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Description du microbiote urinaire par méthodes de culturomics et de métagenomique

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Biologie-Santé, Spécialité Maladies Transmissibles et Pathologies Tropicales

Membres du Jury de la Thèse:

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Avant-propos

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées, qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie « introduction et bibliographie » est remplacée par une revue publiée dans un journal scientifique afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. D'autre part, la thèse est présentée sur articles publiés, acceptés ou soumis associés d'un bref commentaire donnant le sens général du travail. Cette forme de présentation est plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

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Sommaire

Résumé :

L'urine a longtemps été considérée stérile.

Ce travail visait à établir le répertoire des bactéries connues dans les voies urinaires humaines par une revue de la littérature et à implémenter ce répertoire en analysant des échantillons d'urine par culturomics et métagénomique.

562 espèces bactériennes ont été décrites dans des échantillons d'urine humains dans la littérature. 62,6% ont été associées à un cas d'infection humaine.

Parmi les 441 échantillons d'urine analysés par culturomics, 459 espèces bactériennes différentes ont été isolées, dont 264 jamais décrites dans l'urine, 18 nouvelles espèces.

Parmi les 684 espèces bactériennes isolées au moins une fois en culture à partir d'échantillons d'urine, 424 (62%) avaient déjà été isolées du microbiote intestinal.

Parmi les espèces retrouvées uniquement en métagénomique figurent des bactéries extrêmophiles.

Il existe un microbiote des voies urinaires humaines qui peut être décrit par culturomics et métagénomique.

Abstract :

Urine was usually considered sterile.

This work aimed to establish the repertoire of known bacteria in the human urinary tract through a review of the literature and to implement this repertoire by analyzing urine samples by culturomics and metagenomics.

In literature, 562 bacterial species have been described in human urine samples, in which 62.6% were associated with one case of human infection.

Of the 441 urine samples analyzed by culturomics, 459 different bacterial species were isolated, of which 264 never described in the urine, 18 new species.

Of the 684 bacterial species isolated at least once in culture from urine samples, 424 (62%) had already been isolated from the gut microbiota.

Extremophilic bacteria and archaea are identified among the species found only with metagenomics method.

There is a microbiota of the human urinary tract that can be described by culturomics and metagenomics.

1.Introduction :

1.1.Définition :

Le microbiote est l'ensemble des micro-organismes (bactéries, levures, champignons, virus) vivant dans un environnement spécifique (appelé microbiome) chez un hôte (animal ou végétal) en situation saine ou pathologique. Le nombre de bactéries associées à l'homme est estimé à 10^{15}, soit environ 10 fois plus que le nombre de cellules, et environ 1000 fois plus que le nombre de gènes composant le corps humain [1]. Certaines fonctions du microbiote sont déjà bien connues comme le rôle dans la production et la transformation de substances bioactives (acides gras à chaîne courte, vitamine K), la protection contre les infections (Clostridium difficile, E. coli O157), l'homéostasie énergétique, les réactions inflammatoires de l'intestin, le comportement de l'hôte [2, 3].

Suite au projet de séquençage du génome humain appelé « Human Genome Project », achevé en 2003 et par le développement de nouvelles techniques de séquençage de l'ADN, un consortium de chercheurs a lancé un nouveau projet multidisciplinaire international pour le séquençage du métagénome du microbiote humain (microbiome), le « Human Microbiome Project ». Le but de ce projet est de décrire la diversité et de comprendre le rôle du microbiote dans le fonctionnement du corps humain [3].

1.2 Techniques d'analyse du microbiote :

1.2.1.Metagénomique :

Le microbiote a été principalement décrit par des techniques de séquençage haut débit. Ces techniques sont limitées par les biais liés aux techniques d'extraction de l'ADN, aux amorces utilisées pour amplifier l'ADN, mais aussi par le biais de profondeur car seuls les génomes les plus abondants dans l'échantillon peuvent être détectés. Enfin les techniques de métagénomiques ne permettent pas de déterminer si l'organisme était vivant au moment et au lieu où il a été trouvé et ne permettent pas d'isoler les microorganismes identifiés afin d'effectuer des analyses in vitro ou in vivo [4].

1.2.2.Culturomics :

Afin de mieux décrire le microbiote digestif, Jean- Christophe Lagier et al. ont développé la méthode culturomics qui consiste en un ensemble de techniques visant à cultiver des micro-organismes de croissance lente et difficile, combiné à une identification rapide et fiable des micro-organismes par spectrométrie de masse MALDI-TOF [5, 6]. Ces techniques

comprennent notamment l'utilisation de culture en milieux liquides, l'utilisation d'antioxydants, l'utilisation d'un enrichissement par du jus de rumen de mouton, permettant ainsi la culture des *Spirochaetes*, de bactéries aéro-intolérantes et d'*Archae* [7, 8, 9]. Cette méthode de culturomics a permis la découverte de nombreuses nouvelles bactéries dans l'intestin humain qui ont pu être décrite par méthode de taxonogénomique [10].

1.2.3.La taxonogénomique :

Du grec τάξις (taxis) signifiant « placement », « classement », « ordre » et de νομός (nomos) qui signifie « loi », « règle », la taxonomie est une branche de la biologie dont l'objet est de décrire les organismes vivants et de les regrouper en entités appelées taxons afin de les identifier, les nommer et les classer via des clés de détermination. La taxonomie appliquée aux procaryotes (taxonomie microbienne) est l'étude de la diversité des micro-organismes dans le but de les hiérarchiser [11, 12]. Depuis l'introduction de nouveaux outils tels que le séquençage de l'ARN 16S [13], l'hybridation ADN-ADN[14], le MALDI-TOF MS [6] et le séquençage génomique [15] dans l'identification des micro-organismes [16], cette science est devenue taxonogénomique avec l'association de la génomique et la taxonomie. La taxonogénomique est une approche polyphasique qui intégre les données phénotypiques et génotypiques pour la description de nouvelles espèces bactériennes [15]. La culturomics couplée à la taxonogénomique ont augmenté de manière significative le répertoire bactérien associé à l'homme en permettant la description de plus de 500 nouvelles espèces bactériennes [17].

1.3.Histoire du microbiote urinaire :

1.3.1.Intérêt de la communauté scientifique pour les germes dans les urines :

Historiquement, la communauté scientifique s'est intéressée à l'urine afin de confirmer ou d'infirmer les infections des voies urinaires.

L'urine était considérée comme physiologiquement stérile avant d'être contaminée par des bactéries urétrales et périnéales. Des analyses qualitatives et quantitatives étaient effectuées sur le sédiment urinaire (éléments figurés présents dans le culot de centrifugation de l'urine).

1.3.2.Compte d'Addis, compte de Kass :

En 1926, Thomas Addis a défini le « compte d'Addis » comme l'excrétion des composants de sédiments urinaires par minute. Les cellules et les leucocytes des globules rouges des urines de 12 heures étaient comptés. Le nombre normal de globules rouges devait être inférieur à 6.10^5

[18, 19]. Ceci a été adapté par Jacob Hamburger Hartog, et consistait en une analyse quantitative du sédiment urinaire. Le rapport des globules rouges et des globules blancs à la quantité d'urine en mL pendant trois heures devait être inférieur à 2500 globules rouges / mL et 6000 leucocytes / mL. Actuellement, la norme pour le sédiment urinaire est un débit de 2500 globules rouges de sang par minute et nombre de globules rouges inférieur à 5000 / mL, un débit de 6000 leucocytes par minute et un nombre de leucocytes inférieur à 10000 / mL. Les dépassements de ces normes sont des arguments pour les maladies des voies urinaires ou rénales et sont complétés par une mise en culture des urines avec antibiogramme.

En 1957, Edward Kass a défini le compte de Kass pour identifier une infection des voies urinaires. Il consiste à dénombrer les bactéries présentes dans l'urine fraîchement émise (prélevée au cours d'une miction en milieu de jet). Le critère d'infection est un nombre de bactéries supérieur ou égal à 10^5 / mL. Cette technique est fondée sur deux études sur des échantillons de population de petite taille comportant de nombreuses limites et biais [20, 21]. Malgré un niveau de preuve faible, 60 ans plus tard, le critère de 10^5UFC (unité formant une colonie) / mL est encore utilisé dans de nombreux pays pour interpréter le rôle des bactéries dans les différentes symptomatologies cliniques touchant les voies urinaires et non pas seulement pour définir la présence ou non d'une pyélonéphrite. La culture des urines est le test de laboratoire le plus utilisé après la bandelette réactive pour révéler une infection des voies urinaires. Une infection des voies urinaires est définie par la présence de bactéries dans un échantillon d'urine prélevé en amont du sphincter interne de la vessie (cystite si l'infection est limitée à la vessie ou pyélonéphrite en cas d'infection prolongée au rein). La culture d'urine est dans la plupart des cas effectuée sur un échantillon d'urine prélevé en milieu de jet lors d'une miction. Le nombre de leucocytes et de globules rouges contenu dans l'échantillon d'urine est quantifié et une culture de l'échantillon est effectuée après ensemencement sur gélose COS, en condition atmosphérique aérobie, à une température d'incubation située entre 35 et 37°C et pendant 48 heures maximum. Le seuil de détection défini pour cette méthode est de 10^3UFC / ml.

Ceci a progressivement conduit à occulter le microbiote urinaire. Les urines ont été considérées comme stériles sous le seuil 10^3UFC / ml et en dessous de ce seuil les bactéries n'étaient pas analysées. Ce seuil avait une signification clinique en termes de valeur prédictive positive pour les infections des voies urinaires [22]. En outre, l'uroculture qui était effectuée avec un petit volume d'urine ensemencé sur gélose au sang frais en conditions atmosphériques aérobies, à une température d'incubation comprise entre 35°C et 37°C pendant 48 heures de culture ne permettait de trouver que les bactéries aérobies et de culture non fastidieuse. Un autre

biais était que les bactéries connues pour appartenir à la flore vaginale étaient considérées comme une contamination [23].

1.3.3.Changement de paradigme :

En 2004, Marc Anderson et al. ont montré l'existence de bactéries viables mais non cultivables dans l'urine humaine en analysant la présence de membranes cellulaires intactes de bactéries [24].

En 2012, l'équipe de recherche de Wolfe et al. a comparé différentes techniques de collecte d'urine pour l'analyse du microbiote urinaire [25]. Dans des échantillons d'urine prélevés par ponction sus-pubienne ou sondage trans-urétral de la vessie, des techniques de métagénomiques ont permis d'identifier des bactéries chez des patients n'ayant pas d'infection urinaire et pour lesquels les cultures d'échantillons d'urine prélevés en milieu de jet en atmosphère aérobie ou anaérobie ont été négatives. De même, des bactéries ont été retrouvées associées à l'épithélium urinaire en microscopie optique. Et les techniques de métagénomique ont permis l'identification des germes dans des échantillons d'urine de femmes souffrant d'incontinence urinaire ou asymptomatiques [26, 27, 28].

