.ncbi.nlm.nih.gov>) for the genomes registered as *Sma*, *Sma_sak*, *Sne* and *Sur* and found 844 *Sma*, 4 *Sma_sak*, 8 *Sne* and 2 *Sur* genomes (last access: 23 January 2019). From this dataset, we selected 644 genomes with records on country information and isolation sources. In particular, we included the genomes of type strains even if their strain information was not available.

Genome assembly, quality filtering and annotation

The genomes of 171 Japanese and 54 French strains sequenced in this study were assembled using Platanus_B assembler version 1.1.0 [33]. Of the 644 genome sequences obtained from the NCBI database, only assembled sequences were available for 176 genomes. As raw-read sequences were available for the remaining 468 genomes, these sequences were also assembled using the Platanus_B assembler. To remove low-quality sequences from those assembled from short reads, we first removed the sequences if the total number of scaffolds was greater than 300 or the read depth was less than 20 ($n=13$). Then, 79 genomes judged to be of low quality by CheckM [34] with a cutoff of <99% completeness or >3% contamination were removed (see Fig. S1 for the entire process of genome assembly and quality filtering). Two additional genomes were removed because they shared identical core-gene sequences with other strains in the strain set. Thus, we analysed a total of 775 strains (Table S2). For annotation, we used the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (DFAST) [35]. The sequencing statistics of the strains sequenced in this study are shown in Table S3.

ANI-based clustering of Sma complex strains and clade definition

ANI analysis was performed using PYANI version 0.2.7 as described above. Based on the all-to-all ANI matrix, strains were clustered via the complete linkage clustering method, and a dendrogram was generated with the hclust command in R. Clades were defined based on minimum within-node pairwise ANI scores with a 97% threshold.

Phylogenetic analysis and pangenome clustering of the Sma complex strains

Core genes ($n=2,488$), defined as those present in 99% of the strains analysed, were identified using Roary version 3.7.0 with an 80% BLASTP identity cutoff, and a core-gene sequence alignment was generated using MAFFT. Based on the 182520 SNPs identified in this alignment, an ML phylogenetic tree was constructed using RAxML, and displayed and annotated using ggtree version 2.2.4, as described above. Clustering analysis based on the presence/absence matrix of the pangenome (34342 genes) determined by Roary version 3.7.0 was performed using Ward's clustering method [36] and Euclidean distance with the hclust command in R, and the pangenome dendrogram was displayed using ggtree version 2.2.4. The presence/absence matrix of the pangenome is shown in Table S4.

Analysis of the phylogenetic relationships of the strains used in this study with the type strains in the Sma complex

To analyse the phylogenetic relationships of the strains used in this study with the type strains of the Sma complex and its close relatives, we selected all strains in clades 13 ($n=9$) and 14 ($n=1$), the strains that were registered as Sma_sak, Sne, or Sur (strain IDs; DL654, DL655 and DL659, respectively), one representative strain from each clade other than clades 13 and 14, and the type strains of Sma, Sma_sak, Sne, Sur and *S. surfactantfaciens* (Ssu). The type strain of *S. ficalia* was also included as an outgroup. In this strain set, core genes ($n=2790$) defined as those present in all 30 strains were identified using Roary version 3.7.0 with a 60% BLASTP identity cutoff, and a core-gene sequence alignment was generated using MAFFT. An ML phylogenetic tree was constructed based on the SNPs ($n=562767$) identified in the alignment using RAxML as described above. Pairwise ANI scores were also calculated using PYANI as described above.

Analysis of the distribution of the prodigiosin biosynthesis gene cluster

The prodigiosin biosynthetic cluster [37] was identified with DIAMOND version v0.9.21 [38] in BLASTX mode, with the amino acid sequences of the *pigA* - *pigN* gene products of the Sma strain ATCC 274 (GCA_009936295.1) [39] as a reference (Table S5). BLASTX hits with $\geq 50\%$ coverage and $\geq 80\%$ sequence identity were considered to indicate presence. The nucleotide sequence identities of the *pig* gene clusters (from the start codon of *pigA* to the stop codon of *pigN*) were further analysed in all the *pig*-positive strains except for two (DL253 and DL256; both belonging to clade 10) whose clusters were separated into two scaffolds. The all-to-all nucleotide sequence identities of the *pig* gene clusters of 85 strains were determined based on the alignment generated by Clustal Omega with the sequences of strains ATCC 274 and ATCC 39006 (GCA_000463345.3) as references.

Strain clustering based on SNP distances (determination of SNP₁₀ clusters)

The strain set used in this study included many subsets comprising very closely related strains, which could affect statistical analyses. To reduce this potential effect of strain bias, we performed strain clustering based on the SNP distances between each strain. The SNP distances in the core-gene alignment used for phylogenetic analysis were calculated using snp-dists version 0.4 (<https://github.com/tseemann/snp-dists>). Based on the SNP distances, the strains were clustered by Ward's method, and a dendrogram was generated with the hclust command in R. The maximum SNP distance in each cluster was identified, and clusters with a maximum within-cluster SNP distance of ≤ 10 , ≤ 20 or ≤ 30 were defined as SNP₁₀, SNP₂₀ and SNP₃₀ clusters, respectively. We randomly selected one strain from each multimember SNP₁₀ cluster as a representative strain for analyses. The details of the SNP₁₀, SNP₂₀ and SNP₃₀ clusters and the representative strains used are shown in Table S2.

Analyses of plasmid replicons, integrase genes, potentially virulence-related genes and the accessory genes specifically conserved in clades 1 and 2

Plasmid replicons were identified by PlasmidFinder [40]. Integrase genes were identified in the pangenome data generated by Roary. To identify the potentially virulence-related genes described in a previous study that compared the SM39 and Db11 genomes [15], the annotation data of SM39 and Db11 genomes (locus IDs) were converted to those in the DFAST annotation data, and corresponding genes/gene clusters were identified in the pangenome data. To identify the accessory genes specifically conserved in clades 1 and 2, accessory genes that were significantly more frequently present in the two clades than the other clades were identified by Scoary version 1.6.16 (Bonferroni $P < 0.01$) [41], and among these genes, those present in $> 90\%$ of the strains in both clades 1 and 2 were identified.

Statistical analyses

Statistical analyses other than the above-mentioned analysis performed by Scoary were performed in R version 4.1.0 [42]. One-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test was performed for the comparison of genome sizes between clades and that of GC contents between clades. Simple linear regression analysis was performed to analyse the relationship between the genome sizes and GC contents of each strain. Fisher’s exact test [43] with Benjamini–Hochberg correction [44] was performed to determine the difference in the ratio of clinical/hospital environment strains between the entire dataset and each clade.

Identification of AMR genes, FQR mutations and MDR strains

AMR genes were identified by SRST2 [45] using ARG-ANNOT_v3 (<https://www.mediterranee-infection.com/wp-content/uploads/2019/03/arg-annot-nt-v3-march2017.txt>) as a database. To analyse the genome sequences for which short-read sequences were not available, we used the Illumina MiSeq v3 100 bp paired-end simulated short-read data of each genome (insert size; 300 bp with a standard deviation of ± 10 bp, read depth; 100 \times) generated by ART version 2.5.8 [46]. When a gene was predicted as ‘-?’ by SRST2, the presence of the gene was further analysed by a BLASTn search [47] against the assembled genome sequence, and the presence/absence decision was made with the thresholds of sequence identity $\geq 90\%$ and coverage $\geq 80\%$. To search for FQR mutations, the amino acid sequences of the *gyrA*, *gyrB*, *parC* and *parE* gene products of each strain were aligned with those of *Escherichia coli* strain K-12 (NC_000913.3) using Clustal Omega [30], and FQR-associated point mutations in the quinolone-resistance-determining regions (QRDRs) of these gene products were identified [48, 49]. MDR strains were defined according to the definition proposed by Magiorakos *et al.* [50] as strains that were predicted to potentially be resistant to three or more classes/categories of antimicrobials based on the presence of AMR genes and FQR mutations. In this analysis, because *Sma* is known to be naturally resistant to penicillins, penicillins+ β -lactamase inhibitors, nonextended spectrum cephalosporins (first- and second-generation cephalosporins) and polymyxins [51], these classes were excluded from the analysis. In addition, because the presence of the *aac(6)-Ic* gene is not associated with aminoglycoside resistance due to its low expression and *Sma* strains are usually sensitive to aminoglycosides [51], *aac(6)-Ic* was excluded. Therefore, among the 77 AMR genes identified in the entire strain set, those conferring resistance to the following seven classes of antimicrobials were used to define MDR: aminoglycosides, extended-spectrum cephalosporins (third- and fourth-generation cephalosporins), carbapenems, phenicols, tetracyclines, sulfonamides/trimethoprim and fluoroquinolones. We considered a strain as being resistant to an antimicrobial class if the strain possessed at least one resistance gene related to the class (in the case of FQR, at least one FQR mutation in *gyrA*).

Programs, parameter settings and codes used in this study

Programmes, parameter settings and codes to run each analysis pipeline used in this study are listed in Table S6. The in-house programmes and their codes used are available in GitHub (<https://github.com/itTan-git/SMAScript>).

