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Genome Report

Genome of *Superficieibacter maynardsmithii*, a novel, antibiotic susceptible representative of *Enterobacteriaceae*

Gherard Batisti Biffignandi,[1] Marjorie J. Gibbon,[2] Marta Corbella,[3] Harry A. Thorpe,[4] Cristina Merla,[3] Michele Castelli,[5] Teemu Kallonen,[6] Katie Pegrum,[2] Sylvain Brisse,[7] Jukka Corander,[4,8,9] Piero Marone,[3] Edward J. Feil,[2] and Davide Sassera[1,*]

[1]Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia 27100, Italy
[2]The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, Bath BA 27AY, UK
[3]UOC Microbiologia e Virologia, Fondazione IRCCS Policlinico San Matteo, Pavia 27100, Italy
[4]Department of Biostatistics, University of Oslo, Oslo N-0317, Norway
[5]Department of Biology and Biotechnology, University of Pavia, Pavia 27100, Italy
[6]Department of Clinical Microbiology, Turku University Hospital, Turku 20521, Finland
[7]Institut Pasteur, Biodiversity and Epidemiology of Bacterial Pathogens, Paris 75015, France
[8]Parasites & Microbes, Wellcome Sanger Institute, Hinxton CB10 1SA, UK
[9]Department of Mathematics and Statistics, Helsinki Institute for Information Technology, University of Helsinki, Helsinki FIN-00014, Finland

*Corresponding author: Department of Biology and Biotechnology, University of Pavia, Pavia 27100, Italy. davide.sassera@unipv.it

Abstract

During a citywide microbiological screening project in Pavia (Italy) a bacterial strain isolated from the surface of an Automated Teller Machine was classified as a *Klebsiella* sp. by MALDI-TOF spectrometry, and shown to be susceptible to the most antimicrobial classes by phenotypic testing. After Illumina genome sequencing and subsequent assembly, a high-quality draft genome was obtained (size = 5,051,593 bp, N50 = 615,571 bp, largest contig = 1,328,029 bp, N_contig = 17, GC content = 51.58%, coverage = 141.42), absence of antimicrobial resistance genes was confirmed, but the strain resulted to be highly divergent from all *Klebsiella*, and more related to other *Enterobacteriaceae*. The higher values of 16S rRNA identity were with members of the genera *Citrobacter*, *Salmonella*, and "*Superficieibacter*." An ortholog-based phylogenomic analysis indicated a sister group relationship with "*Superficieibacter electus*," in a distinct clade from other members of the *Enterobacteriaceae* family. In order to evaluate whether the novel genome represents a new species of "*Superficiebacter*," average nucleotide identity (ANI) and Hadamard analysis were performed on a dataset of 78 *Enterobacteriaceae*. The novel genome showed an ANI of 87.51% with *S. electus*, which compared on identity values between other members of the family, clearly indicates that the genome represents a new species within the genus "*Superficieibacter*." We propose for the new species the name "*Superficieibacter maynardsmithii*."

Keywords: *Superficieibacter*; *Enterobacteriaceae*; ANI; Hadamard

Introduction

The family *Enterobacteriaceae* (*Gammaproteobacteria*), which includes a large spectrum of Gram-negative, facultatively anaerobic, nonspore-forming, and rod-shaped bacteria (Octavia and Lan 2014). From a biochemical standpoint, members of this family are in general catalase positive and oxidase negative, with the ability to reduct the nitrate to nitrite, and product acid starting from glucose fermentation (Brenner and Farmer 2015).

Many members of this family, such as *Citrobacter koseri*, *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Enterobacter* spp. are well-known pathogens, of which the last two belonging to the ESKAPE group (Rice 2008). Due to the massive antibiotics use in the last three decades, an increased prevalence of resistant strains was observed within the *Enterobacteriaceae* family, enabling a rapid worldwide spread of MDR clones (Wilson and Török 2018). Several mechanisms are involved in these antimicrobial resistances, acquired due to gene acquisitions or by point mutations. The most diffused β-lactamase resistance related genes are *bla*-TEM, *bla*-SHV, *bla*-VEB, or *bla*-CTX-M (Nicolas-Chanoine *et al.* 2007; Bonomo 2017) . The most diffused carbapenemase coding genes are *bla*-KPC, *bla*-OXA-48, *bla*-IMP, *bla*-NDM, and *bla*-VIM (Logan and Weinstein 2017), while other carbapenem resistance mechanisms can be conferred by point mutations causing porin loss, such as those on the *ompK* gene, when coupled with the expression of extended-spectrum β-lactamases (ESBLs) or AmpC-type β-lactamases gene (Hamzaoui *et al.* 2018). Other *Enterobacteriaceae* are classified as commensal, but in general members of this family tend to be widespread, capable to colonize many environmental niches, such as water and soil (Brenner and Farmer 2015). The isolates not associated with human hosts are in general more susceptible to antibiotics such as cefotaxime, aztreonam, gentamicin, nalidixic acid, and ciprofloxacin (Österblad *et al.* 1999). However, recent works have observed that