1.3.4.Renaissance de la culture de l'urine :

En 2014 Evann E. Hilt et al. ont étudié le microbiote urinaire des patients adultes atteints d'hyperactivité vésicale par rapport à des témoins indemnes de symptomatologie urinaire sur des urines recueillies par sondage trans-urétral de la vessie, en utilisant des techniques améliorées et diversifiées de culture d'urine et l'identification des microorganismes par spectrométrie de masse MALDI-TOF. Ils ont inoculé de plus grands volumes d'urine que le volume standard d'une culture d'urine (de 100µL à 1 ml contre 1µL fait jusqu'alors) pour mettre en évidence des bactéries dans les urines sous le seuil de 10^3 UFC / ml. Ils ont incubé les échantillons dans diverses conditions atmosphériques pendant une période supérieure à 48 heures (jusqu'à 5 jours) [29]. Ils ont signalé la détection de bactériurie par ces techniques dans 80% des échantillons d'urine de patients sans infection urinaire contre 8% avec les techniques standard et le seuil de détection de 10^3 UFC / mL. Il y avait aussi une prédominance de bactéries anaérobies obtenues par les méthodes de culture utilisant une atmosphère riche en CO2 et un temps d'incubation prolongée. Par la suite, d'autres laboratoires ont expérimenté des techniques de culture d'urine améliorées afin d'isoler des bactéries exigeantes et moins exigeantes dans l'urine et ont confirmé qu'un microbiote urinaire existe [30, 31, 32]. L'urine n'est pas stérile [33].

1.4.Objectifs de ce travail de these :

1.4.1.Inventaire des bactéries connues dans les urines humaines :

Nous avons commencé notre travail en réalisant une revue de la littérature des bactéries décrites dans les urines humaines que ce soit par des méthodes de culture ou par une approche de biologie moléculaire.

1.4.2.Description du microbiote urinaire par méthodes de culturomics et de métagénomique et implémentation du répertoire des bactéries connues dans le tractus urinaire humain :

Nous avons ensuite utilisé la double approche culturomics et métagénomique pour décrire la composition d'échantillons d'urine de patients et de volontaires sains afin d'implémenter le repertoire des bactéries connues dans les urines humaines.

1.4.3. La culturomics comme outil pour faire progresser la taxonomie bactérienne :

Enfin nous avons décrit les nouvelles espèces bactériennes isolées dans les échantillons d'urine par culturomics en utilisant la méthode taxonogénomique.

2.Partie I : Inventaire des bactéries connues dans les urines humaines :

Avant-propos :

L'objectif de ce travail était de faire le point sur la connaissance du microbiote (bactéries et archées) du tractus urinaire humain. Pour cela nous avons fait une revue de la littérature afin d'identifier les bactéries déjà décrites par méthode de culture et par biologie moléculaire dans les urines humaines. Cela nous a ensuite conduit à comparer ce répertoire avec les autres répertoires de bactéries humains connus (tube digestif et vagin).

Nous avons conduit une recherche bibliographique automatisée et une recherche bibliographique manuelle. Au total 562 espèces bactériennes ont été rapportées dans la littérature comme faisant partie du microbiote urinaire humain. Sur les 562 espèces, 322 ont été décrites uniquement par culture, 101 par culture et métagénomique et 139 par métagénomique. 352 espèces (62,6%) ont été associées à au moins une déclaration de cas d'infection humaine, dont 225 (40,0%) ont été décrites comme agent causal d'infection des voies urinaires. Le répertoire bactérien des voies urinaires contient 21,4% de la diversité de procaryote connue comme étant associée à l'être humain (464 en commun) et partage 23,6% des espèces bactériennes avec le microbiote intestinal humain (350 en commun, 62,3% de l'urine) [4, 5, 10, 17, 34]. Les 4 *Phyla* les plus représentés dans les urines humaines sont les Proteobactéries (35,5%), les Firmicutes (31,3%), les Actinobactéries (22,4%) et les Bacteroidetes (6,4%), ce sont aussi les 4 *Phyla* les plus représentés dans le microbiote digestif et dans le microbiote global humain déjà décrit. Le microbiote urinaire ainsi décrit partage une différence significative avec le microbiote digestif en ce qui concerne entre les espèces bactériennes aéro-intolérantes, (100/562; 17,8% et 505/1484; 34,0% respectivement; $p < 0,001$; OR = 9,0 [7,0-11,4]). Les études utilisant le séquençage à haut débit montrent une proportion plus élevée de bactéries aéro-intolérantes dans l'urine que les études utilisant la culture (74/240, 30,8%).

Il est à noter que la plupart des bactéries pathogènes font partie des bactéries commensales du tractus urinaire humain. Leur pouvoir pathogène peut survenir à la suite d'un déséquilibre de ce microbiote urinaire.

Article I : Revue

Human Bacterial Repertoire of the Urinary Tract: a Potential Paradigm Shift.

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MINIREVIEW

Human Bacterial Repertoire of the Urinary Tract: a Potential Paradigm Shift

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ABSTRACT The aim of this article is to review the human repertoire of bacteria in urine already described by culture and metagenomic techniques and published in the literature. Our study led us to compare this repertoire with other available human repertoires. We followed automatic and manual bibliographical methods and found 562 bacterial species reported in the literature as part of the human urinary microbiota. Of the 562 species, 322 were described only by culture, 101 by both culture and metagenomics, and 139 only by metagenomics. A total of 352 species (62.6%) have been associated with at least one case report of human infection, of which 225 (40.0%) have been described as causative agents of urinary tract infection. The urinary tract bacterial repertoire contains 21.4% of the known prokaryotic diversity associated with human beings (464 species in common), and it shares 23.6% species with the human gut microbiota (350 species in common, 62.3% of the urine species). The urinary repertoire shares a significant difference in aerointolerant species compared with those of the gut microbiota (100/562 [17.8%] and 505/1,484 [34.0%], respectively; $P < 0.001$; odds ratio [OR] = 9.0 [7.0 to 11.4]). Studies using high-throughput sequencing show a higher proportion of aerointolerant bacteria in urine (74/240 [30.8%]) than studies using culture techniques (40/423 [9.5%]). Most pathogenic bacteria are part of the commensal human urinary tract bacteria, and their pathogenicity may occur following any imbalance of this microbiota. The restoration of urinary tract health can occur following a fecal transplantation. The potential gut origin of the human bacterial microbiota has to be explored.

KEYWORDS bacteria, culture, human, microbiota, repertoire, urine

Historically, urine has always been considered a sterile fluid, and scientists have studied urine to predict and confirm urinary tract infection. In 1926, Thomas Addis defined the "Addis count" as the excretion of urinary sediment components per minute (1), and it was further adapted by Hartog Jacob Hamburger. Currently, a number of red blood cells greater than 5,000/ml and a number of leukocytes greater than 10,000/ml from urinary sediments are arguments for urinary tract or renal diseases, the diagnosis of which must be completed by bacterial culture. The latter is interpreted using the Kass criterion, published in 1957 by Edward Kass, which consists of the counting of bacteria cultured from fresh urine. Thus, the number of bacteria superior or equal to 10^5 CFU/ml was predictive of urinary tract infection (UTI). Those criteria, resulting from two studies with small sample sizes (2), remain in current use 60 years later. Therefore, the study of the urinary microbiota fell into oblivion.

In 2004, Anderson et al., analyzing intact bacterial cell membranes, showed the existence of viable but uncultured bacteria in the urine of women with and without

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urinary tract infection (3). Similarly, *Escherichia coli* cells were associated with urinary epithelium in a patient with aseptic leukocyturia (4). With the development of the high-throughput sequencing techniques, in 2012, Wolfe et al. identified bacteria in urine samples of patients without urinary infection taken by subpubic puncture and transurethral catheterization (5). Other studies were performed, using metagenomic techniques that allowed for the identification of bacteria in the urine samples of women or men, asymptomatic or with urinary disorders (6–8). In 2014, Hilt et al. studied the urinary microbiota of adult patients with an overactive bladder versus that of controls, using enhanced urine culture techniques and identification by mass spectrometry on the urine collected by transurethral bladder catheterization. Inoculation of a large volume of urine (1 ml instead of 1 μl), combined with prolonged incubation in various atmospheres (instead of aerobic only over 48 h), allowed recovery of bacteria present at a concentration level lower than 10^3 CFU/ml (9). Such methods evidenced bacteria in 80% of urine samples from patients without urinary infection versus 8% in samples analyzed with standard techniques, with a predominance of aerointolerant bacteria. Other laboratories have experimented with enhanced urine culture techniques and have confirmed the existence of a urinary microbiota (10).

As a matter of fact, in many cases, urine is not sterile. The role of the urinary microbiota is currently debated (11), and there is no existing database, exhaustive or specific, listing all bacterial species associated with the urinary tract of human beings. Here, we propose to establish, through a systematic literature review, a comprehensive human repertoire of urinary bacteria detected by culture and/or metagenomic techniques.

BIBLIOGRAPHICAL METHODS

Automated research. We decided to perform an automated search using the list of identified prokaryotic species with standing in nomenclature using the "List of prokaryotic names with standing in nomenclature" (LPSN; www.bacterio.net) and taxonomy on the NCBI website (https://www.ncbi.nlm.nih.gov/guide/taxonomy/) (20,660 species of bacteria and archaea as of 15 February 2018) comprising 2,172 prokaryotes isolated from human beings established by the work of Hugon et al. (11) and supplemented by the list of bacteria isolated since its publication (Data Set S1). The following query was automatically performed between 15 and 17 February 2018 in the Medline database using the PubMed search engine with the search parameters #3 (Name of the prokaryotes or its synonymes/derivatives), MeSH, TW (Text Words), and SH (Subheadings): (#3[tw] OR #3[MesH]) AND (("Urologic Diseases"[Mesh] OR "Urine"[Mesh] OR "Urinary Tract"[Mesh] OR "urinalysis" [Mesh] OR "Anti-Infective Agents, Urinary"[Mesh] OR "Bacteriuria"[Mesh] OR "Urinary Tract Infections"[Mesh]) OR ("Urologic Diseases"[TW] OR "Urine"[TW] OR "Urinary Tract"[TW] OR "urinalysis" [TW] OR "Urinary Anti-Infective Agents"[TW] OR "Bacteriuria"[TW] OR "Urinary Tract Infections"[TW])) AND (("Metagenomics"[Mesh] OR "microbiology"[SH] OR "isolation and purification"[SH] OR "DNA, Bacterial"[Mesh] OR "RNA, Ribosomal, 16S"[Mesh] OR "Bacteriological Techniques"[MeSH] OR "Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization" OR "Molecular Diagnostic Techniques"[Mesh] OR "Sequence Analysis"[Mesh] OR "Polymerase Chain Reaction"[Mesh] OR "Culture Media"[Mesh]) OR ("Metagenomics"[TW] OR "microbiology"[TW] OR "isolation"[TW] OR "purification"[TW] OR "Bacterial DNA"[TW] OR "Ribosomal RNA 16S"[TW] OR "Bacteriological Techniques"[TW] OR "Mass Spectrometry"[TW] OR "Matrix-Assisted Laser Desorption-Ionization"[TW] OR "Molecular Diagnostic Techniques"[TW] OR "Sequence Analysis"[TW] OR "Polymerase Chain Reaction"[TW] OR "Culture Media"[TW])) AND (Humans[Mesh] OR Human[TW] OR Patient[TW] OR Patients[TW] OR Humans[TW] OR Child[TW] OR Children[TW] OR Infant[TW] OR Man[TW] OR Woman[TW] OR Men[TW] OR Women[TW]).