RESULTS

Phylogenetic relationships of *Sma* with closely related *Serratia* species

To investigate the phylogenetic relationships between *Sma* and its close relatives in the genus *Serratia*, we performed a core-gene-based phylogenetic analysis of the type strains of 17 *Serratia* species/subspecies and two completely sequenced *Sma* strains (SM39 and Db11) using the type strain of *Y. enterocolitica* as an outgroup (Table S1). In the constructed ML tree, the type strains of *Sma*, *Sma_sak*, *Sne*, *Sur* and *Ssu* formed a cluster distinct from those of other *Serratia* species. Strains SM39 and Db11 were included in this cluster, but they did not form a subcluster with the *Sma*-type strains (Fig. 1a). Although the type strain of *Ssu* showed ANI scores lower than 95% to all other strains, the pairwise ANI scores between the other strains were over 95%, except for that between SM39 and the type strain of *Sur* (94.5%) (Fig. 1b). Based on these results, we tentatively defined the three species, *Sma*, *Sne* and *Sur*, as the ‘*Sma* complex’, and we collected and analysed the genome sequences of strains belonging to this group.

Strain dataset for the phylogenetic analysis of the *Sma* complex

We collected the genome sequences of strains belonging to the *Sma* complex from various geographic regions and isolation sources (see Methods and Fig. S1). We isolated 108 *Sma* strains from soil samples collected in Japan and France and 117 *Sma* clinical strains from multiple hospitals in Japan and France and obtained their draft genome sequences. In addition, we collected the genome sequences of 644 strains from the NCBI database that were registered as species/subspecies belonging to the *Sma* complex along with their metadata (countries and sources). The type strains of the *Sma* complex species and completely sequenced strains were included in the dataset even if sufficient metadata were not recorded. After quality filtering and removing strain redundancy, the final dataset included 775 strains (225 sequenced in this study and 550 from the NCBI database; Table S2). These strains were isolated in 29 countries (divided into seven geographic regions in Fig. 2a). Approximately 43% of the strains were isolated in Europe ($n=336$), of which more than half of them were from the UK ($n=195$), many of which were analysed in a population study of *Sma* isolated from cases of bacteremia [9]. Regarding the isolation sources, 68.8% were clinical isolates ($n=533$), and

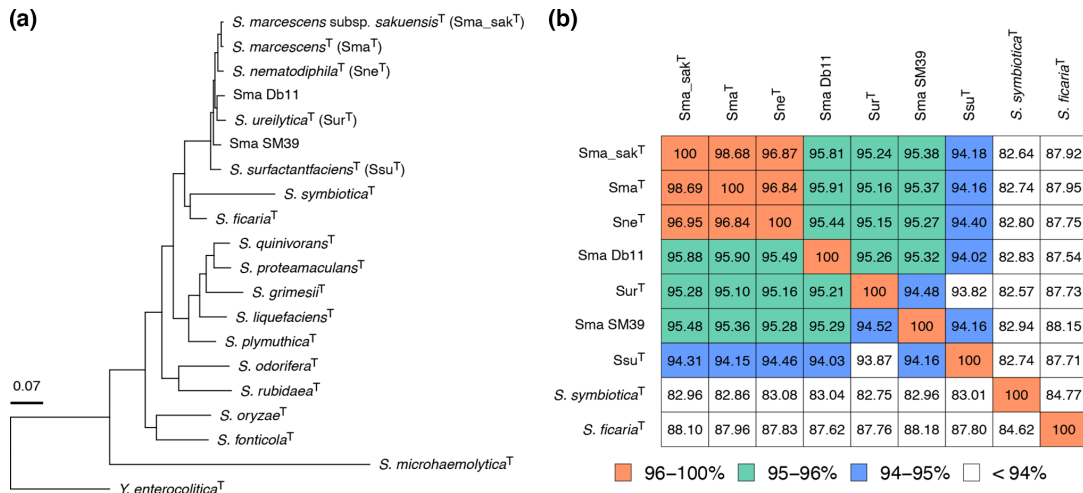


Fig. 1. Phylogenetic relationships between *Serratia* species. (a) An ML phylogenetic tree based on the core gene SNPs (296627 sites in 801 genes) of the type strains of 17 *Serratia* species/subspecies and two completely sequenced *S. marcescens* strains (SM39 and Db11), with the *Y. enterocolitica* type strain as an outgroup. The core genes that are present in all strains were identified using Roary with a 60% BLASTP identity cutoff. (b) ANI matrix among the nine strains of *S. marcescens* (Sma) and its close relatives. All-to-all ANI values were calculated using PYANI in ANIb mode, and cells are coloured according to ANI values. Strains other than Db11 and SM39 are type strains of each species/subspecies.

9.5% were isolated from hospital environments ($n=74$). The remaining 21.7% ($n=168$) were isolated from other sources, mostly from natural environments (Fig. 2b). The strains sequenced in this study represented 64.7% of the last group, thus markedly expanding the genome resources of environmental strains in the Sma complex.

ANI-based clustering of the Sma complex strains

We first classified the 775 strains based on their pairwise ANI scores. By using the minimum within-node ANI of 97% as a threshold, which was employed because the ANI score between the type strains of Sma and Sne was 96.84%, the strains were divided into 14 clades (Fig. 3). In this study, we isolated and performed genome sequencing of the strains whose 16S rRNA

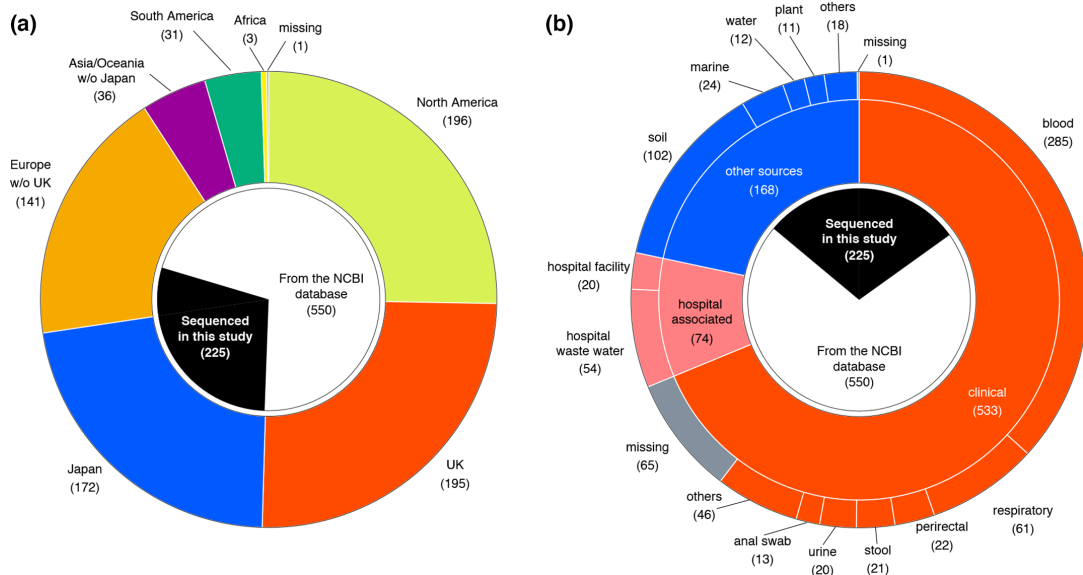


Fig. 2. Regions and sources of the isolation of the Sma complex strains. (a) Distribution of the regions where strains were isolated. Regions were classified into seven geographic regions: the UK, Japan, North America, South America, Europe other than the UK (Europe w/o UK), Asia/Oceania other than Japan (Asia/Oceania w/o Japan), and Africa. (b) Distribution of isolation sources. Subcategories that contained <10 strains were grouped as 'others'. The proportion of strains that were sequenced in this study is indicated in the most inner circles in both panels.

