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To cite this version:
Gherard Batisti Biffignandi, Marjorie Gibbon, Marta Corbella, Harry Thorpe, Cristina Merla, et al.. Genome of Superficieibacter maynardsmithii, a novel, antibiotic susceptible representative of Enterobacteriaceae. G3, Genetics Society of America, 2021, 11 (2), pp.jkab019. 10.1093/g3journal/jkab019. pasteur-03198313

HAL Id: pasteur-03198313
https://hal-pasteur.archives-ouvertes.fr/pasteur-03198313
Submitted on 14 Apr 2021

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

Gherard Batist Batiffignandi, Marjorie J. Gibbon, Marta Corbella, Harry A. Thorpe, Cristina Merla, Michele Castelli, Teemu Kallonen, Katie Pegrum, Sylvan Brisse, Jukka Corander, Piero Marone, Edward J. Feil, and Davide Sassera

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 Enterobacteriacae family. In order to evaluate whether the novel genome represents a new species of “Superficieibacter,” 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 reduce 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 Torok 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 (Osterblad et al. 1999). However, recent works have observed that...
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 (Alnajjar and Gupta 2017). The low efficiency of 16S rRNA 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 16S rRNA similarity with S. enterica, and in the description of Klebsiella africanaensis sp. nov., Klebsiella varicola subsp. tropicalensis subsp. nov., and K. varicola subsp. varicola 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 a city of Pavia, Northern Italy, with the aim to study the diversification 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 cm² 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 QIAasympol 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/teeemann/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/teeemann/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 sets 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:

\[ c_{ij} = a_{ij} b_{ij} \]

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. enteritidis" (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. enteritidis," 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.

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 the 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.

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

| Antibiotic                      | Susceptibility |
|--------------------------------|----------------|
| Amikacin                       | ≤4, S          |
| Amoxicillin | clavulanic acid | ≤2, 1, 2, S |
| Ampicillin                      | 4, S           |
| Aztreonam                      | ≤1, S          |
| Cefalexin                      | ≤4, S          |
| Cefepime                       | ≤1, S          |
| Cefixime                       | ≤0.5, S        |
| Ceftazidime                    | ≤0.5, S        |
| Ceftriazone                    | ≤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, 1, 4, S |
| Ticarcillin | clavulanic acid | ≤4, 1, 2, S |
| Tobramycin                     | ≤1, S          |
| Trimethoprim                   | ≤1, S          |
| Trimethoprim | sulfamethoxazole | ≤1, 19, S |
This work was funded by the SpARK project “The rates and routes of transmission of multidrug resistant Klebsiella clones and genes into the clinic from environmental sources,” which has received funding under the 2016 JPI-AMR call “Transmission Dynamics” (MRC reference MR/R00241X/1), by Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022)—Dept. of Biology and Biotechnology “L. Spallanzani,” University of Pavia to D.S.

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

**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.

**Funding**

This work was funded by the SpARK project “The rates and routes of transmission of multidrug resistant Klebsiella clones and genes into the clinic from environmental sources,” which has received funding under the 2016 JPI-AMR call “Transmission Dynamics” (MRC reference MR/R00241X/1), by Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022)—Dept. of Biology and Biotechnology “L. Spallanzani,” University of Pavia to D.S.

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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Communicating editor: R. Reyes