Results were sorted, bacteria reporting no result were eliminated, and bacteria reporting at least one result were sorted by title, abstract, and full text of the scientific article if available. The full text was not systematically investigated if the title or abstract was sufficiently explicit. We did not retain bacteria for which only the antigen was detected. Data analysis was performed using Microsoft Excel 2007 software.

Manual research. We conducted a literature search in the Medline database using the PubMed search engine and reviewed the articles from 1950 to 1 October 2018 that dealt with the urinary microbiota in adults and children, using the following keywords: "microbiota," "urine," "urinary tract," ((("Microbiota" [Mesh] AND "Urine" [Mesh]) OR ("Microbiota" [Mesh] AND "Urinary Tract" [Mesh])) OR (("Microbiota" [TW] AND "Urine" [TW]) OR ("Microbiota" [TW] AND "Urinary Tract" [TW]))) AND (("Urine/microbiology" [MAJR]) AND (("Humans" [TW]) OR ("Humans" [TW] AND ("infant" [TW] OR "child" [TW] OR "adolescent" [TW])) AND ("Urine/microbiology" [MAJR]) AND (("Humans" [Mesh]) OR ("Humans" [Mesh] AND ("infant" [MeSH] OR "child" [MeSH] OR "adolescent" [MeSH]))).

Analysis of articles and references was used to select articles of interest with a list of bacteria found in human urine by culture or metagenomics.

Then, the clinical and research bacteriology laboratory database of the Hospital-University-Institute (IHU) Méditerranée-Infection (Marseille, France) was also checked, starting with records from 1 January 2002. For all of the bacterial species isolated at least once in the human urine in our laboratory, a literature search with keywords consisting of the name of the bacterium AND ("Urine" OR "Urinary Tract") AND ("Human") was conducted for records through 1 October 2018, via PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Google Scholar (https://scholar.google.fr/) websites, in order to determine if each bacterium had been previously isolated in a human urine sample and to decide whether to add each bacterium to the repertoire or not.

Determination of the main characteristics of the bacteria. We classified the bacteria by phylum and genus with the help of NCBI taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy).

We used the "List of bacteria according to their aerotolerant or aero-intolerant metabolism" (https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/list-of-prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism/) to define the tolerance to oxygen and genus of each bacterial species.

The risk group classification was obtained according to German Technical Rules for Biological Agents (TRBA; https://www.baua.de/EN/Service/Legislative-texts-and-technical-rules/Rules/TRBA/pdf/TRBA-466.pdf) completed with the American Biological Safety Association (ABSA) Risk Group Database (https://my.absa.org/Riskgroups) and with the "List of prokaryotic names with standing in nomenclature" (LPSN; http://www.bacterio.net). The risk group classification (12) reflects the risk of biological agents for laboratory staff security, community security, and human health: risk group 1, risk group 2, risk group 3, and risk group 4. However, risk group classification does not provide information about the incidence of infectious diseases at an individual level. We therefore looked for the pathogenicity of each bacterial species, using the PubMed database and Google Scholar. We considered as commensal a microorganism that colonizes its host without causing disease. Some commensal bacteria could have mutualistic relationships with humans and have roles in protecting us from external pathogens or contributing to metabolic pathways (13, 14). However, human bacterial pathogens can be commensals, as they are able to colonize human body sites without causing any infection. This observation has led microbiologists to reconsider their view of the nature of commensals and pathogens. Bacteria currently considered beneficial for health were first isolated as commensals but were later recovered from clinical specimens as disease-causing agents. Therefore, we considered as potentially pathogenic a bacterium with at least one human clinical infection case reported in the literature; however, being pathogenic did not exclude the possibility of a species of being commensal under other conditions. The manual request of records through 1 October 2018 used as keywords the name of the bacterium AND "human" AND "infection."

HUMAN URINARY REPERTOIRE

Here, we established the first repertoire of bacterial species isolated in urine samples through a comprehensive review of the scientific literature, constituting a starting point

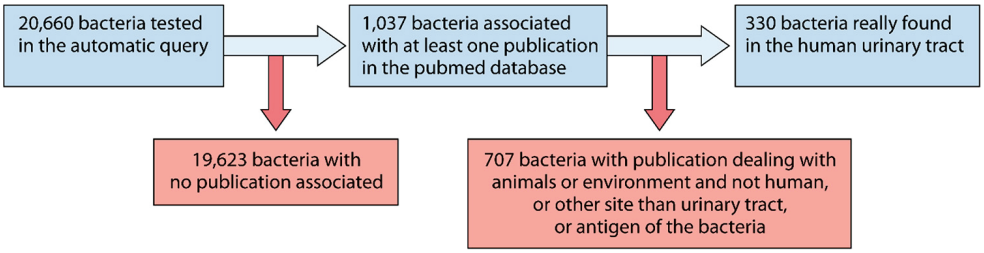

FIG 1 Flow chart of the automated bibliographical request. This figure represents the flow chart of the bacterial species found associated with at least one publication in PubMed database, based on the automatic query and then manual triage of all the publications to keep only prokaryotic species found in the human urinary tract.

for describing the components of the urinary microbiota in physiological or pathological conditions.

Thanks to the automated request, 1,037 different bacterial taxa reported from 192,391 publications were extracted from the PubMed database. A total of 704 species were excluded because the corresponding publications reported (i) bacteria exclusively found in animals or the environment, (ii) bacteria found in human microbiota other than the urinary microbiota, and (iii) bacteria for which only the antigen was found. Overall, 330 bacterial taxa were associated with one or more publications, showing that they were found in human urine samples by culture and/or metagenomics (see flow-chart in Fig. 1). Manual bibliographic research has increased the repertoire with 232 other bacterial taxa recovered from human urine samples. Consequently, a total number of 562 different bacterial taxa were identified as being found in human urine (Data Set S2).

The 562 bacterial species found in the urinary tract belong to 9 phyla (Fig. 2 and Data Sets S2 and S3). A total of 210 different genera were identified (Fig. 3 and Data Sets S2 and S3).

According to their aerotolerant or aerointolerant metabolism, 100 species (17.8%) were aerointolerant.

The 8 more commonly found species in the literature were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus faecalis* (Table S1, Fig. S1). All are considered pathogenic.

Of the 562 species listed, 322 were described only by culture, 101 by both culture and metagenomics, and 139 by metagenomics only (Fig. S2 and Data Set S2). The

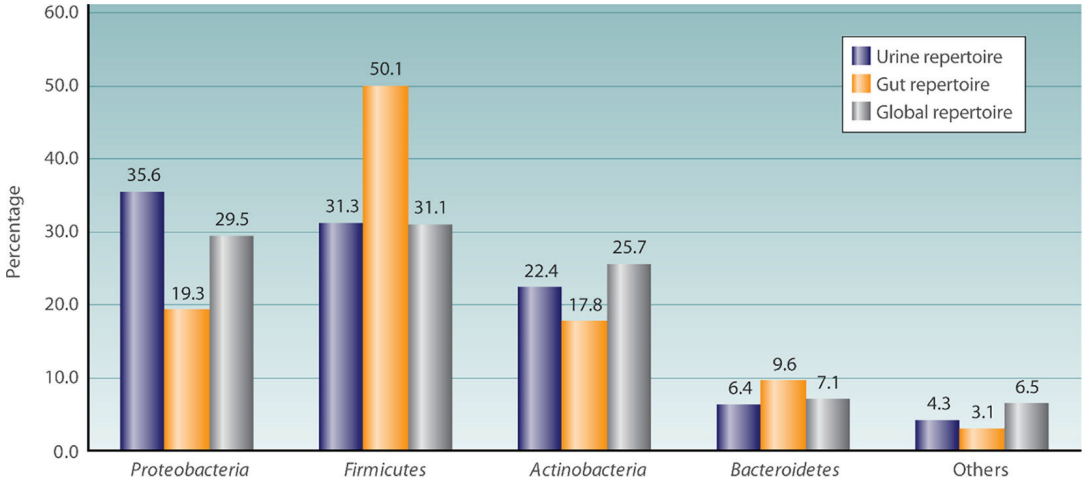

FIG 2 Comparison of the percentage of repartition of the most-represented phyla in the human urinary tract bacterial repertoire, the human gut repertoire, and the human global repertoire. This histogram represents the percentage of taxa in the main phyla in the human urinary tract repertoire, compared to those in the human gut and human global repertoires.

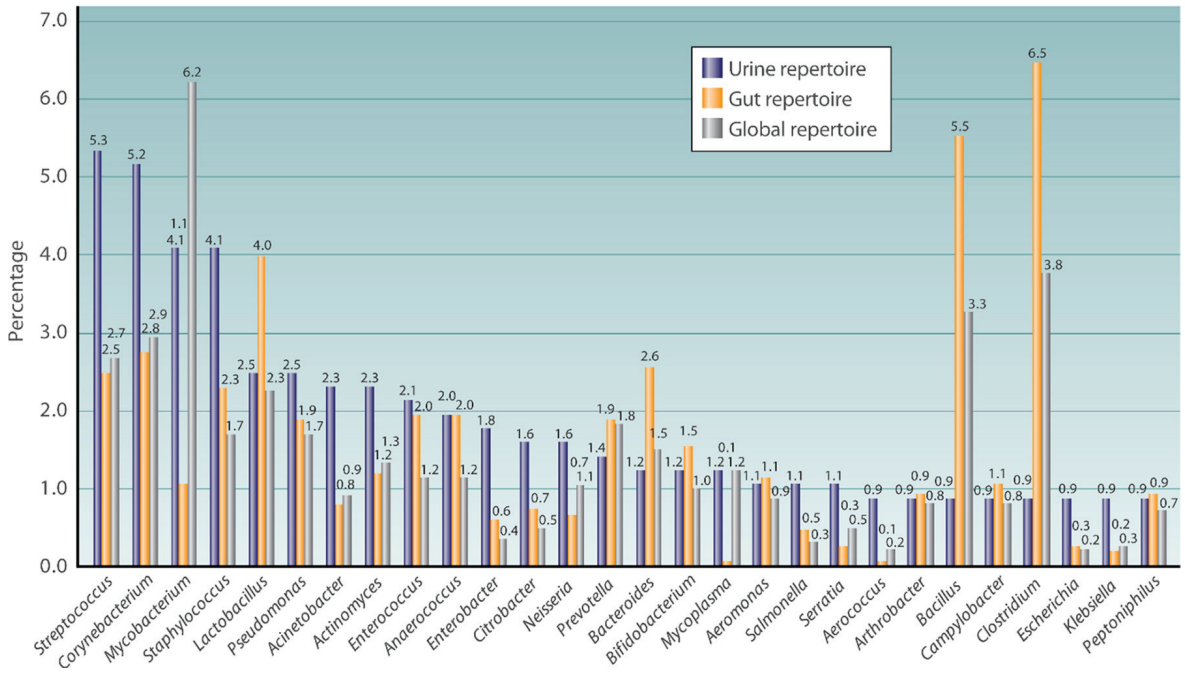

FIG 3 Comparison of the percentage of repartition of the most represented genera in the human urinary tract bacterial repertoire, the human gut repertoire, and the human global repertoire. This histogram represents the percentage of taxa in the main genera in the human urinary tract repertoire, compared to those in the human gut and human global repertoires.

manual bibliographic research allowed the finding by high-throughput sequencing methods of additional operational taxonomic units (OTUs) not identified at the species level. Among the metagenomic studies, most authors used shotgun metagenomics techniques (integrated next-generation sequencing of 16S rRNA genes) or broad-range 16S rRNA gene PCR and mainly found OTUs; 229 bacterial taxa (95.4%) were identified at a species level. Ten other bacterial taxa were described by the PCR method using a targeted gene, and one study used a DNA microarray method and found one bacterial taxon (Data Set S2).