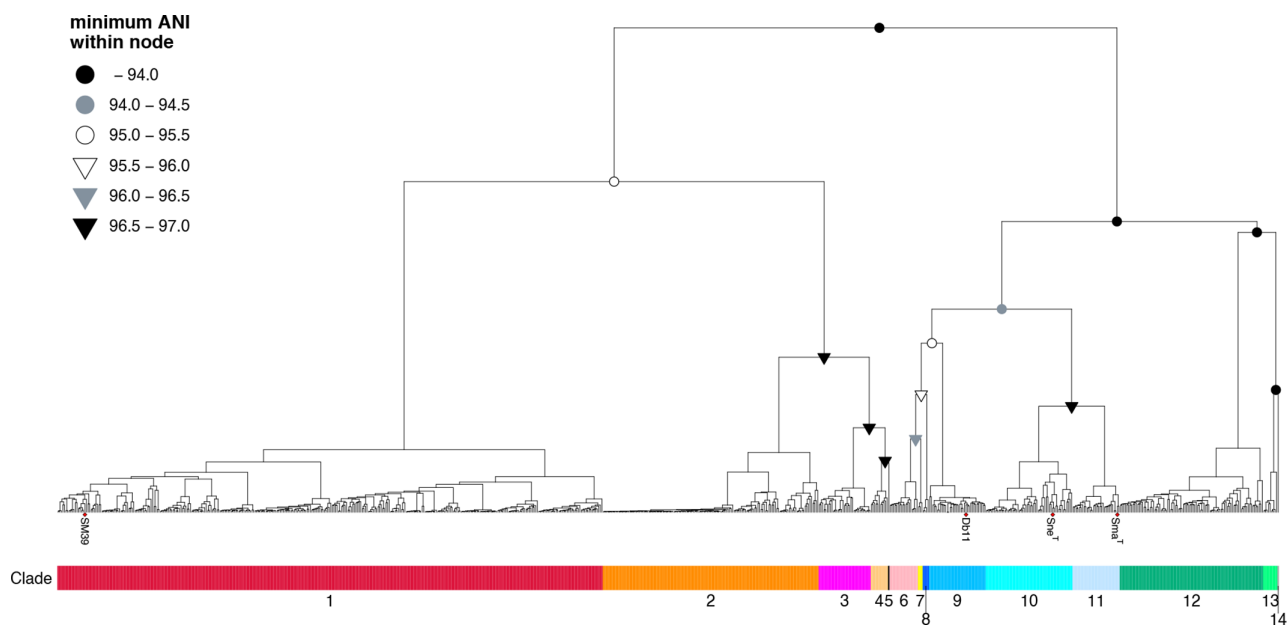


Fig. 3. ANI-based clustering of the Sma complex strains. Complete-linkage unsupervised hierarchical clustering of the Sma complex strains was performed based on their all-to-all ANI analysis. The type strains of *S. marcescens* (Sma¹), *S. nematodiphila* (Sne¹), and two Sma strains (Db11 and SM39) are indicated by their names. Nodes whose minimum pairwise ANI scores were <97% are indicated by coloured symbols according to minimum within-node ANI scores. Fourteen clades defined according to the minimum within-node ANI threshold of 97% are indicated under the tree.

sequences showed >99% identity to that of strain SM39, and we collected the genome sequences of strains registered in the NCBI database as the species/subspecies belonging to the initially defined Sma complex using a >95% ANI threshold. However, the minimum ANI scores between clades were below 95% in several cases (indicated by black and grey filled circles in Fig. 3). This result indicated that our strain set included more diverse strains than we initially expected and highlighted the difficulty of the species identification of strains belonging to the Sma complex and its close relatives.

Core-gene-based phylogenetic analysis of the Sma complex and the relationships between clades and known species

Next, we performed a core-gene-based phylogenetic analysis of our strain dataset (Fig. 4). By mapping the clade information to the phylogenetic tree, we confirmed that the strains belonging to the same clade clustered together and formed monophyletic clusters.

The type strains of Sma (ATCC 13880^T) and Sne (CGMCC 1.6853^T) belonged to clades 11 and 10, respectively. Although the type strains of Sma_sak (DSM 17174^T) and Sur (CCUG 50595^T) were not included in the strain set because their sequences were not available when we designed this study, the phylogenetic analysis of these type strains with representative strains of each clade revealed that the Sma_sak type strain belonged to clade 11 together with the Sma-type strain and indicated that the Sur-type strain belonged to clade 12 (Fig. S2). The former finding is consistent with a recent report [22]. Notably, strain CRK0003, which was registered as Sne in the database, belonged to clade 3 (not to clade 10) and showed 94.95% ANI to the Sne-type strain belonging to clade 10 (Fig. S2). On the other hand, strain K27, which was registered as Sma_sak, belonged to clade 10 and showed 98.44% ANI to the Sne-type strain but 96.72 and 96.69% to the type strains of Sma and Sma_sak, respectively.

Clades 13 ($n=9$) and 14 ($n=1$) were distantly related to the other clades; their ANI scores relative to all other clades were lower than 94%. The phylogenetic analysis of these strains with representative strains of other clades and the type strains of species closely related to the Sma complex (*Ssu* and *S. ficalia*) revealed that clade 13 corresponded to *Ssu* (Fig. S2), while the 16S rRNA sequences of the clade 13 strains and the *Ssu*-type strain showed >99.44% identity to the sequences of the Sma complex (Fig. S3). This analysis also revealed that clade 14 (single-member clade) represented a lineage that was more distantly related to the initially defined Sma complex (clades 1–12) than *Ssu* (clade 13) and was most closely related to *S. ficalia* among the 14 clades, but the 16S rRNA sequence also showed >99.03% identity to those in the finished genomes in the Sma complex and 98.77% to that of the *S. ficaria*-type strain. The clade 14 strain was isolated from *Rhyncholacis pedicillata* in the Guayana region of Venezuela [52] and was shown to have anti-oomycete, antifungal and antibacterial activities [53].

Based on these findings, we redefined the Sma complex to include *Ssu* (clade 13) and clade 14.

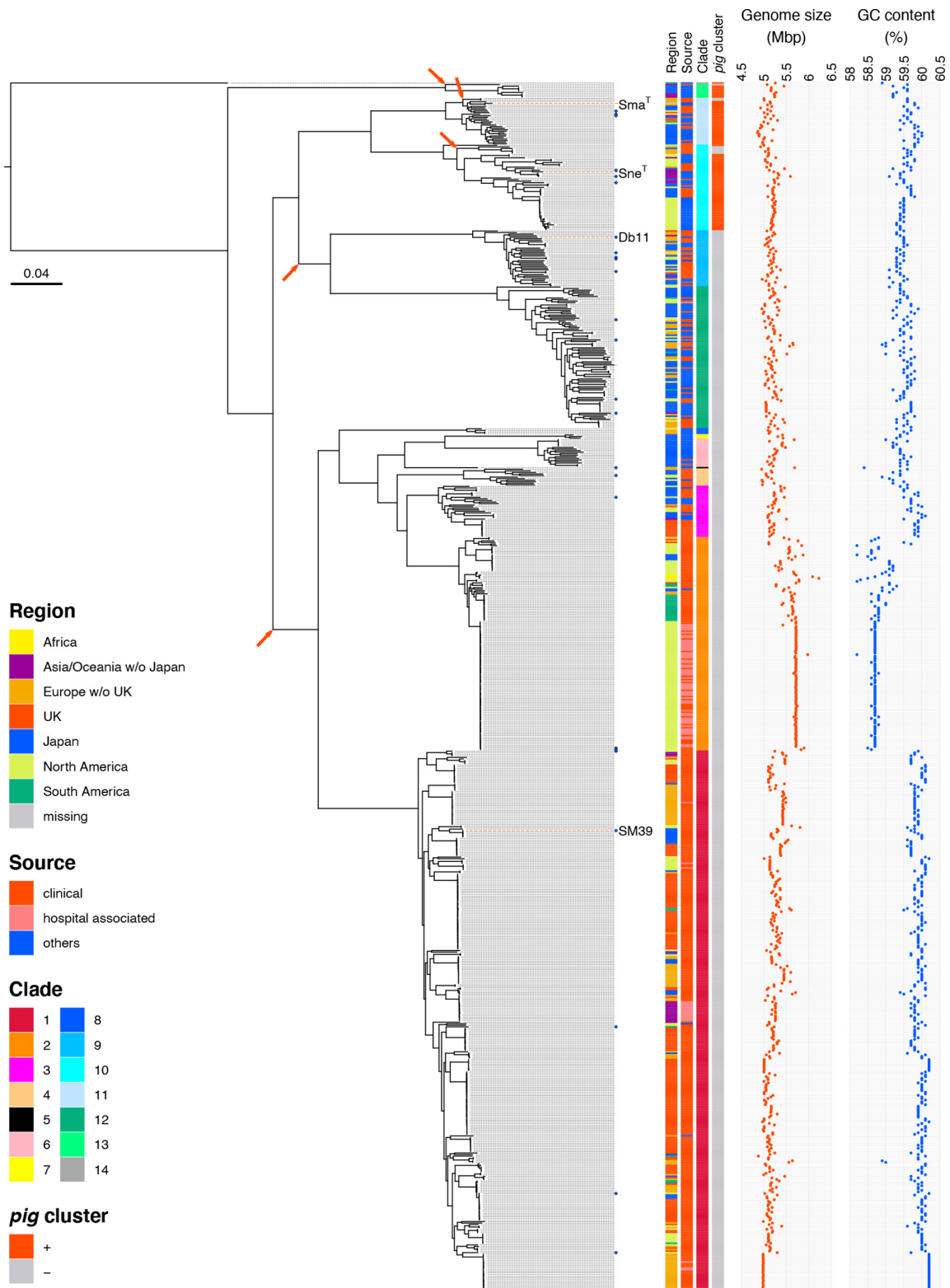


Fig. 4. Phylogenetic relationships among the *Sma* complex strains. The phylogenetic relationship between the *Sma* complex strains analysed in this study ($n=775$) is shown. An ML tree was constructed based on the 182520 SNP sites identified in 2488 core genes. The root was defined by a similar phylogenetic analysis using the type strain of *S. ficalia* as an outgroup. Blue diamonds indicate the strains whose complete genome sequences are available. The type strains of *Sma* and *Sne* and two *Sma* strains (Db11 and SM39) are indicated by their names and red dotted lines. For each strain, the isolation region, isolation source, clade, presence/absence of the *pig* gene cluster, total genome size and GC content are shown. Branches where the deletion of the *pig* gene cluster occurred are indicated by arrows.