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also the environmental isolates can harbor several antibiotic resistance genes (Berglund 2015; Caltagirone *et al.* 2017).

The family *Enterobacteriaceae* was revisited by Adeolu in 2016 to include 22 officially recognized and seven "additional" (not validated by the International Committee on Systematics of Prokaryotes) genera (Adeolu *et al.* 2016). Recently, the family has expanded to include novel genera, for a current total of 34 recognized and 19 additional genera (bacterio.net accessed September 28, 2020). "*Superficieibacter*" (Potter *et al.* 2018) is one of these novel, not officially recognized ones. Indeed, the high level of phenotypic and genomic heterogeneity of this family has made taxonomic classification difficult through the years. In fact, although the 16S rRNA gene is widely used in bacterial phylogeny and systematics, it has been shown to be an unreliable marker for the order *Enterobacteriaceae*, due to highly variable levels of intra and inter genus identity and polyphyly of well-established taxa, such as *Escherichia*, *Enterobacter, Kluyvera, and Klebsiella* (Alnajar and Gupta 2017). The low efficiency of 16sRNA in placing the members of *Enterobacteriaceae* in a proper taxonomic context was evidenced within *Klebsiella* genus, as previously described (Boye and Hansen 2003; Naum *et al.* 2008). Also, more recent works on *Klebsiella* noticed this issue, such in the case of *Klebsiella indica* sp. nov (Gujarati *et al.* 2020) in which it was observed a higher 16sRNA similarity with *S. enterica*, and in the description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. *tropicalensis* subsp. nov., and *K. variicola* subsp. *variicola* subsp. nov (Rodrigues *et al.* 2019). Thus, the necessity of more reliable frameworks to assign the correct taxonomy arose. Multiple authors have shown that multilocus sequence analysis is able to improve phylogenetic resolution, assigning a more reliable clade allocation within this family (*e.g.*, Brady *et al.* 2016; Tambong 2019). Moreover, massive whole genome sequencing has allowed to use high throughput approaches such as core genes phylogeny (Segata *et al.* 2011) and overall genomes relatedness techniques such as the Overall Genetic-Related Index (OGRI) (Chun and Rainey 2014) and the average nucleotide identity (ANI) (Goris *et al.* 2007; Chun *et al.* 2018).

Among the recently described genera of *Enterobacteriaceae*, the genus "*Superficieibacter*" currently comprises only one species, "*Superficieibacter electus,*" proposed by Potter and collaborators in 2018, after recovering two highly similar isolates on surfaces in an intensive care unit in Pakistan. The described bacterium is facultative aerobic Gram-negative bacillus capable of fermenting arabinose, amygdalin, melibiose, saccharose, rhamnose, sorbitol, lactose, glucose, and mannitol. The two isolated strains of "*S. electus*" resulted negative for arginine dihydrolase, indole production, acetoin production and motility, and positive for sorbitol fermentation (Potter *et al.* 2018). It is important to note that both strains resulted to possess two extended-spectrum beta-lactamase genes: *bla*-SHV12 and *bla*-TEM-1B, while retaining susceptibility to carbapenems.

During a microbiological sampling performed in and around the city of Pavia, Northern Italy, with the aim to study the diversity of genus *Klebsiella*, a strain isolated from the surface of an Automated Teller Machine, named 1612_C1, was observed and further investigated.

Materials and methods

Bacterial isolation and antibiotic tests

An area of 10 cm^2 from the surface of an Automated Teller Machine in Pavia, Northern Italy, was swabbed using a sterile swab. After the collection, the swab was enriched in Luria Bertani broth with amoxicillin (10 mg/ml) at 36 ± 1°C for 24 h. The enrichment was plated onto Simmon's Citrate Agar with 1% Inositol (SCAI) and plates were incubated at 36 ± 1°C for 48 h. Species identification was performed using MALDI ToF-MS (Bruker). Antibiotic susceptibility was tested through Phoenix automated system (Becton Dickinson).