To describe the ethnicities of the population analyzed in the different publications considered for the human urinary tract bacterial repertoire, we did an ancillary study of all the scientific publications retained. The twenty most relevant publications reported 375 (66.7%) of all the bacterial taxa described. Nine studies took place in the United States (295 bacterial taxa [52.5%]), 9 in Europe (74 bacterial taxa [13.2%]), 1 in China (3 bacterial taxa [0.5%]), and 1 in Africa (3 bacterial taxa [0.5%]) (Data Set S5).

COMPARISON WITH WHOLE HUMAN REPERTOIRE AND GUT REPERTOIRE

The number of identified bacterial and archaeal species with standing in nomenclature was 20,660 species on 15 February 2018. Here, we identified 562 bacterial taxa from urine specimens. Compared to the repertoire of 2,172 prokaryotes cultured from human beings that was published in 2015 by Hugon et al. (11) and with the 1,484 prokaryotes isolated from human gut microbiota that was published in 2016 by Lagier et al. (15), urine harbors 21.4% of the known prokaryotic diversity associated with human beings (464 common species) and shares 23.6% of species in the human gut microbiota (350 common species, 62.3% of the urine species) (details in Fig. S3 and Data Sets S3 and S4). Similarly to the global human repertoire and to the human gut microbiota, the 4 most represented phyla in the human urinary tract are *Proteobacteria* (35.5%), *Firmicutes* (31.3%), *Actinobacteria* (22.4), and *Bacteroidetes* (6.4%) (Fig. 2 and Data Sets S3 and S4). The proportion of the different genera represented appears to be different between the global human repertoire, the human gut repertoire, and the human urinary tract repertoire, with a higher proportion of *Mycobacterium* (6.2%) in the global human repertoire, *Clostridium* (6.5%) in the human gut repertoire, and *Strepto-*

coccus (5.3%) in the human urinary tract repertoire (Fig. 3 and Data Sets S3 and S4). More aerointolerant species are described in the human gut microbiota than in the global human repertoire and in that of the human urinary tract, with 505/1,484 (34.0%), 386/2,172 (17.8%), and 100/562 (17.8%) aerointolerant species, respectively. By the Fisher chi-square analysis, the urine repertoire shares a significant difference in aerointolerant species with the gut microbiota ($P < 0.001$; OR = 9.0 [7.0 to 11.4]). This could be biased by the longtime occultation of the urinary microbiota and the use of only aerobic culture techniques for urine samples. In this sense, the studies using high-throughput sequencing show a higher proportion of aerointolerant bacteria in urine (74/240 [30.8%]). Few studies have evaluated the vaginal bacterial repertoire, but in 2007, Fredricks et al. (16) showed the absence of some major urinary tract species, such as *Escherichia coli* and *Enterococcus faecalis*, which goes against the hypothesis of a vaginal source of urine colonization.

COMMENSAL MICROORGANISMS VERSUS PATHOGENS

Because of the difficulty in defining commensal, opportunistic, or strictly pathogenic species, we first grouped species affecting human beings on the basis of their risk group, defined as the risk of biological agents for laboratory staff security, community security, and human health (risk group 1: a biological agent is most unlikely to cause human disease; risk group 2: a biological agent may cause human disease and might be a hazard to laboratory workers but is unlikely to spread in the community, laboratory exposure rarely produces infection, and effective prophylaxis or treatment is available; risk group 3: a biological agent may cause severe human disease and present a serious hazard to laboratory workers and it may present a risk of spread in the community but there is usually effective prophylaxis or treatment; risk group 4: a biological agent causes severe human disease and is a serious hazard to laboratory workers, it may present a high risk of spread in the community, and there is usually no effective prophylaxis or treatment). Most of the species found in urine belonged to risk group 2 (336 [59.8%]), 89 (15.8%) belonged to risk group 1, and 12 (2.1%) belonged to risk group 3. No species belonged to risk group 4, but 125 (22.2%) were not yet classified. To know the impact of the different bacterial taxa in causing infectious diseases, especially urinary tract infection, at an individual level, we looked at the pathogenicity of each bacterial taxa of the human urinary tract repertoire. According to the literature, 352 out of 562 species (62.6%) have been associated with at least one case report of human infection, including 225 (40.0%) reported as causative agents of urinary tract infection (Data Set S2). At least 60.0% of the urine microbiota is not reported in the literature as causing human urinary tract infection and could really be considered commensal until new cases are reported.

HUMAN URINARY MICROBIOTA AND CLINICAL MICROBIOLOGY IMPLICATIONS

Several roles could be attributed to the urinary tract microbiota. Urinary emergency and other chronic urinary tract symptoms have been associated with modification of bacterial components of urine (5, 9, 10). Patients who tend to have kidney stones seem to have different gut and urinary microbiota compared to that of healthy control patients (17). Difference in stone formation prevalence is also seen between vegetarians and meat eaters, which has been attributed to the difference in protein consumption but could result from different urinary microbiota (18). Urinary tract microbiota may also influence bladder cancer. In fact, *Mycobacterium bovis* (in *Mycobacterium bovis* BCG therapy) is used for treating urothelial bladder cancer (8, 19), as is *Mycobacterium indicus* subsp. *pranii* (20). In a double-blind, placebo-controlled randomized trial, oral administration of *Lactobacillus casei* decreased superficial bladder cancer recurrence (21).

Urinary tract microbiota influences urinary tract infections. Commensal bacteria might outcompete pathogens for common resources and act as a barrier to uropathogens by secreting inhibitory or bactericidal molecules. Decreased diversity of the urinary flora may be a risk factor for urinary tract infection (22). Most human bacterial

pathogens are commensals, as they are able to colonize human body sites without causing any infection and can become pathogenic in response to some host factors (immunosuppression favoring opportunistic infection, local foreign material favoring proliferation of microorganisms, or antibiotic pressure favoring part of the global bacterial population) (13, 14), and uropathogens could be present in the urinary tract before infection (23). Indeed, evidence of a permanent urinary microbiota associated with modifications of these biochemical and physical parameters could explain the development of urinary tract infections. On the other hand, some *Escherichia coli* and *Enterococcus faecalis* strains causing urinary tract infections are foodborne pathogens and are considered zoonosis (24). In 2007, Manges et al. described a statistical link between meat eaters and multidrug-resistant *Escherichia coli* urinary tract infections (25). Urinary tract infection can develop with commensals due to dysbiosis (22), but can also be promoted by biochemical, hormonal, or mechanical disorders or after the introduction of a pathogen through the food that passes through the intestinal reservoir and joins the urinary tract (24). Moreover, urinary tract infection can occur because of some particular virulent bacterial strain that is able to grow very fast and to give an important inoculum (26, 27).

In 2017, Tariq et al. (28) reported, in a small-size case-control study, the decrease of recurrent urinary tract infections and the antibiotic resistance of urinary bacteria in a patient with recurrent *Clostridium difficile* infection during the year following fecal microbiota transplantation. Patients who did not benefit from fecal microbiota transplantation had no modification of the urinary tract infection frequency or of the antibiotic resistance profile of urinary bacteria. Restoration of healthy gut microbial communities with fecal microbiota transplantation may decolonize enteric multidrug-resistant organisms and decrease the risk of recurrent urinary tract infections and urinary multidrug-resistant organisms. Similarly, frequent consumption of fermented milk products containing probiotic bacteria has been described as reducing the risk of recurrent urinary tract infection (29).

Additional investigations of bacteria with unknown pathogenicity are required in order to improve diagnostic assays and better understand the diversity and epidemiology of infections. The constitution of a comprehensive repertoire is the first essential step before considering the association between some bacterial strains and some clinical involvements. A recent study has demonstrated that the extension of the prokaryotic repertoire associated with humans, by performance of high-throughput culture studies (13–15), had enlarged the spectrum of prokaryotes known to be involved in infectious diseases (14). Longitudinal studies that include genome sequencing of the strains will elucidate if, in a single individual, a bacterial species can be commensal and, following changes in the ecosystem, can become pathogenic (30). This work constitutes an essential starting point by objectively listing all the bacterial taxa found at least once in urine without surmising their role in human physiology and/or pathology.

CONCLUSION: A POTENTIAL PARADIGM SHIFT?

Work on the microbiota, especially that reported here, suggests that a number of bacteria from other mucus membranes and probably from the digestive tract are likely to colonize the urinary tract, especially the bladder.

This microbiota can persist without causing symptoms, which is increasingly recognized in older people, in whom a significant percentage may carry a germ without obvious infections. It appears that pathogens can be part of a consortium, but the role this consortium plays in the control of urinary tract infection is unknown. It has been suggested that some probiotics, or even changes in acidity or consumption of cranberries, may lead to better control of the microbiota and bladder-infecting bacteria.

We believe, as previously described (8), that urinary tract infections are often the consequence of a change in the bladder ecosystem caused either by traumatic aspects such as urinary catheters, by metabolic changes (pregnancy, acidity), or by mechanical

stasis conditions (prostatic adenoma, constipation). In practice, it appears that in a large number of cases, pathogens are present in the bladder without causing diseases (30).

This work constitutes an essential starting point that is made necessary by the fact that urine is not sterile (5, 6, 8, 9). Coupled studies using metagenomic and culturomic techniques (13–15) to test urine samples of patients with urinary tract infections or diverse other clinical involvements and urine samples from healthy individuals will enable us to elucidate the relationships between the urine microbiota and the human health, including the physiopathology of urinary tract infections.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00675-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.01 MB.

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3.Partie II : Description du microbiote urinaire par méthodes de culturomics et de métagénomique et implémentation du répertoire des bactéries connues dans le tractus urinaire humain :

Avant-propos :

L'objectif de ce travail était par une approche mixte de culturomics et de métagénomique d'analyser la composition bactérienne d'échantillons d'urine de sujets malades et de volontaires sains afin d'implémenter le répertoire de bactérie connues dans le tractus urinaire humain [35].

L'urine humaine a été considérée comme stérile pendant longtemps. Cependant, 423 espèces ont déjà été cultivées et décrites dans la littérature, dont seulement 40 espèces anaérobies (9,4%). Ici, nous avons utilisé la méthode culturomics, en ciblant particulièrement les anaérobies, comme cela a été rapporté précédemment pour l'intestin [5, 10, 17], afin de mieux comprendre le microbiote urinaire.