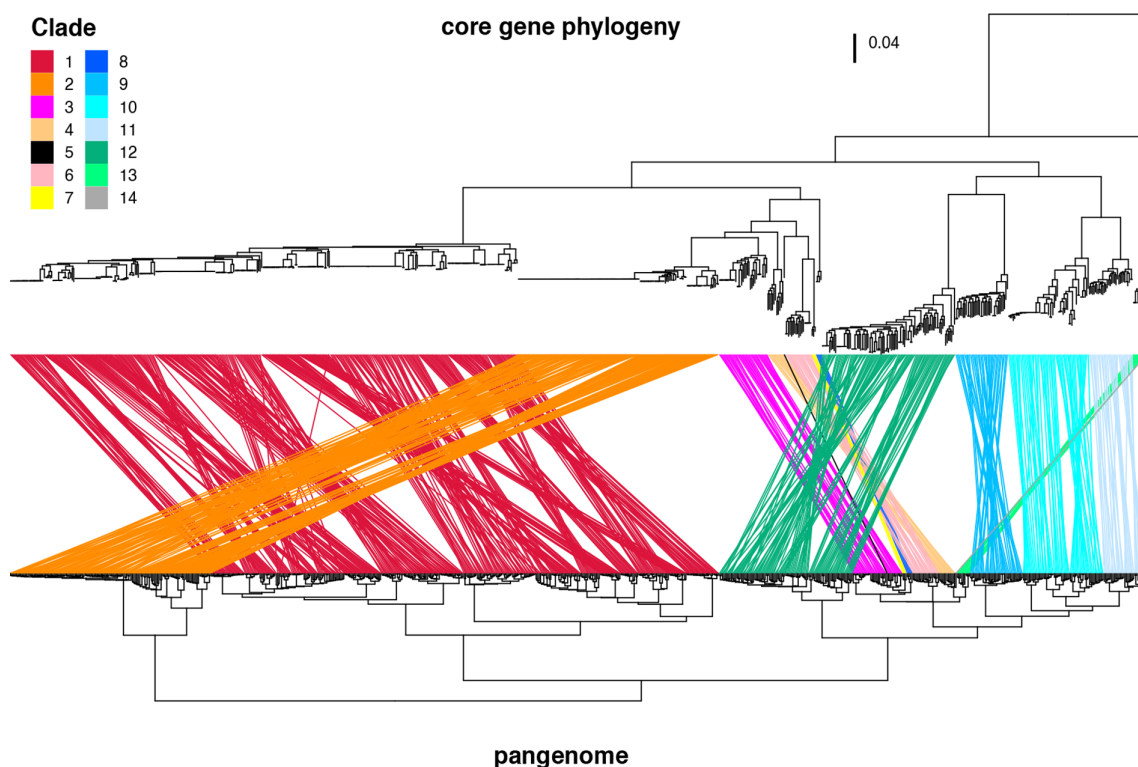


Fig. 5. Relationship between the core-gene-based phylogeny and the pangenome-based clustering of the Sma complex strains. The upper tree is the core-gene-based ML tree shown in Fig. 4, and the lower dendrogram was constructed based on the cluster analysis of the pangenome ($n=34342$) identified by Roary. Ward's clustering method with Euclidean distance was used for the cluster analysis. The same strains present in the two trees are connected by coloured lines according to their clades.

Pangenome clustering of Sma complex strains

To examine the differences and similarities of the accessory gene contents within each clade and between clades, we performed a pangenome-based clustering analysis of the Sma complex strains (Fig. 5). The clustering of the strains by this method was consistent with that obtained via the ANI-based approach; strains belonging to the same clade clustered together. This result indicates the presence of accessory genes unique to each clade or shared by clades in an evolutionary context and corroborated the presence of the 14 clades defined based on the results of ANI analysis.

Distribution of the *pig* gene cluster

The production of a red pigment known as prodigiosin is a well-known characteristic of Sma [1], but it was reported that the prodigiosin biosynthesis gene cluster (*pigA* - *pigN*) is not present in all Sma strains [54]. The analysis of the distribution of the *pig* gene cluster in our strain dataset revealed that the *pig* gene cluster was distributed only in clades 10 (corresponding to Sne), 11 (Sma and Sma_sak), 13 (Ssu) and 14 (Fig. 4). All *pig*-positive strains contained a full set of *pig* gene cluster comprising 14 genes in the chromosome region upstream of the *copA* gene encoding a copper-translocating P-type ATPase. The nucleotide sequences of these gene clusters were highly conserved, showing >98.2% identity to the cluster of Sma strain ATCC 274 [39], except for the genes of clade 14 (92.4%) and one clade 10 strain (ADJS-2C_Purple, SAMN08373108; 80.4%), but notably lower similarities (<60%) to the *pig* gene cluster found in the *Serratia* sp. strain ATCC 39006 [55]. The low sequence identity of the ADJS-2C_Purple genes appeared to be due to low sequence quality of this chromosome region. These findings indicate that the *pig* gene cluster was acquired by the common ancestor of the Sma complex and has been deleted from most clades in the Sma complex. The distribution of *pig*-negative strains in the phylogenetic tree shown in Fig. 4 indicated that the deletion of the *pig* gene cluster occurred at least five times in the strain set analysed in this study (indicated by arrows in Fig. 4, including the deletions in three sublineages/strain in clades 10, 11 and 13).

Inter- and within-clade variations in genome size and GC content related to horizontal gene transfer

Notable interclade differences in genome sizes and GC contents were observed (Fig. 4). However, clades 1 and 2 contained many subclades comprising very closely related strains. To reduce such strain bias, we grouped strains with less than a 10-SNP distance