Genome sequencing and assembly

The genomic DNA of isolate 1612_C1 was extracted using the QIAsymphony automated instrument (QIAGEN) with the DSP Virus/Pathogen kit (QIAGEN). DNA was then sequenced using Illumina HiSeq X Ten platform with a 2 × 150 nt paired-end protocol. The resulting sequencing Reads were then subjected to assembly using Spades (Bankevich *et al.* 2012). Genome annotation was performed using PROKKA (Seemann 2014). The genome of isolate 1612_C1 was screened for resistance and virulence genes using Abricate (https://github.com/tseemann/abricate v1.0.1), using all the resistance databases available (cutoffs 90% coverage and 90% identity). The 16S rRNA sequence was detected using Barrnap (https://github.com/tseemann/barrnap v.0.9) and then extracted manually. 16S rRNA sequence similarity was searched using Blastn against the nr NCBI online database.

Ortholog gene phylogeny

The dataset for the phylogenetic analysis was constructed based on a set of 78 representative members of the family *Enterobacteriaceae*, plus a *Pasteurella multocida* as outgroup for the analysis (see Supplementary Table S1 for a complete list). All genomes were retrieved from NCBI in February 2020. The protein sequences of ORFs from all genomes were predicted through Prodigal (Hyatt 2010). Then, single copy orthogroup calling was performed using Orthofinder (Emms and Kelly 2019). Obtained orthologues were aligned with Muscle (Edgar 2004) and filtered using Gblocks (Talavera and Castresana 2007) to remove highly variable positions. After concatenation, the best evolutionary model (PROTGAMMAILG) was assessed via Modeltest-ng (Darriba *et al.* 2020) and used to infer the phylogeny using RAxML (Stamatakis 2014) with 100 bootstraps. The resulting tree was visualized using iTOL (Letunic and Bork 2019).

Average nucleotide identity calculation

The ANI was measured on the 79 genomes dataset through the tool pyANI (Pritchard *et al.* 2016), choosing the Mummer option. Specifically, two set of values were analyzed: ANI identity and Hadamard dot product values, the second being the matrix dot product between percentage identity and alignment length for each pairwise genome comparison; starting from *a*, *b* dimensionally identical matrices (coverage and identity, respectively), each element *i, j* of the Hadamard matrix represented by *c* is the result between the product of *i, j* element of the two starting matrices:

$$cij = aij\ bij.$$

ANI and Hadamard matrices were analyzed in R Software version 3.5.3 and two heatmaps were generated using the "Pheatmap" package (Kolde 2015).

Data availability

All sequencing data are available at NCBI under Bioproject PRJNA670827, Biosample SAMN16442788.

Supplementary material is available at G3 online.

Results and discussion

Genome characterization

A high-quality draft genome assembly was obtained (size = 5,051,593 bp, N50 = 615,571 bp, largest contig = 1,328,029 bp, N_contig = 17, GC content = 51.58%, coverage = 141.42) from the 1612_C1 isolate. A total of 4721 coding sequences were predicted. The 16S rRNA (1539 bp) similarity found as best hits the 16S rRNA genes of *Enterobacteriaceae*: *S. enterica* with identity of 98.70%, followed by *C. koseri* (identity 98.57%), *Salmonella bongori* (97.99%) and "*S. electus*" (97.75%). *In silico* screening of resistance and virulence genes was performed using Abricate, and none was found. The possible presence of virulence genes was checked also in the two available genomes of "*S. electus*," which resulted devoid of them as well. Additional studies could investigate whether these isolates are indeed not virulent or if they present highly divergent factors, not recognizable just by sequence comparison with known genes. The absence of resistance genes is coherent with the performed susceptibility tests (Table 1).

Phylogenetic analysis

The initial MALDI-ToF MS identification identified the isolate as a member of the genus *Klebsiella*, but a blast search on the 16S rRNA appeared to disagree with this result. Thus, in order to place the 1612_C1 isolate within the proper taxonomic context, phylogenetic placement was inferred using a concatenate of single copy orthologous ($n = 612$), using a dataset of 78 *Enterobacteriaceae*, plus a *P. multocida* as outgroup.