En testant 441 échantillons d'urine, nous avons isolé 459 espèces bactériennes différentes, dont 264 jamais décrites dans l'urine, parmi lesquelles 18 nouvelles espèces. Parmi les espèces isolées, 161 étaient des espèces anaérobies, nos techniques de culture nous ont ainsi permis de trouver plus de bactéries aérointolérantes (35%) que celles qui avaient été précédemment décrites dans l'urine dans la littérature (17,8%). Dans l'ensemble, cette étude a augmenté de 39% le répertoire bactérien des voies urinaires humaines connu. Parmi les 684 espèces bactériennes isolées au moins une fois à partir du microbiote d'urine, 424 (62%) avaient déjà été isolées du microbiote intestinal [10], alors que seules 218 (32%) avaient déjà été isolées du vagin [36]. D'autre part il n'a pas été mis en evidence une difference majeure de composition du microbiote urinaire entre les sexes féminin et masculin en dehors d'une fréquence plus importante d'*Escherichia coli* ainsi qu'un nombre d'uropathogènes par échantillons plus important chez les femmes. Nos résultats conduisent à un changement de paradigme dans la compréhension du microbiote urinaire, soulignant que son origine est l'intestin plutôt que le vagin.

Nous avons de plus mené une étude en sous groupe sur la population pédiatrique de notre cohorte. Nous avons prélevé les échantillons d'urine de patients âgés de moins de 3 mois suspects d'avoir une infection des voies urinaires, grâce à un cathétérisme de la vessie ou par recueil sur urinocol après désinfection périnéale avec un antiseptique chloré (Amukine®) et des échantillons d'urine de patients âgés de 2 à 18 ans ayant une propreté urinaire acquise et ayant subi un examen systématique d'urine grâce à un prélèvement d'urine en milieu de jet après une

désinfection minutieuse du périné. Ces échantillons ont été analysés par méthodes de culturomics et par métagénomique. Au total, 31 échantillons d'urine de nourrissons de moins de 3 mois et 12 échantillons d'urine d'enfants de 2 à 18 ans ont été recueillis. Nous avons identifié une moyenne de 11,16 bactéries par échantillon, une moyenne d'indice de Shanon de 2,09 par échantillon et un total de 160 espèces bactériennes différentes, dont 58 n'avaient jamais été identifiées dans l'urine humaine auparavant, 72 n'ont pas été retrouvées par la méthode métagénomique et 37 étaient uniquement retrouvées par la culturomics (ni dans la littérature ni par méthode de métagénomique). Le métabolisme de ces bactéries était anaérobie dans 38,75% des cas. Les échantillons d'urine étaient significativement plus riches en espèces bactériennes différentes et en index de Shannon chez les enfant plus âgés et de poids plus important. Les 31 échantillons d'urine de nourrisson de moins de 3 mois et 8/12 échantillons d'urine d'enfants âgés de 2 à 18 ans ont été analysés par la technique de métagénomique. La métagénomique a permis de révéler 433 OTU au niveau de la sous-espèce, soit 422 espèces différentes, dont 264 n'avaient jamais été décrites dans l'urine auparavant et 334 n'étaient pas cultivées selon nos méthodes de culturomics, 278 n'ont été trouvées que par la technique métagénomique dans notre étude (ni dans la littérature, ni par licrobial Culturomics). Dans ces espèces figuraient certaines bactéries extrêmophiles.

Nous concluons qu'il existe bien chez les enfants comme chez les adultes, chez les femmes comme chez les hommes, chez des sujets sains comme des sujets malades un microbiote urinaire qui peut être décrit par les techniques de culturomics en association à une approche par métagénomique.

La standardisation des techniques de culturomics et l'utilisation de supports plus spécifiques pour la culture de certaines espèces bactériennes fastidieuses ou extrémophiles permettraient une approche complémentaire de la métagénomique pour décrire le microbiote urinaire et comprendre son rôle en physiologie et en pathologie.

Article II : letter

Deciphering urinary microbiota repertoire by culturomics reveals mostly anaerobic bacteria from the gut

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Deciphering the urinary microbiota repertoire by culturomics reveals mostly anaerobic bacteria from the gut

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Authors contributions:

D.R. conceived and designed the experiments; V.M., M.T., and E.L., actively participated in the specimen collection and the study design; A.M., F.M., R.G., A.C., A.Y., A.D., F.C., M.C., S.B. performed the experiments. G.D., A.M., J.C.L., and D.R. analysed the data; G.D., A.M., J.C.L., and D.R. wrote the manuscript. All authors read and approved the final manuscript.

SUMMARY

Human urine was considered sterile for a long time. However, 423 species have been previously cultured including only 40 anaerobic species. Here, we used culturomics, particularly targeting anaerobes to better understand the urinary microbiota.

By testing 435 urine samples, we isolated 459 different bacterial species, including 264 never described in urine, of which 18 were new species. Among the bacterial species identified, 161 were anaerobes (35%). This study increased the known urine repertoire by 39%. Among the 684 bacterial species isolated at least once from urine microbiota, 424 (62%) were previously isolated from gut microbiota, while only 218 (32%) were previously isolated from vagina. In parallel with *Lactobacillus*, for which passage from the digestive tract to urine or milk has been demonstrated, our results lead to a paradigm shift in the understanding of the urinary microbiota, highlighting that its origin is the gut rather than the vagina.

The study of the urinary microbiota is recent and has been subjected to many biases. Indeed, since urine has been considered naturally sterile[1,2] due to methodological biases, the techniques developed to detect bacteria of urinary origin have led to the consideration of only dominant bacteria from easily, rapidly and aerobically cultured[3 4] urinary specimens. In addition, the higher frequency of urinary tract infections in women than in men has led to the consideration that the source of bladder colonization is genital, due to the small size of the female urethra[5]. By analogy, this has led to the consideration that the bladder microbiota, apart from urinary tract infections, is of vaginal origin, neglecting the fact that men also have urinary tract infections[6] and a urinary microbiota.

The aim of this work was therefore to evaluate the urinary microbiota of men and women by anaerobic culture techniques[7,8] developed to recover bacteria potentially shared with the gut microbiota.

We have recently constituted a database of bacteria isolated from the urinary tract containing 416 cultured bacterial species, except those isolated as a part of culturomics studies[9]. Herein, the study was segmented in thirteen different projects, including 435 urine specimens from 279 patients. (Supplementary Table 1). A total of 17 different culture conditions were designed for this purpose (Supplementary Table 2), but the number of conditions per project ranged between 3 and 11 culture conditions. All culture conditions were carried out by inoculating a volume of urine ranging from 100µL to 1mL (Supplementary material). Overall, by analysing 143,689 colonies, we cultured a total of 435 urine specimens and identified 466 microorganisms, of which 7 were fungi. Among the 459 bacteria cultured, 195 were already known from a previously established repertoire of bacteria cultured from the urinary tract. Indeed, to date, 264 prokaryotes were not identified from urine specimens (Figure 1), of which 234 were previously identified from humans. Thanks to the shared databases between different MALDI-TOF devices used in the laboratory, 47 species

that were previously discovered as a part of culturomics studies were identified in urine specimens (Supplementary Table 4). Most of these taxa were first isolated from the human gut (32/47, 68%), 14 were isolated from vaginal specimens and one from sputum samples. Of the 30 species that were not previously detected in humans, 12 were previously recognized taxa, of which one was a new species we previously isolated from the gorilla gut (i.e., *Microbacterium marseillegorillensis*)[10]. Among the main discoveries, the present study was able to isolate 18 different new species and genera. Of these, seven were only recovered once, while more than half were detected in several projects. Indeed, *Actinomyces urinae* was cultured in 5 different projects, highlighting the probable high prevalence of the species in the urinary tract. Due to these culturomics studies, the number of bacteria known in the urinary tract is now 684, thereby extending the prokaryotic urinary repertoire by 39%. When focusing on these 264 bacteria added to the repertoire, Firmicutes were mainly represented (134 species, 50%) followed by Actinobacteria (65 species, 26%). Species from rare phyla were also added, such as Fusobacteria (i.e., *Fusobacterium naviforme and F. necrophorum*) and, more interestingly, two species from Synergistetes, including *Pyramidobacter piscolens*, which has been isolated from the oral cavity and the gut microbiota to the best of our knowledge[11], and *Jonquetella anthropic*, which was initially recovered from clinical specimens[12]. More specifically, the family *Peptoniphilaceae* contributed to extending the repertoire the most, as it represents 10% of the species added. This family contains mainly anaerobes; therefore, we looked at the tolerance of the oxygen of the bacteria recovered in this study. Among the 264 additional species, 133 were strict anaerobes (50%). In the previously established repertoire[9], only 9.4% of the cultivated species were anaerobes, and the number of anaerobes was 35% when considering only the present study (Figure 2), highlighting that anaerobes were so far ignored from the urinary tract. We looked at the prevalence of uropathogens (see supplementary material, section 4.1) in a subset of 406 urinary samples

with corresponding gender information. We found a non-significant difference regarding the presence of at least one uropathogen between male (107/195, 54.8%) and female (129/212, 60.8%) specimens (Fischer exact test, *p=0.12*) (Supplementary Figure 1A). Nevertheless, the number of uropathogens cultured per urinary sample was different between males and females (Mann and Whitney test, *p=0.032*) (Supplementary Figure 1B), and *E. coli* was more frequently found in specimens from women (58/212, 37.9%) than in those from men (36/195, 18.5%) (Fischer exact test *p=0.03*) (Supplementary Figure 1C). We attempted to identify the potential source of the microbes inhabiting the urinary tract by comparing the current updated repertoire of bacteria cultured from urine[9] with those established from the gut[7], the respiratory tract[13] and the vagina[14]. Strikingly, the majority of the 657 species (i.e., 62%) cultured from urine were shared with the human gut repertoire (Figure 3), while less than half were shared with vaginal and respiratory/oral cavity microbiomes (i.e., 30% and 40%, respectively). We also looked at the 10 most prevalent bacteria retrieved from urine specimens in clinical microbiology laboratories over a five-year period. Compared to males, females were found to have more *S. agalactiae* and *Staphylococcus saprophyticus* (Supplementary Table 3). In addition, 6 and 7 bacteria from this ranking list from male and female specimens, respectively, are common residents of the digestive tract. Finally, when comparing the species recovered from male and female subjects in this study, a substantial proportion (i.e., 48%) was found in both groups (Figure 4).

Herein, we report the culture of 466 microorganisms from urinary specimens by a culturomics approach, of which 459 were prokaryotes. The current work thereby enriches the current human microbiota repertoire by a dramatic amount of 39%. In addition, the current work shows that men have a microbiota as diverse as women (Figure 4) and, as a result, raises the question about the exclusive vaginal origin of the female bladder microbiota, even if some microorganisms are found in common in the vagina and urine (Figure 4). The source of

126 vaginal bacteria can be both urinary and faecal in origin. Anatomically, it seems more directly
127 related to a urinary source than to a faecal source. In addition, we found herein bacteria that
128 have been ignored until now because they are strict anaerobes (Figure 2), and there was no
129 systematic protocol dedicated to their culture because their impact on urinary tract infections
130 was considered negligible. Again, the fact that studies of the urinary microbiota were deduced
131 from urinary tract infections led to a poor choice of strategy to discover the real microbiota.
132 This was recently illustrated by the fact that *Methanobrevibacter smithii*, which is a
133 very strict anaerobic Archae, was found in urine by two teams[15]. This is inconsistent with a
134 recent study suggesting a shared microbiota between the vagina and bladder by culturing 149
135 bacterial strains, of which several strains displayed a high level of similarity, although the
136 bacteria were cultivated from two sites[16]. Indeed, the authors did not perform extensive
137 anaerobic cultures as the media were only kept for 48 hours, which can lead to erroneous
138 conclusions.
139 Our study constitutes a paradigm shift demonstrating that the origin of the urinary
140 microbiota is the digestive tract. As a matter of fact, gut microbiota contributing to the
141 diversity of prokaryotes inhabiting the urinary tract was suggested by a recent study
142 highlighting the reduction in the recurrence of urinary tract infections (UTI) following faecal
143 microbiota transplantation (FMT)[17,18]. It has also been recently suggested that the composition
144 of the intestinal microbiota could impact the occurrence of UTI in children[19]. These data
145 suggest that UTI are in fact the consequences of ecosystem disruptions and that uropathogens
146 could be acquired from the environment, particularly from animals[20–23]. Supporting this, a
147 systematic review demonstrated that half of the bacteria cultured from human milk have a
148 probable digestive source[24], and the passage of *Lactobacillus* species directly through urine
149 following yogurt ingestion has recently been demonstrated[25]. Recent studies dedicated to the
150 influence of urinary microbiota on bladder cancer nevertheless incriminate bacteria mostly

151 derived from the gut[26,27]. It therefore appears that tissue microbiota considered, until recently,
152 sterile are in fact colonized by bacteria that are often tedious and anaerobic and that have
153 passed through the digestive tract.