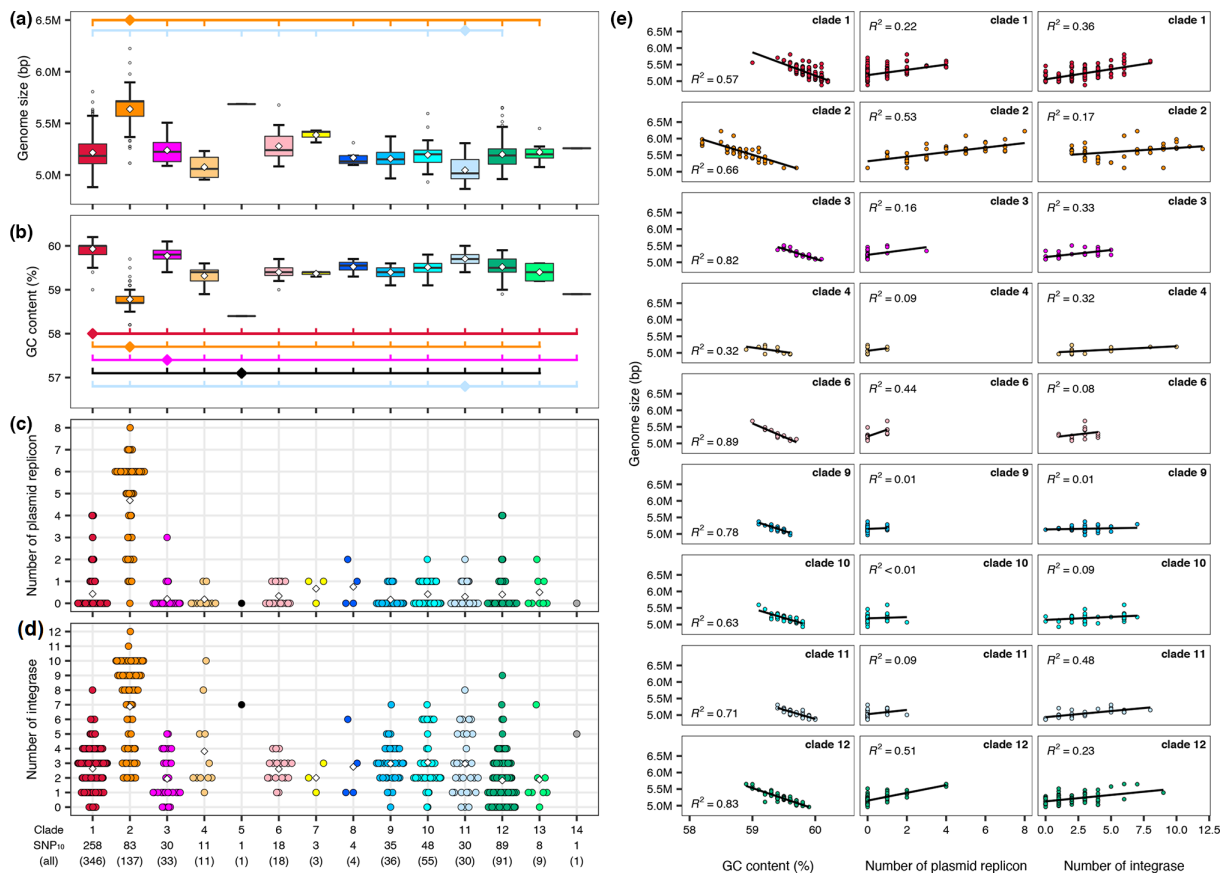


Fig. 6. Interclade differences in genome size and GC content. Genome size ranges (a) and GC content ranges (b) of SNP₁₀ clusters belonging to each clade. For multimember SNP₁₀ clusters, one strain was randomly selected from each cluster. Open diamonds in the boxplot indicate the mean values of each clade. Clades that show significant differences ($P < 0.01$) compared to the clades marked by diamonds are indicated by short vertical lines. Lines are coloured according to the clade indicated by diamonds. Only the combinations that show significant interclade differences are shown. The numbers of total strains and SNP₁₀ clusters in each clade are shown at the bottom of (d). (c, d) The numbers of plasmid replicons and integrase genes found in the SNP₁₀ clusters belonging to each clade. Diamonds indicate the averages in each clade. (e) Correlations between genome size and GC content (left) and between genome size and the numbers of plasmid replicons (middle) or integrase genes (right) in nine clades that contained >10 members. Linear regression lines and R-squared results are shown. In all panels, colours for each clade are the same as those in Figs. 3–5.

into one cluster (named the SNP₁₀ cluster) and then randomly selected one strain from each multimember cluster for clade comparison. Among the 619 SNP₁₀ clusters identified, 515 were single-member clusters and 93 contained two or three members, while 11 clusters contained four or more members (up to seven). Notably, limited numbers of SNP₁₀ clusters showed marked within-cluster variations in genome size and GC content (Fig. S4). In addition, there were no clusters that contained strains isolated in different countries while several clusters comprised strains that were isolated 8–10 years apart in different countries when the SNP₂₀ distance metric was used (see Table S2 for all strain-clustering information).

Statistical analysis using representative strains from the 619 SNP₁₀ clusters revealed that the genome size of clade 2 was significantly larger than those of other clades (excluding clades 5, 7 and 14, which were single- or three-member clades), and clade 11 contained significantly smaller genomes than the seven other clades (Fig. 6a). Conversely, the GC content of clade 2 was significantly lower than those of all other clades except for two single-member clades, and the GC contents of clades 1 and 3 were higher than those of most other clades (Fig. 6b). The GC contents of the clade 11 genomes were lower than those of clades 1 and 3 but higher than those of seven clades. Further analysis of nine clades containing >10 members revealed a negative correlation between genome size and GC content in all nine clades ($R^2 = 0.32\text{--}0.89$) (Fig. 6e).

Because the Sma complex members showed relatively high GC contents (ranging from 58.2–60.2% in this study), it is most likely that the lower GC contents of large genomes are due to the acquisition of more genes from other species via horizontal gene transfer [56, 57]. To confirm this possibility, we analysed plasmid replicons and integrase genes (genetic markers of prophages and other integrative elements) in each SNP₁₀ cluster. As shown in Fig. 6(c, d) (see Figs. S5 and S6, Table S7 for the distribution of the identified 35 plasmid replicons and 130 integrase genes in the Sma complex), clade 2 contained apparently more plasmids

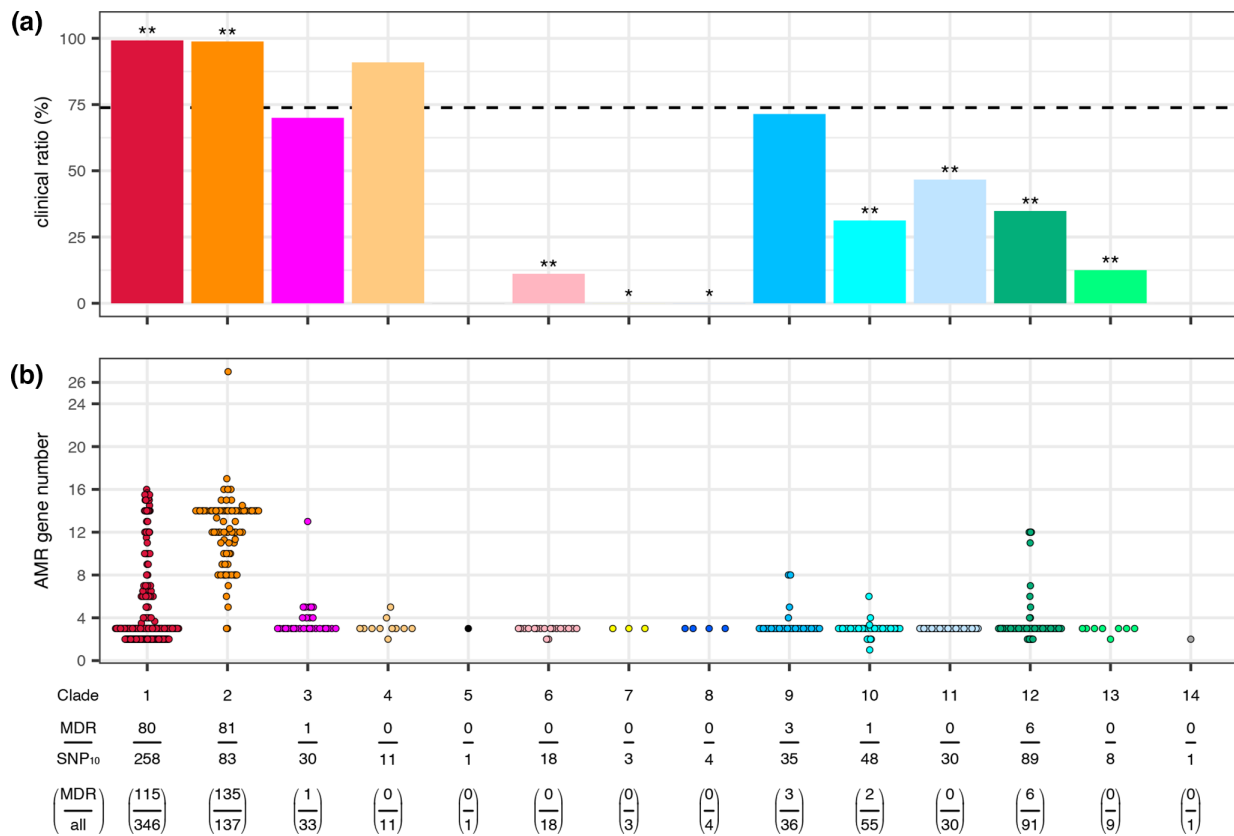


Fig. 7. Proportions of the SNP₁₀ clusters isolated from clinical/hospital environmental sources in each clade and numbers of AMR genes in each SNP₁₀ cluster belonging to each clade. (a) The proportions of the SNP₁₀ clusters isolated from clinical/hospital environment samples in each clade. The dotted line indicates the proportion of clinical/hospital environment clusters in the entire strain set (73.8%). Note that the isolation sources of the strains belonging to the same SNP₁₀ clusters were the same for all multimember clusters. *, $P < 0.05$; **, $P < 0.01$. (b) The numbers of AMR genes in each SNP₁₀ cluster belonging to each clade. The numbers of AMR genes in multimember SNP₁₀ clusters were calculated as the average numbers in each SNP₁₀ cluster. The numbers of MDR strain-containing SNP₁₀ clusters, total SNP₁₀ clusters, MDR strains and total strains belonging to each clade are indicated at the bottom. In both panels, colours for each clade are the same as those in Figs. 3–6.

and prophages (and other integrative elements) than the other clades, indicating that the larger genome size of clade 2 is attributed considerably to the acquisition of these mobile genetic elements. In addition, notable within-clade variations in the numbers of plasmid replicons and integrase genes, particularly that of integrase genes, were also observed. Further analysis of the nine clades revealed a positive correlation between genome size and the numbers of plasmid replicons (clades 2, 6 and 12) or those of integrase genes (clades 1, 3, 4 and 11) in seven of the nine clades ($R^2 > 0.32$) (Fig. 6e). This result indicates that the acquisition of different types of mobile genetic elements contributed to the generation of observed within-clade variations in genome size (and GC content) in each clade.

Difference in isolation sources between clades and identification of hospital-adapted clades

We next analysed the differences in isolation sources among clades using the 619 strains representing each SNP₁₀ cluster (Fig. 7a). Importantly, when the SNP₁₀ distance was used as the threshold for strain clustering, 33.8% of the hospital environment isolates (25/74) formed SNP₁₀ clusters with clinical isolates while no isolates from the other sources did not form SNP₁₀ clusters with any clinical isolates (note that, when the SNP₂₀ distance was used as the threshold, 79.9% of the hospital environment isolates clustered with clinical isolates while only two strains from the other sources clustered with clinical isolates; see Table S2 for the details). Therefore, hospital environment isolates in our strain set were unable to be distinguished from clinical isolates. More importantly, nearly all SNP₁₀ clusters in clades 1 and 2 were clinical or hospital environment isolates (99.2 and 98.8%, respectively), and these ratios were significantly higher than the average ratio for the entire strain set (73.8%) ($P < 0.01$ by Fisher's exact test after false discovery rate correction by the Benjamini–Hochberg method). In contrast, the ratios of such SNP₁₀ clusters in seven clades (clades 6–8 and clades 10–13) were significantly lower ($P < 0.01$ for clades 6, 10, 11, 12 and 13; $P < 0.05$ for clades 7 and 8). As mentioned before, clades 10, 11, 12 and 13 correspond to Sne, Sma (and Sma_sak), Sur and Ssu, respectively. This result suggests that clades 1 and 2 represent hospital-associated clades in the Sma complex.