The single copy orthologous phylogenomics presents an overall tree topology congruent with the established taxonomic distribution of the different genera of the family (Figure 1). The tree clearly shows that the 1612_C1 isolate clusters with the two available genomes of *S. electus*, resulting in a distinct, well supported clade from other *Enterobacteriaceae*. The evolutionary distance between the two previously published *S. electus* strains is very low as expected, while the branch leading to the 1612_C1 isolate is clearly longer. A similar situation in terms of evolutionary distance between species of the same genus can be found in other genera on the tree, such as *Kosakonia*, *Citrobacter*, and *Kluyvera*, suggesting that 1612_C1 could represent a novel species of the "*Superficieibacter*" genus.

Table 1 Results of the antibiotic susceptibility test performed on Phoenix automated system (Becton Dickinson)

Antibiotic	Susceptibility
Amikacin	≤4, S
Amoxicillin | clavulanic acid	≤2 | 2, S
Ampicillin	4, S
Aztreonam	≤1, S
Cefalexin	≤4, S
Cefepime	≤1, S
Cefixime	≤0.5, S
Ceftazidime	≤0.5, S
Ceftriaxone	≤0.5, S
Cefuroxime	4, S
Ciprofloxacin	≤0.25, S
Ertapenem	≤0.25, S
Gentamicin	≤1, S
Imipenem	0.5, S
Levofloxacin	≤0.5, S
Meropenem	≤0.125, S
Nitrofurantoin	≤16, S
Norfloxacin	≤0.5, S
Piperacillin	≤4, S
Piperacillin | tazobactam	≤4 | 4, S
Ticarcillin | clavulanic acid	≤4 | 2, S
Tobramycin	≤1, S
Trimethoprim	≤1, S
Trimethoprim | sulfamethoxazole	≤1 | 19, S

Average nucleotide identity analysis

Isolate 1612_C1 exhibited the highest ANI with *S. electus* with a score of 87%. The currently accepted general ANI threshold to consider two strains as belonging to the same species is >95% (Jain *et al.* 2018), indicating a novel species for 1612_C1.

Due to the lack of specific taxonomic boundaries for ranks higher than species, especially in the context of *Enterobacteriaceae*, both the ANI percentage identity and Hadamard score were considered, comparing the results among genera within a representative dataset of members of this family, in order to evaluate the possible presence of an empirical threshold to identify a novel putative genus/species. The comparative ANI analysis allowed a more weighted comparison, and showed that the average ANI score for species within the same genus varies greatly within *Enterobacteriaceae*. For example, this value ranges from 83% to 96% in *Enterobacter* genus, from 85% to 94% in *Klebsiella* and from 89% to 92% in *Escherichia* (Figure 2A). The Hadamard score, a result of the product between the percentage identity and the alignment length, allowed a more weighted comparison, and also varied similarly in the different genera (Figure 2B). In fact the observed range varies greatly, from an average of 50% among representatives of the *Escherichia* genus and 34% among the *Citrobacter* genus up to 70–80% in the *Klebsiella, Citrobacter*, and *Escherichia* genera. The Hadamard score between the isolate 1612_C1 and the two *S. electus* strains was 59%.

The ANI similarity is a well-established method to verify identities between organisms in a taxonomic context. When the first two "*S. electus*" were identified (Potter *et al.* 2018), the same methodology was used to confirm that the two isolates belonged to the same, novel, species (ANI > 95%). In this work we show that the ANI between 1612_C1 and the two "*S. electus*" strains was 87% and thus much less than the accepted threshold to consider two organisms as belonging to the same species. Moreover, since that ANI is based only on identity between similar regions, the Hadamard score was considered, allowing to weight the percentage identity on coverage between pairwise genome alignments. The observed ANI and Hadamard scores highlighted the boundaries and so the acceptable intervals between the organisms among the heterogeneous dataset of representative *Enterobacteriaceae* genera. Thus, both ANI and Hadamard scores between 1612_C1 and *S. electus* are clearly within the accepted intervals for different species in a genus of *Enterobacteriaceae* (Figure 2, A and B).

To summarize, the 16S rRNA similarity values were not useful to place the novel 1612_C1 isolate in the proper taxonomic context, a well-documented issue in the *Enterobacteriaceae* family. Thus, an orthologues gene phylogeny was inferred, placing isolate 1612_C1 as sister group of the two *S. electus* strains, outlining a relation between these organisms. Further genomic analysis based on ANI similarity, indicated that the isolate 1612_C1 represents a novel species within the genus "*Superficieibacter*," for which we propose the name "*Superficieibacter maynardsmithii*" referring to John Maynard Smith, British mathematical and theoretical geneticist, important contributor to experimental and evolutionary biology. This, albeit with only one isolate, can be currently considered a putative environmental bacterium devoid of known mechanisms of antibiotic resistance and virulence, contributing to expand the known diversity within the genus "*Superficieibacter*" and the family of *Enterobacteriaceae*.