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156 03**.**

157 **Conflict of interest**

158 The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Evolution over time of the urinary repertoire according to culturomics studies. The bacterial species are represented in five categories: Known in urines, prokaryotes isolated by other laboratories but not by culturomics; Identified in urines by culturomics, taxa recovered by culturomics studies and already known to belong to the bacterial urinary repertoire; New species culturomics, new taxa discovered as a part of other culturomics studies; Unknown in humans, prokaryotes first isolated in humans; and New species culturomics Urines, species isolated from urinary tract as a part of this study.

Figure 2. Proportion of anaerobes recovered by culture when considering the urinary repertoire of prokaryotes previously established[9] (2A) and as a part of this study (2B).

Figure 3. Venn diagram showing the shared cultured species between urinary tract, gut[7], respiratory tract/oral cavity[13] and vagina[14].

Figure 4. Comparison between species recovered from urine specimens from male and female individuals. Each node represents a bacterial species. Nodes on the left are bacterial species recovered only from male specimen while those on the right were cultured only from female specimen. Nodes in the middle are bacterial species recovered from both male and female specimen. Color edges represent the putative origin of these bacterial species, as indicated by the legend on the left of the figure.

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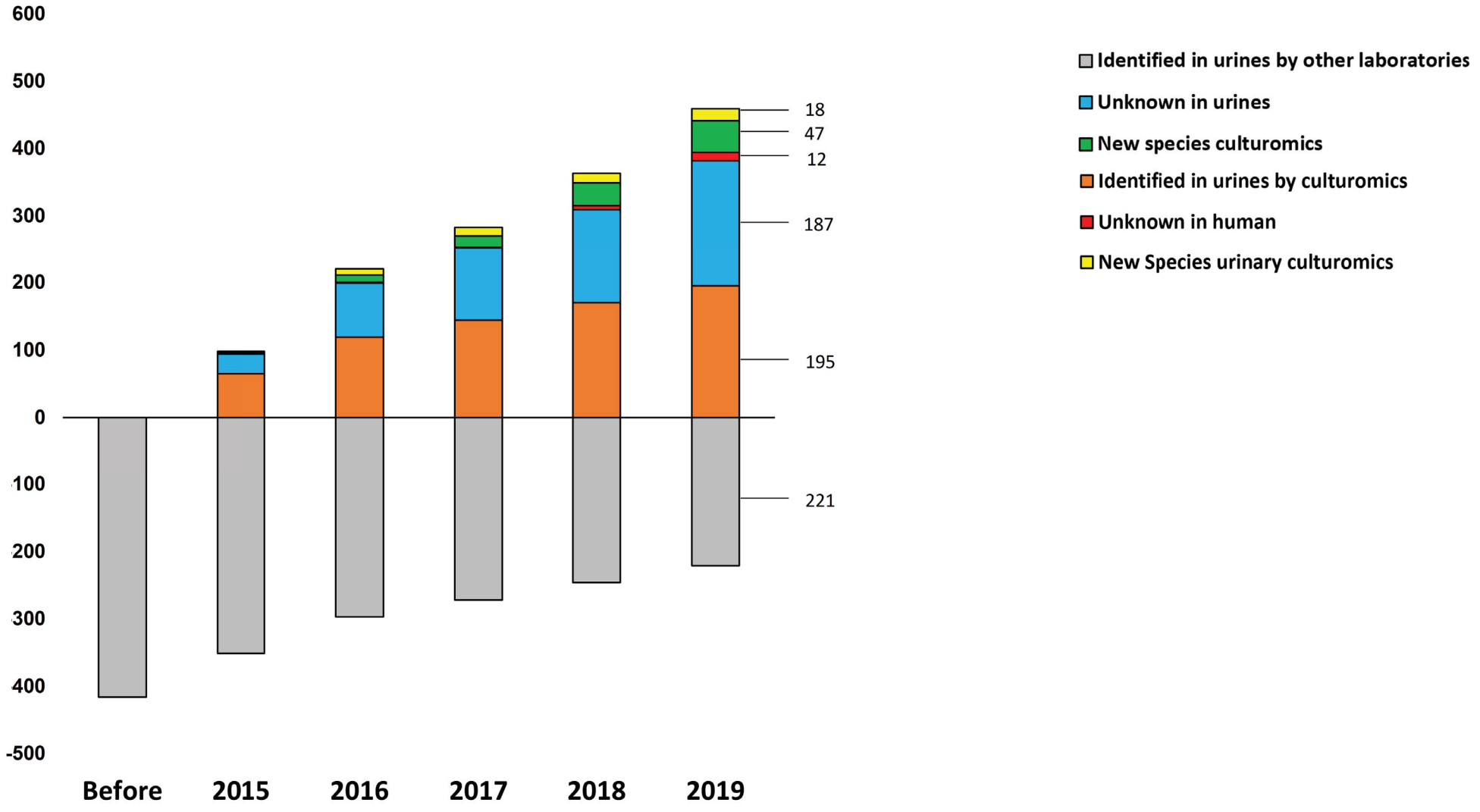
600
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Before
2015
2016
2017
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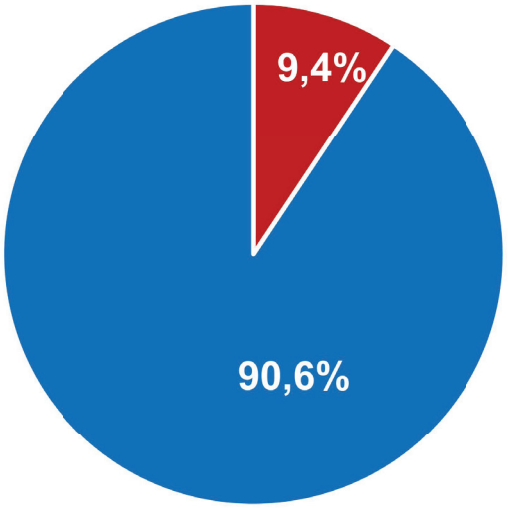
2019
18
47
12
187
195
221
Identified in urines by other laboratories
Unknown in urines
New species culturomics
Identified in urines by culturomics
Unknown in human
New Species urinary culturomics

A

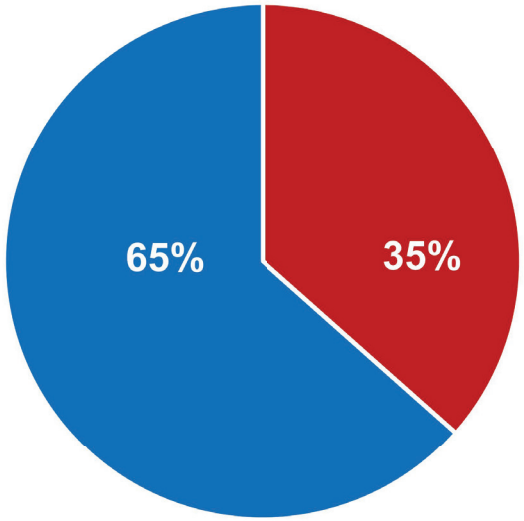
Repertoire of bacterial species associated with urinary tract