Accumulation of AMR genes and fluoroquinolone target-site mutations in hospital-adapted clades

As two hospital-associated clades were identified, we analysed the distribution of AMR genes and FQR-associated mutations (FQR mutations) in the *gyrA*, *gyrB*, *parC* and *parE* genes [48, 49] in our strain set (Fig. 7b, Fig. 8, Tables 1 and 2 and S8). In this section, although 23 out of the 104 multimember SNP₁₀ clusters showed some variation in the repertoire of AMR genes and FQR mutations (Table S9), a SNP₁₀ cluster was counted as positive for the gene/mutation of interest if one of the cluster members contained it.

AMR genes:

We detected 77 AMR genes in the entire strain set. Among these, *bla*_{SRT-SST} (*ampC*) and *aac(6)-Ic*, which are known to be chromosomally encoded in *Sma* [1], were highly conserved in the set, including clades 13 and 14. The *tet41* gene, encoding a tetracycline-specific efflux pump [58], was also widely distributed but was absent in all but one strain of clade 1, indicating that *tet41* was lost from clade 1. Many of the SNP₁₀ clusters in clade 1 (190/258) contained additional AMR genes that were probably acquired horizontally (up to 13). More strikingly, nearly all clade 2 clusters (81/83) contained additional AMR genes (up to 23). Thus, AMR genes have undergone marked accumulation in these two clades. In contrast, in the other clades, a small number of clusters contained additional AMR genes (28 clusters in five clades). All 28 clusters were clinical isolates except for a bovine isolate in clade 12 (Fig. S7).

FQR mutations:

As summarized in Table 1, FQR mutations in *gyrA* were detected in 264 SNP₁₀ clusters. Mutations in *parC* (30 clusters) and *parE* (13 clusters) were also detected, but all of these clusters contained *gyrA* mutations. Similar accumulation of FQR mutations is often found in Gram-negative bacteria and is known to enhance FQR [59]. Among the identified *gyrA* mutations, the majority were located at Ser83, Ser83Ile (186 clusters) or Ser83Arg (78 clusters). In five clusters, an additional *gyrA* mutation was present at Asp87. Such double mutations are also associated with high levels of FQR [59].

Notable accumulation of FQR mutations also occurred in clades 1 and 2. In clade 1, 67.4% of the 258 clusters contained *gyrA* mutations, with 23 and 11 clusters additionally carrying *parC* or *parE* mutations. The five aforementioned clusters that contained double *gyrA* mutations all belonged to clade 1. In clade 2, 85.5% of the 83 clusters contained *gyrA* mutations, but only one or two clusters additionally contained mutations in *parC* and *parE*, respectively. In the other clades, while *gyrA* mutations were detected in nine clade 3 clusters (9/30), including five clusters that additionally contained mutations in *parC*, only a few or no clusters contained FQR mutations in the remaining clades.

In addition to FQR mutations, four FQR-associated AMR genes (*oqxB*, *qnrA*, *qnrB* or *qnrS*) were found in 19 clusters, but most of them (15/19) contained *gyrA* mutations.

MDR strains:

The accumulation of horizontally acquired AMR genes and FQR mutations in clades 1 and 2 resulted in a high proportion of MDR strains in these clades; 31.0% of the SNP₁₀ clusters in clade 1 and 97.6% of the clade 2 SNP₁₀ clusters were genotypically defined as MDR (resistant to three or more categories of antimicrobials; see Methods for the definition of MDR). The higher proportion of MDR clusters in clades 1 and 2 was not a simple reflection of a higher proportion of clinical and hospital environment isolates in these clades because the proportions of MDR strains among the clinical isolates of the other clades were much lower than those of clades 1 and 2 (11 out of the 202 clinical clusters in all other clades).

ESBL and carbapenemase genes:

We analysed these genes in more detail because they are the clinically most important genes worldwide (Table 2). We detected five ESBL genes in 122 clusters and seven carbapenemase genes in 122 clusters, among which 82 clusters carried both ESBL and carbapenemase genes. These clusters, particularly ESBL/carbapenemase double-positive clusters (77/82), frequently contained *gyrA* mutations (Fig. 8).

Among the detected ESBL genes, *bla*_{TEM-1D} was the most prevalent (104 clusters), followed by *bla*_{SHV-OKP-LEN} (52 clusters) and *bla*_{CTX-M1} (25 clusters), with a few clusters containing *bla*_{CTX-M2} and *bla*_{CTX-M9}. A notable number of clusters contained multiple ESBL genes (62 clusters, one of which contained three ESBL genes). Among the carbapenemase genes detected, *bla*_{KPC-1} was the most frequently found (68 clusters), followed by *bla*_{NDM-1} (14 clusters), *bla*_{SME-1} (14 clusters), *bla*_{GIM-1} (12 clusters) and *bla*_{OXA-48} (10 clusters), with a few clusters containing *bla*_{IMP-1} or *bla*_{VIM-1}. Notably, one cluster in clade 12 contained two carbapenemase genes (*bla*_{NDM-1} and *bla*_{OXA-48}).

Strains carrying ESBL and/or carbapenemase genes were highly enriched in both clades 1 and 2, but a clear difference in the repertoire of these genes was observed between the two clades (Table 2). In clade 1, among the 47 ESBL-positive clusters, *bla*_{TEM-1D} (37 clusters) and *bla*_{CTX-M1} (20 clusters) were predominant, and 11 clusters contained both of these genes (one of them additionally contained *bla*_{SHV-OKP-LEN}). Among the 45 carbapenemase-positive clade 1 clusters, *bla*_{SME-1}, *bla*_{GIM-1}, *bla*_{NDM-1} and *bla*_{OXA-48} were predominant (found in 11 or nine clusters). In addition, 21 clusters contained both ESBL and carbapenemase genes. In clade