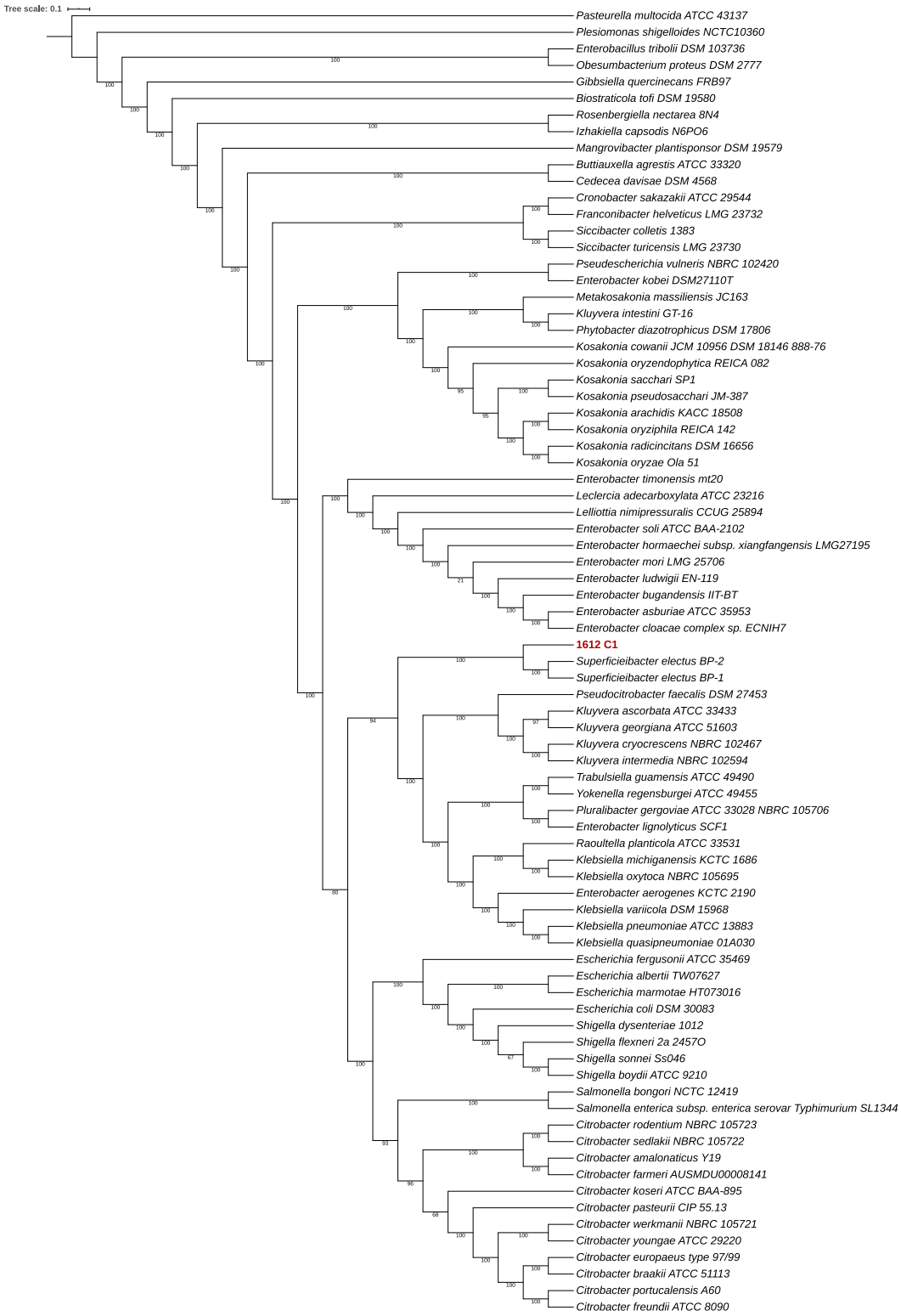

Figure 1 Phylogenetic reconstruction based on a concatenate of 612 single copy orthologs present in the dataset of 78 members of the family *Enterobacteriaceae*, plus *Pasteurella multocida* as outgroup. The phylogeny was reconstructed using RaXML with 100 bootstrap replicates.