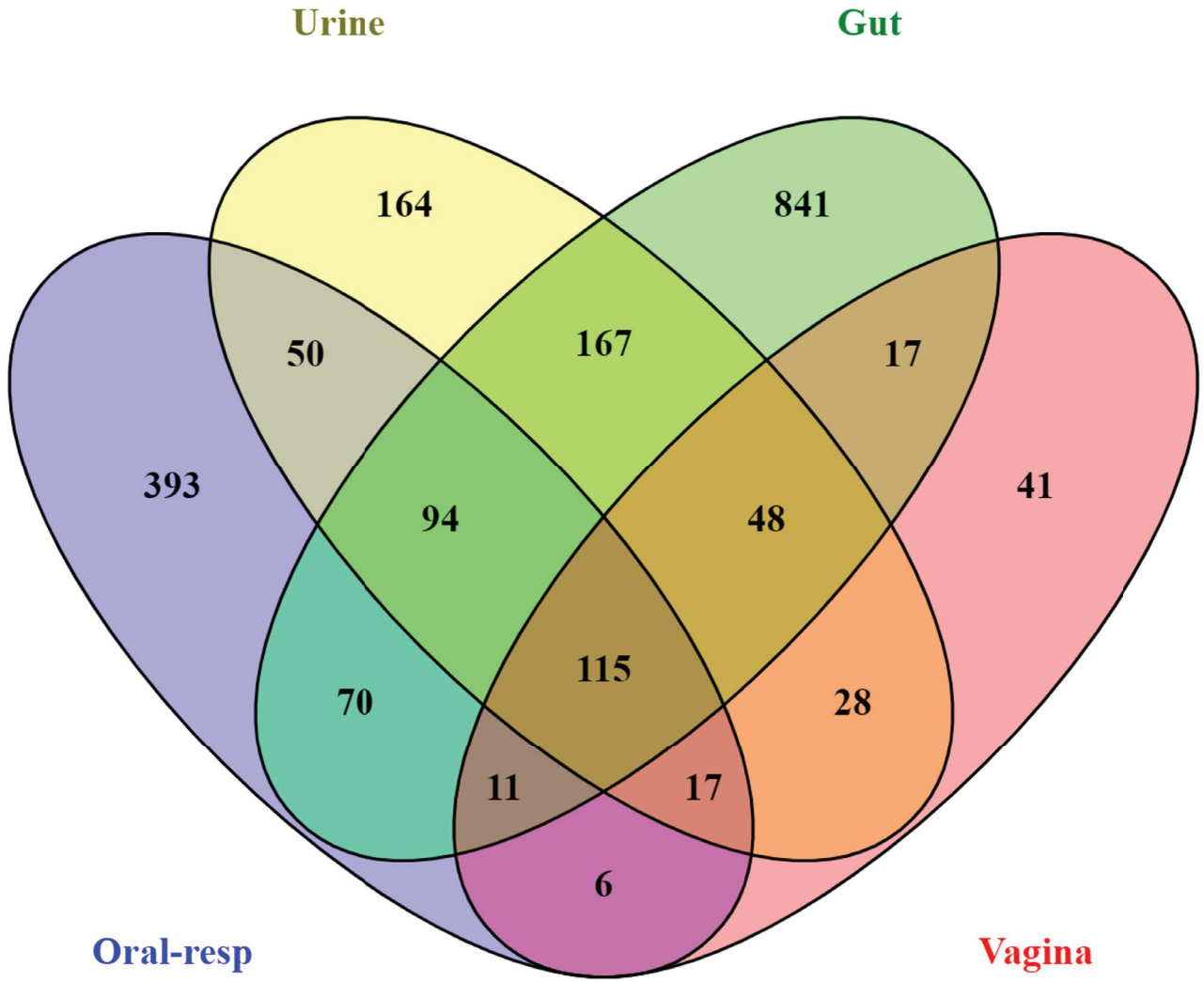
N=418 species

B

This study



N=459 species

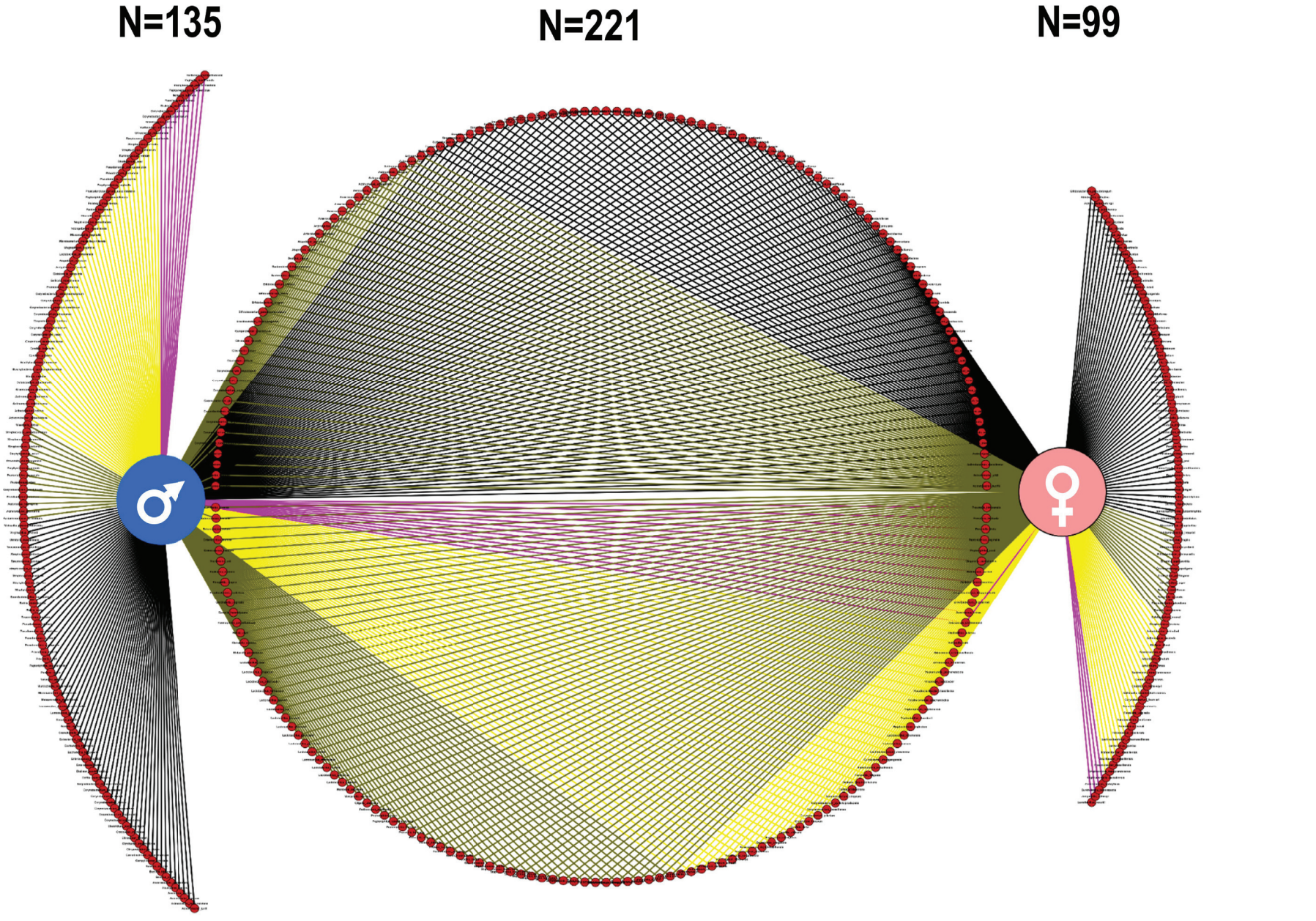


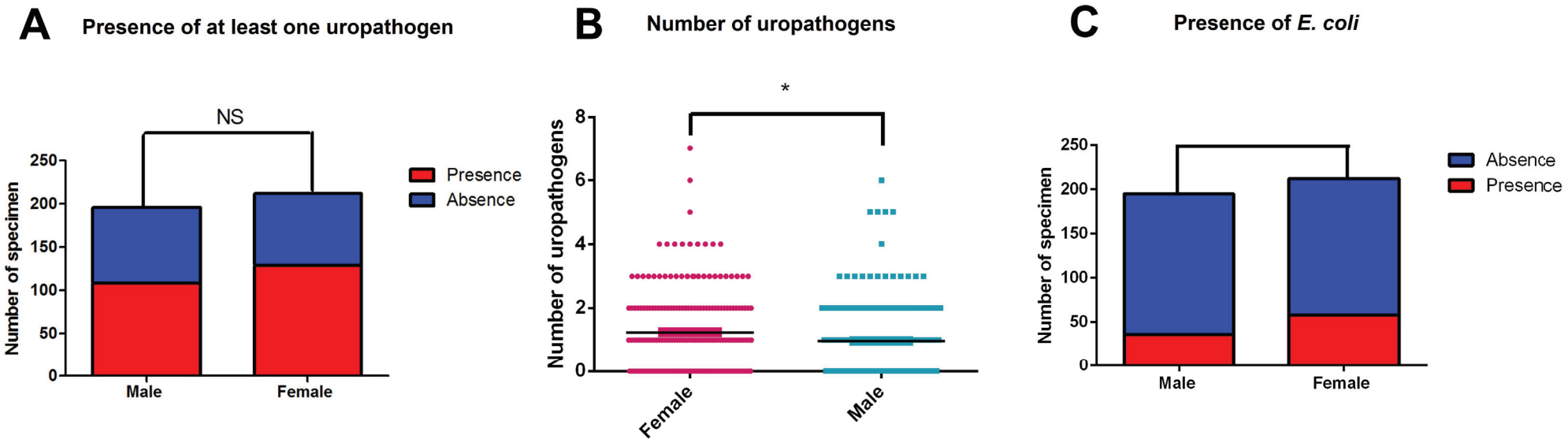
Species cultured from urines : N=684

Shared with gut : 424 (62%)

Shared with vagina : 208 (30%)

Shared with oral-respiratory tract : 276 (40%)


N=135
N=221
N=99
♂
♀
Microbes also cultured from the gut
Microbes also cultured from the vagina
Microbes also cultured both from the gut and the vagina
Microbes only cultured from urine


A
Presence of at least one uropathogen
NS
Number of specimen
250
200
150
100
50
0
Male
Female
Presence
Absence
B
Number of uropathogens
*
Number of uropathogens
8
6
4
2
0
Female
Male
C
Presence of E. coli
Number of specimen
250
200
150
100
50
0
Male
Female
Absence
Presence

Supplementary Methods

1. Ethics

Ethics committee was obtained for the UTI project under the number 2015-A00884-45. The ethics committee of the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection validated the study under number 2016-01 and 2016-011. Regarding the inclusion of children, the study was explained to the parents, and a consent form was given to the parents.

2. Samples and patients

2.1 Urine sample of children with nephrologic or urologic diseases and urinary sample of children without any nephrologic or urologic disease history (control)

Void urine midstream samples obtained from children aged 0 to 18 years old were analysed. Those children were hospitalized or consulted at the Timone University Hospital, Marseille, France, from December 2014 to July 2015 and had one of the following pathologies:

Enuresis or other micturition disorders, nephritic syndrome, end-stage renal disease (glomerular filtration flow < 80 mL/min/1.73 m²) with dialysis (hemodialysis or peritoneal dialysis) or not, repeated urinary tract infections with sterile urine culture between each infection. A control group was also analysed. Those children had to be hospitalized in orthopaedic paediatric surgery for an acute surgical problem and without any serious medical history. A form of non-opposition was given and signed by the parents. Administrative data and clinical information were obtained from the parents, the medical staff and the patient medical documents: weight, height, sex, pathology, date and time of the urine collection. The sampling technique consisted of collecting void urine midstream samples after a perineal wash with a sterile compress moistened with chloric antiseptic (Amukine®) in a sterile jar.

The samples could be collected by the parents who were informed of the sampling technique or by the medical staff.

2.2 Urine sample of human adults after kidney transplant

The first project involved adults who received one kidney transplant without any urinary symptoms, were hospitalized or were seen in consultation during their follow up in the Nephrology and Transplantation Centre of the Conception University Hospital, Marseille, France, from February 2014 to July 2015. The patients declared in written form not to be opposed to a urine collection for the study. Void urine midstream samples were obtained after a peritoneal wash with a sterile compress moistened with chloric antiseptic (Amukine®) and collected in a sterile jar.

2.3 Urine sample from human adults before and after one kidney transplant and of healthy alive adults who were kidney donors

Three other projects were carried out on adults who received one kidney transplant *de novo* one kidney at the Nephrology and Transplantation Centre of the Conception University Hospital, Marseille, France, from February to May 2019 (Supplementary Table 1). For each patient, one to five urine samples were collected. One sample was collected before or the day of renal graft transplantation (named D0). One sample was collected two days after renal transplantation (D2), and one sample was collected each Monday following renal transplantation for three weeks (W1, W2, W3) for cohorts 1 and 2. Samples were collected monthly for cohorts 3 and 4 ten days (J10), one month (M1), and three months (M3) after renal transplantation. An additional sample was collected six months (M6) after renal transplantation for cohort 4. Transplanted patients experienced antibiotic treatment with cefamandole (cohorts 1 and 2) or cefazolin (cohorts 3 and 4) from day zero to day five after transplantation to reduce the risk of operating site infection. The patients also received

cotrimoxazole from day two to 6 months after transplantation to prevent *Pneumocystis jiroveci* pulmonary infection. Moreover, if the graft kidney came from a living adult donor, a urine sample from this donor before kidney extraction was also collected and analysed. Void urine midstream samples or urine samples collected by vesical catheterization were obtained after a perineal wash with a sterile compress moistened with chloric antiseptic (Amukine®) and collected in a sterile jar.

2.4 Urine sample of infants under 3 months of age

Void urine midstream samples obtained from children under three months old were analysed. Those children were hospitalized or consulted at the Timone University Hospital, Marseille, France, from February to June 2016, and sterile urine samples were collected for one of the following reasons: fever or other clinical symptoms compatible with maternofoetal infection (jaundice, poor weight curve, vomit, etc.), urine catheterization for retrograde urethrocystography, urine catheterization for sedation, systematic urine sampling for controlling urine chlorine excretion of patients affected by pylorostenosis waiting for surgery.