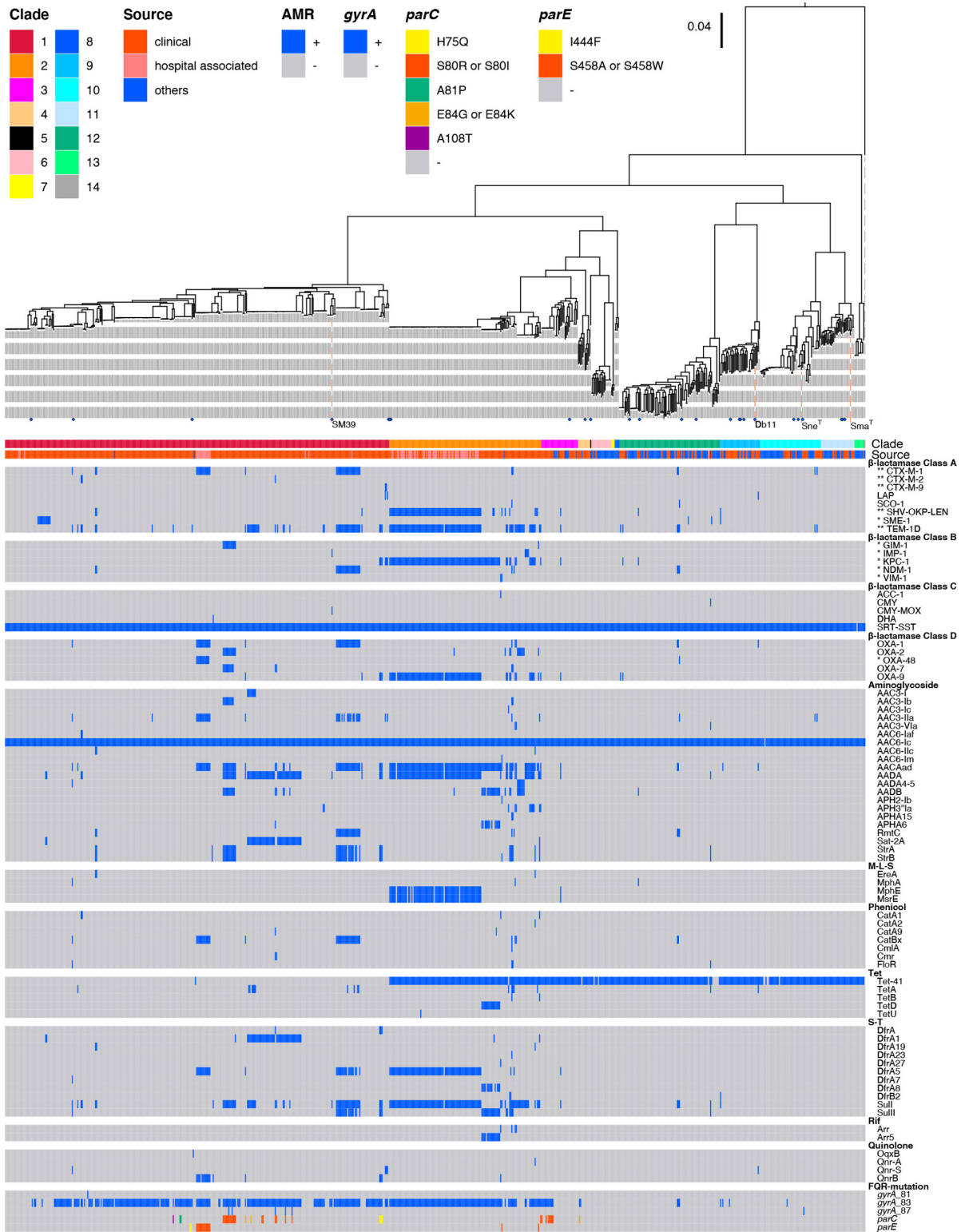


Fig. 8. Distributions of AMR genes and FQR-conferring mutations in the Sma complex strains. The same ML tree and strain information on the clade and isolation source presented in Fig. 4 are shown here. Different mutations in the *parC* and *parE* genes are indicated by different colours. Carbapenemase and ESBL genes are marked by * and **, respectively.

Table 1. Distribution of FQR mutations

		Entire strain set	Clades						
			1	2	3	4	9	10	12
	Number of SNP ₁₀ clusters (number of strains)	619 (775)	258 (346)	83 (137)	30 (33)	11 (11)	35 (36)	48 (55)	89 (91)
<i>gyrA</i>	total	264 (393)	174 (248)	71 (124)	9 (11)	2 (2)	3 (3)	2 (2)	3 (3)
	Gly81Asp	1 (1)	1 (1)	0	0	0	0	0	0
	Ser83Ile	186 (268)	106 (134)	69 (121)	9 (11)	0	2 (2)	0	0
	Ser83Arg	78 (123)	68 (113)	3 (3)	0	2 (2)	1 (1)	1 (1)	3 (3)
	Asp87Tyr	3 (3)	3 (3)	0	0	0	0	0	0
	Asp87Asn	2 (2)	1 (1)	0	0	0	0	1 (1)	0
	Asp87Gly	1 (1)	1 (1)	0	0	0	0	0	0
	double mutations in <i>gyrA</i> *	total	5 (5)	5 (5)	0	0	0	0	0
	Ser83Ile + Asp87Tyr	3 (3)	3 (3)	0	0	0	0	0	
	Ser83Ile + Asp87Asn	1 (1)	1 (1)	0	0	0	0	0	
	Ser83Ile + Asp87Gly	1 (1)	1 (1)	0	0	0	0	0	
<i>parC</i>	total	30 (35)	23 (26)	1 (1)	5 (7)	1 (1)	0	0	0
	His75Gln	2 (3)	2 (3)	0	0	0	0	0	0
	Ser80Ile	22 (25)	16 (17)	1 (1)	5 (7)	0	0	0	0
	Ser80Arg	1 (1)	1 (1)	0	0	0	0	0	0
	Ala81Pro	1 (2)	1 (2)	0	0	0	0	0	0
	Glu84Lys	2 (2)	1 (1)	0	0	1 (1)	0	0	0
	Glu84Gly	1 (1)	1 (1)	0	0	0	0	0	0
	Ala108Thr	1 (1)	1 (1)	0	0	0	0	0	0
<i>parE</i>	total	13 (17)	11 (15)	2 (2)	0	0	0	0	0
	Ile444Phe	2 (2)	2 (2)	0	0	0	0	0	0
	Ser458Trp	9 (13)	9 (13)	0	0	0	0	0	0

Continued

Table 1. Continued

		Entire strain set	Clades						
			1	2	3	4	9	10	12
	Ser458Ala	2 (2)	0	2 (2)	0	0	0	0	0
<i>gyrA</i> + <i>parC</i>	total	30 (35)	23 (26)	1 (1)	5 (7)	1 (1)	0	0	0
<i>gyrA</i> + <i>parE</i>	total	13 (17)	11 (15)	2 (2)	0	0	0	0	0
<i>OqxB</i> , <i>QnrA</i> , <i>S</i> , or <i>B</i>	total	19 (25)	16 (22)	2 (2)	0	0	1 (1)	0	0
	without FQR mutations	4 (4)	3 (3)	0	0	0	1 (1)	0	0

All of the five strains additionally contained Ser80Ile or Ser80Arg mutations in *parC*.

*The clusters/strains containing double *gyrA* mutations were also included in the count of each mutation.

2, much higher proportions of clusters contained ESBL and carbapenemase genes than in clade 1. ESBL genes were found in 79.5% of the clade 2 SNP₁₀ clusters. *bla*_{TEM-1D} (60 clusters) and *bla*_{SHV-OKP-LEN} (47 clusters) were the most prevalent of these genes and were frequently present together (41 clusters). Carbapenemase genes were found in 68 clusters (81.9%), and most of them (61/68) contained *bla*_{KPC-1}. The proportion of ESBL/carbapenemase double-positive clusters was also much higher than in clade 1 (65.1 vs. 8.1%).

The distribution of potentially virulence-related genes in the Sma complex and the identification of accessory genes specifically conserved in the hospital-adapted clades

The virulence factors of Sma have not been well defined except for several factors, such as the ShyA haemolysin [60]. However, a set of potentially virulence-related genes/operons were listed up in a previous study where a detailed genomic comparison of strains SM39 and Db11 [15]. We therefore analysed the distribution of these genes/operons in the strain set to search for potentially virulence-related genes/operons specifically conserved in clades 1 and 2 (Fig. S8 and Table S10). Although this analysis identified the genes/operons that are relatively well conserved across the Sma complex and those exhibited variable distributions, only an operon encoding a haemolysin or contact-dependent inhibition (CDI)-related protein and a protein for its secretion (*cdiA2* and *shlB_3* in Fig. S8) and two fimbriae operons (represented by *ecpC* and *yehB* in Fig. S8) showed a distribution somewhat biased to clades 1 and 2.

To further explore the genetic features potentially underlying the hospital adaptation of clades 1 and 2, we searched for accessory genes that are highly conserved in both clades 1 and 2 (present in >90% of SNP₁₀ clusters) but significantly less frequently present in the other clades (Bonferroni $P < 0.01$) and identified 287 such genes (Table S11; the distribution of top 100 genes in the Sma complex is shown in Fig. S9). This gene set included not only the genes in the above-mentioned three operons but also many genes/operons for various metabolic functions, but it is currently unknown whether or how these genes are involved in the adaptation of clades 1 and 2 to hospital-related environments.

DISCUSSION

In this study, we performed a large-scale genomic analysis of Sma and its close relatives (the Sma complex), including newly sequenced 108 soil and 117 clinical isolates, and obtained several taxonomically, biologically or medically important findings.

First, our analysis clarified the global population structure and complex phylogenetic relationships of Sma and its close relatives. According to the results of ANI-based clustering, we defined 14 clades in which the minimum within-clade ANI values were >97% (Fig. 3). This clade structure was supported by core-gene-based phylogenetic analysis and pangenome-based clustering (Figs. 4 and 5). Among the 14 clades, clades 10, 11, 12 and 13 included the type strains of Sma, Sma, Sur and Ssu, respectively (Fig. S2); thus, the four clades represent these species. The type strain of Sma_sak belonged to clade 11, together with the Sma type strain, confirming their very close relationship pointed out recently [61]. The other clades each represent distinct lineages in this complex. These results indicate not only the marked diversity but also the insufficient classification status of the Sma complex, thus providing an important basis for reconsidering the classification of this complex in the future. It should also be emphasized the difficulty