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Conflicts of interest: The authors have no conflicts of interest to declare.

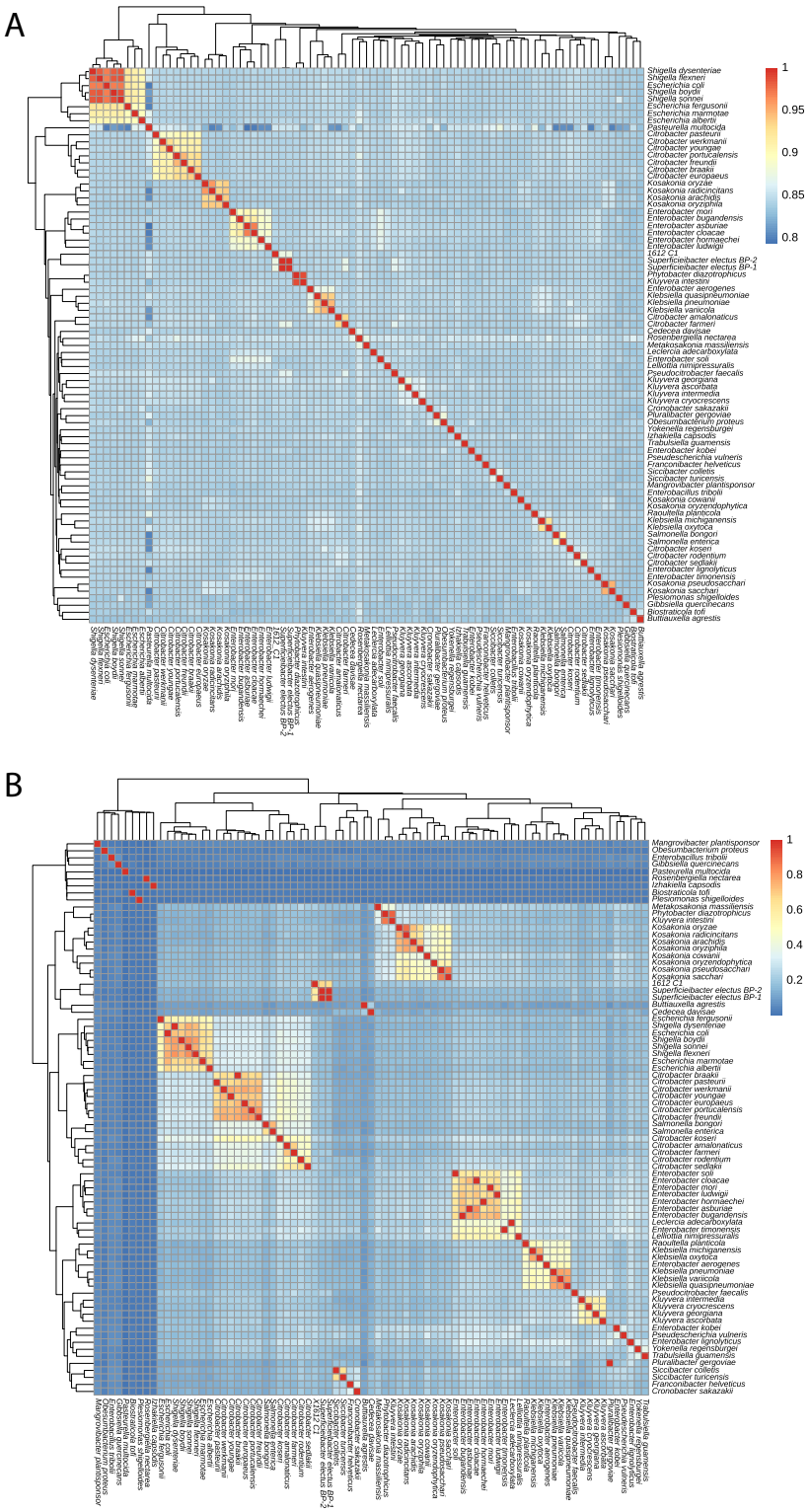

Figure 2 Heatmap showing pairwise identity between pairs of genomes in all members of the genomic dataset of 78 members of the family *Enterobacteriaceae*. (A) Average nucleotide identities. (B) Hadamard identities.

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