Table 2. Distribution of ESBL and carbapenemase genes

		Entire strain set	Clades						
			1	2	3	9	10	12	
Number of SNP ₁₀ clusters (number of strains)		619 (775)	258 (346)	83 (137)	30 (33)	35 (36)	48 (55)	89 (91)	
ESBLs	total	122 (191)	47 (69)	66 (112)	1 (1)	1 (1)	1 (2)	6 (6)	
	TEM-1D	104 (167)	37 (54)	60 (105)	1 (1)	0	1 (2)	5 (5)	
	SHV-OKP-LEN	52 (97)	2 (3)	47 (91)	1 (1)	1 (1)	0	1 (1)	
	CTX-M1	25 (45)	20 (39)	2 (2)	0	0	1 (2)	2 (2)	
	CTX-M2	3 (3)	3 (3)	0	0	0	0	0	
	CTX-M9	1 (2)	1 (2)	0	0	0	0	0	
	multiple ESBLs*	total	62 (121)	15 (30)	43 (86)	1 (1)	0	1 (2)	2 (2)
	TEM-1D + SHV-OKP-LEN	43 (86)	1 (1)	41 (84)	1 (1)	0	0	0	
	TEM-1D + CTX-M1	15 (30)	10 (24)	2 (2)	0	0	1 (2)	2 (2)	
	TEM-1D + CTX-M2	3 (3)	3 (3)	0	0	0	0	0	
	TEM-1D + SHV-OKP-LEN + CTX-M1	1 (2)	1 (2)	0	0	0	0	0	
carbapenemases	total	122 (198)	45 (67)	68 (122)	1 (1)	1 (1)	0	7 (7)	
	KPC-1	68 (122)	4 (6)	61 (113)	1 (1)	0	0	2 (2)	
	NDM-1	14 (29)	9 (24)	2 (2)	0	0	0	3 (3)	
	SME-1	14 (15)	11 (12)	0	0	1 (1)	0	2 (2)	
	GIM-1	12 (13)	11 (12)	1 (1)	0	0	0	0	
	OXA-48	10 (13)	9 (12)	0	0	0	0	1 (1)	
	IMP-1	3 (5)	1 (1)	2 (4)	0	0	0	0	
	VIM-1	2 (2)	0	2 (2)	0	0	0	0	
	multiple carbapenemases*	total	1 (1)	0	0	0	0	0	1 (1)
		NDM-1 + OXA-48	1 (1)	0	0	0	0	0	1 (1)
ESBLs + carbapenemases*	total	82 (148)	21 (41)	54 (100)	1 (1)	1 (1)	0	5 (5)	

Continued

Table 2. Continued

	Entire strain set	Clades					
		1	2	3	9	10	12
Number of SNP₁₀ clusters (number of strains)	619 (775)	258 (346)	83 (137)	30 (33)	35 (36)	48 (55)	89 (91)
TEM-1D & KPC-1	13 (15)	2 (3)	10 (11)	0	0	0	1 (1)
CTX-M1 & OXA-48	9 (12)	9 (12)	0	0	0	0	0
CTX-M9 & KPC-1	1 (2)	1 (2)	0	0	0	0	0
SHV-OKP-LEN & KPC-1	4 (4)	0	3 (3)	0	0	0	1 (1)
SHV-OKP-LEN & VIM-1	1 (1)	0	1 (1)	0	0	0	0
SHV-OKP-LEN & SME-1	1 (1)	0	0	0	1 (1)	0	0
TEM-1D + CTX-M1 & NDM1	11 (25)	8 (22)	1 (1)	0	0	0	2 (2)
TEM-1D + SHV-OKP-LEN & KPC-1	42 (85)	0	41 (84)	1 (1)	0	0	0
TEM-1D & NDM-1 + OXA-48	1 (1)	0	0	0	0	0	1 (1)
TEM-1D & NDM-1 + OXA-49	1 (2)	1 (2)	0	0	0	0	0

*The clusters/strains containing these combinations of genes were also included in the count of each gene.

to accurately identify the species in the Sma complex via 16S rRNA sequence analyses that has a much lower resolution power than whole-genome-ANI analysis; sequence identities of 16S rRNA genes within the Sma complex were over 99%.

Second, the comparison of the 14 clades revealed notable interesting inter- and within-clade variations in genome size and GC content, and we found that the acquisition of mobile genetic elements such as plasmids and prophages is related to the generation of these variations (Figs. 4 and 6). In particular, clade 2 strains contained larger genomes with lower GC contents than the other clades, which was attributable to the acquisition of more plasmids and prophages (and other integrative elements) than the other clades. The within-clade variation in genome size and GC content and a negative correlation of these two genomic parameters were observed in not only clade 2 but also other clades, and interestingly, it appears that different types of mobile elements contributed to the generation of the variations observed in different clades, which may be related to the difference in main environments where each clade inhabits or in other biological or genetic features of each clade. However, some cautions are required to interpret the current results because our preliminary results of plasmid analysis suggest that considerable numbers of plasmid replicons are unable to be detected by PlasmidFinder used in this study (one third of plasmids was not detected in some strains) and because the genetic features of each mobile element are largely unknown at present.

The medically most important finding was the identification of two clades (clades 1 and 2) that comprised almost exclusively clinical or hospital environment isolates (Figs. 4 and 7). As the higher ratios of such strains in these clades were statistically significant, they can be regarded as hospital-adapted lineages in the Sma complex. In this context, the marked accumulation of a wide range of AMR genes and FQR mutations observed in the two clades is interesting (Figs. 7 and 8, Tables 1 and 2). Multiple genes encoding ESBLs and carbapenemases were found in many strains in these clades, and strains carrying both ESBL and carbapenemase genes were also frequently identified, particularly in clade 2. The accumulation of AMR genes was also observed in several strains of the other clades, but all such strains were clinical isolates with a single exception. Thus, it is most likely that the accumulation of AMR genes in clades 1 and 2 is the result of repeated exposure to multiple antibiotics due to their long persistence in clinical settings. This notion is supported by the wide distribution of the Ser83Phe (or Leu) substitution in *gyrA* and the presence of additional mutations in *parC* and *parE* in notable numbers of strains of these clades. The accumulation of AMR genes in clades 1 and 2 also suggests that these clades can be important reservoirs of AMR genes in hospitals. Regarding the

mechanisms underlying the adaptation of clades 1 and 2 to hospital-related environments, we have identified a set of accessory genes that were specifically conserved in both clades (Table S11 and Fig. S9) although it is currently unknown whether or how these genes are involved in this adaptation. It is also unknown whether clades 1 and 2 have a higher potential virulence than other clades. However, our data will be a basis for investigating these important issues in future studies. Considering that many potentially virulence-related functions are encoded by core genome (Table S10 and Fig. S8) as previously suggested [15], it will be also important to search for core genes that are under positive selection in clades 1 and 2 [62].

Finally, we should mention the relationships of the clades defined in this study and the (sub)clades/clusters/lineages defined in the recently published three genome-based analyses [16–18]. In the study by Abreo and Altier [16], 45 strains analysed were classified into two clades (clades 1 and 2) each containing several subclades based on the data of whole-genome multilocus strain typing (wgMLST) analysis, and the two clades were mostly comprised of environmental (clade 1) or clinical (clade 2) isolates. In particular, subclades 2c and 2d included only clinical strains. Of the 45 strains, 34 were included in our strain set and it appears that subclades 2c and 2d correspond to clades 2 and 1 of this study, respectively (see Table S12 for more details of the comparison). On the other hand, Saralegui *et al.* divided 452 strains into nine clusters by ANI-based clustering [18]. Of the 452 strains, 338 were included in our strain set and it appears that cluster 1 corresponds to clades 10/11 of this study; clusters 3–5 to clades 2, 9 and 12, respectively; clusters 6/7/8 to clade 1; and cluster 9 to clade 10, but cluster 2 included strains from multiple clades of this study (see Table S12 for more details). More recently, Matteoli *et al.* analysed 642 genomes and divided them into 12 lineages, three of which (lineages Sm5, Sm6 and Sm7) were comprised of only human-associated strains [17]. In addition, the authors reported several interesting findings, such as the presence of more AMR genes in lineages Sm5 and Sm7 as well as lineage Sm9, which is also a human-associated strain-enriched lineage, the wide distribution of *bla*_{KPC-2} in lineage Sm7, and the presence of more plasmids in lineage Sm9. Although 340 out of the 601 strains analysed by Matteoli *et al.* were included in our strain set, we could not analyse the relationships between their lineages and the clades of this study because the strain compositions of the 12 lineages were not provided in their paper.

In conclusion, through a large-scale genome analysis, we revealed the global population structure of the Sma complex, comprising 14 distinct clades, which will be an important basis for its reclassification in the future, and we further identified two hospital-adapted clades. Although the potential virulence of these clades are currently unknown, special attention needs to be paid to these clades because of the marked accumulation of a wide range of AMR genes, including ESBL and carbapenemase genes, and mutations that confer FQR.

Funding information

This work was supported by KAKENHI from the Japan Society for the Promotion of Science (18K16175) granted to TH. DSN was supported by a Japanese Government Scholarship from MEXT (Ministry of Education, Culture, Sports, Science and Technology).

Acknowledgements

We thank Mai Horiguchi, Yuriko Satoh and Kie Ozaki for providing technical assistance and the members of the T. Itoh and E. Oswald laboratories for collecting soil samples.

Author contributions

Conceptualization: N.G., T.H. Methodology: T.O., I.T., D.Y., T.I., T.H. Validation: T.O., I.T., K.N., Y.G., Y.O., T.H. Formal analysis: T.O., I.T., M.P.S., T.H. Investigation: T.O., I.T., D.S.N., M.P.S., A.I., K.M., A.S., T.H. Resources: T.O., R.N., K.M., D.Y., T.I., A.S., D.D., E.O., N.G., T.H. Data curation: T.O., I.T., D.S.N., T.H. Writing – original draft: T.O., I.T., T.H. Writing – review and editing: T.O., I.T., K.N., Y.O., E.O., N.G., T.H. Visualization: T.O., I.T., T.H. Supervision: K.N., Y.G., Y.O., N.G., T.H. Project administration: N.G., T.H. Funding acquisition: T.H.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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