A consent form was given and signed by the parents. The following administrative data and clinical information were obtained from the parents, the medical staff and the patient medical documents: weight, sex, pathology, information about pregnancy, delivery, neonate history, breastfeeding or not, history of antibiotherapy, date and time of the urine collection. The sampling technique consisted of collecting void urine samples in a sterile jar by vesical catheterization, suspubian punction, midstream urine collection or Urinocol® (B. Braun Medical SAS, Melsungen, Hessen, Germany) after a perineal wash with a sterile compress moistened with chloric antiseptic (Amukine®). The sample had to be collected by the medical staff.

2.5 Subjects who have taken probiotics

Subjects were apparently healthy volunteers who have taken probiotics during 7 days and a second urine specimen was collected at this time.

2.6 Patients who underwent Fecal Microbiota Transplantation (FMT)

To reduce the mortality of severe *Clostridium difficile* infections, early FMT has been proposed as a first line treatment[1]. Urine specimen were collected at different time points following transplantation. Patient's consent and agreement from the local ethics committee were obtained (Agreement N° 2016-011, Marseille, France).

2.7 Patients suffering UTI

Urine specimen were collected as a part of the diagnosis urinary tract infections (UTI) in the routine laboratory and prescribed in the form of Urinary Microbiota Kit. The latter was set up in 2014 in our hospital center (IHU Méditerranée Infection, Marseille, France) with the aim of comprehensively describing the resident flora of the urinary tract from samples of patients consulting or being hospitalized within our hospital center. Patient's consent and agreement from the local ethics committee were obtained (Agreement N° 2016-011, Marseille, France).

2.8 Patients with suspected bladder cancer

Patients needing cystoscopy were recruited to the Urology Department (Conception University Hospital, Marseille, France). Among them, patients had a suspicion of bladder cancer, a haematuria report, or a report of repeated urinary tract infections. The urine collection took place just before this cystoscopy was performed. A non-opposition form was collected.

3. Transport, repartition of the urine and urine qualitative analyses

All the urine samples were quickly transported to the microbiology laboratory of the Timone University Hospital to be inoculated in culture media in the 6 hours following the urine collection. All the samples were separated into three specimens: one for the routine microbiology laboratory, one for the special culture laboratory (where culturomics was performed) and one aliquot of 1 mL was frozen at -80°C in the microbiology laboratory of the Timone Medical University, Marseille, for metagenomic analyses. The pH of the samples was measured with reactive strips (pH-Fix 4.5-10 reference 92120 (Macherey-Nagel®, Düren, Germany), and the salinity of the samples was measured with a refractometer PR-100SA (Atago®, Tokyo, Japan).

4. Culture Methods

All manipulations were performed in a laboratory with biosafety containment level 2, and bacterial manipulation was performed under a domestic microbiological safety cabinet with a hood and laminar flow.

4.1 Standard culture in routine laboratory

One specimen of each sample was analysed by standard culture methods in the routine microbiology laboratory of the University Hospital, Timone, Marseille. A cytobacteriological examination with standard culture and antibiotic susceptibility testing was performed. The number of leukocytes and red blood cells contained in the sample was quantified. A small volume of urine (10 µL) was seeded on 5% sheep blood-Columbia agar medium (COS agar) (bioMérieux, Marcy l'Etoile, France), and the sample was cultured aerobically between 35 and 37°C for 48 hours. Each colony was identified by MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry) (Bruker, Billerica, USA)[2]. Standard bacterial culture was reported following expert recommendations[3]. The detection limit of this method was dependent on the sex of the patient and the bacterial species:

- 10^3 colony forming units (CFU)/mL for *Escherichia coli* and *Staphylococcus saprophyticus* from samples from men and women

- 10^3 CFU/mL for *Enterobacteriaceae* other than *E. coli* from samples from men

- 10^4 CFU/mL for *Enterococcus faecalis*, *Enterococcus faecium, Corynebacterium urealyticum*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* from samples from women.

Four conclusions were available after laboratory analyses:

- Sterile urine

- No significant microbial development ($<10^3$ or $<10^4$ CFU/mL)

- Polymicrobial development ($>10^3$ or $>10^4$ CFU/mL)

- Bacterial identification and antibiotic susceptibility testing

4.2 Culturomics

4.2.1 Analyses on urine from children with nephrologic or urologic diseases

The first step was to seed fresh urine on six different solid culture media. Five solid culture media were home-made. One millilitre of fresh urine was seeded on solid media and incubated in aerobic conditions at 37°C for 24 to 96 hours. The fresh urine was plated twice on the commercial medium COS (bioMérieux) to incubate it in aerobic and anaerobic conditions. In a second step, urine samples were preincubated in 4 different liquid media. In each bottle, 1 mL of fresh urine was inoculated. At day 1, day 3, day 7, day 10, day 14, day 21 and day 30, 1 mL of the suspension was sampled and diluted from 1/1 up to 1/10 in PBS (Dulbecco). Then, 1 ml of each dilution was seeded on COS agar (bioMérieux) for all four liquid conditions: for conditions for 1 and 2, the dilutions were seeded on commercial COS agar (BD Diagnostics Becton Dickinson GmbH®, Heidelberg, Germany); and for the liquid

conditions for 3 and 4, the dilutions were seeded on home-made medium with Columbia agar enriched with 5% rumen fluid, which was sterilized by 0.2 µm pore microfiltration, and 5% defibrinated sheep blood. These cultures were incubated at 37°C for 24-96 hours in an aerobic atmosphere for the liquid conditions for 1 and 3 and 72-120 hours in anaerobic atmosphere for the liquid conditions for 2 and 4.

4.2.2 Analyses on urine from adults after a one kidney transplant

A large quantity of urine (1 mL) was seeded in two solid media. Then, urine was cultured for 10 days under aerobic conditions at 37°C. Colonies obtained were then quadrant streaked on 5% sheep blood-Columbia agar medium (bioMérieux) on days 1, 3, 7 and 10.

4.2.3 Analyses on urine from adults before and after a one kidney transplant and of healthy alive adults who were kidney donors

This project was divided into two steps. First, the urine was directly seeded on two solid media. For each urine specimen, 1 mL was sampled and then serially diluted (from 1/1 up to 1/10) in phosphate buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline Gibco® Life Technologies™ Paisley, United Kingdom). Then, 100 µl of each dilution was seeded on solid media and incubated at 37°C in aerobic conditions on R-medium and both in aerobic and anaerobic conditions on COS agar (bioMérieux) using an anaerobia generator (AnaeroGenTM, Oxoid Ltd, Dardilly, France).

Second, the urine was used to inoculate liquid medium. After 48 hours, 96 hours and 10 days of incubation, 1 ml of the suspension was sampled and diluted from 1/1 up to 1/10 in PBS (Dulbecco). Then, 100 µl of each dilution was seeded on COS agar (bioMérieux) and incubated at 37°C for 24-96 hours in an anaerobic atmosphere.

4.2.4 Analyses on urine from infants < 3 y.o.

The first step consisted of seeding fresh urine in three different solid culture media. Two Solid culture media were home-made. For each urine specimen, 1 mL was sampled, and then serial dilutions (from 1/1 up to 1/10) were performed in PBS (Dulbecco). A 100 µl volume of each dilution was seeded on solid media and incubated in anaerobic atmospheric conditions at 37°C for 24 to 96 hours in the two home-made media. Each dilution was plated twice on the commercial medium COS (bioMérieux) to be incubated at 37°C for 24 to 96 hours in aerobic and anaerobic atmospheric conditions. The second step consisted of sub-culturing urines in two liquid media. For each subculture specimen, 1 mL was sampled and then serially diluted (from 1/1 up to 1/10) in PBS (Dulbecco). Then, 100 µL of each dilution was seeded on the different solid media and incubated 24-96 hours at 37°C in the four conditions described above.

4.2.5 Subjects who have taken probiotics

One milliliter of urine was directly inoculated onto two media: MRS agar and Columbia + 5% sheep blood. The two media were incubated in both anaerobic and aerobic conditions. Following two days of incubations, colonies were subjected to MALDI-TOF analysis.

4.2.6 Patients who underwent FMT

One milliliter of urine was directly inoculated onto two media: MRS agar and Columbia + 5% sheep blood. The two media were incubated in both anaerobic and aerobic conditions. Following two days of incubations, colonies were subjected to MALDI-TOF analysis.

4.2.7 Patients with UTI

One milliliter of each sample was inoculated into a liquid enrichment medium for 30 days for follow-up at day 1, day 3, day 7, day 10, day 15, day 21 and day 30 after seeding. On each day of monitoring 1 mL was taken for serial dilutions (1/1 to 1/10) in DPBS (Dulbecco). 200

µl of each dilution was inoculated on Columbia agar + 5% sheep blood (bioMérieux) and then incubated for 24-96 hours at 37 ° C. under aerobic and anaerobic atmospheric conditions.

4.2.8 Patients with suspected bladder cancer

Nine specimen were processed as described for UTI specimen (4.2.7 section). Eleven urine samples were analysed using fast culture protocol which associate three culture conditions: (i) Direct; (ii) Yeast extract, casitone and fatty acid (YCFA) liquid medium[4] and Anaerobic blood culture bottle supplemented with Rumen 2ml and 5% Sheep Blood[5]. For the two latter culture conditions, 2 ml of each sample was inoculated in liquid medium and incubated at 37°C in anaerobic atmosphere. At 3Hours, 6Hours, 9Hours, Day1, Day3, Day5, Day7, and Day10, 10-fold serial dilutions of the culture were plated directly onto Columbia agar with sheep blood (Columbia agar + 5% sheep blood, bioMérieux, Marcy l'Etoile, France) and YCFA agar. In parallel, urine samples were directly inoculated on 5% Columbia agar with sheep blood after serial dilutions.

4.3 Isolation by quadrant streaking and identification by mass spectrometry (MALDI-TOF)

Colonies were isolated by quadrant streaking on COS agar (bioMérieux) and incubated at 37°C in the same atmospheric conditions as before and identified after 24-96 hours of growth by MALDI-TOF MS (Bruker) as previously described [2]. Only qualitative analysis was performed. Each deposit was covered with 2 mL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% aceto-nitrile and 2.5% trifluoroacetic acid). A Microflex spectrometer (Bruker Daltonics) was used to perform this analysis according to the manufacturer's recommendations. A maximum of 100 peaks were used for each spectrum, and those peaks were compared with the computer databases from Bruker and the Timone University Hospital. The new bacterial species cultured in previous studies were previously added to our database.

An isolate was considered correctly identified at the species level when at least one spectrum had a score≥1.9 and a spectrum had a score≥1.7

4.4 16S identification of species not identified by MALDI-TOF MS (Bruker)

If the isolate was not accurately identified using MALDI-TOF MS (Bruker), a pure culture of the species was obtained on COS agar (bioMérieux). A new attempt of identification by MALDI-TOF MS (Bruker) was performed if the identification score was inferior to 1,9, and if the quality of the spectra was good based on flexAnalysis software (Bruker), the pure culture of the bacterial species was sent to the microbiology laboratory of the Timone Medical University, Marseille, to perform 16S rRNA gene sequencing as previously described [6].

The 16S rRNA gene was sequenced using the fD1-rP2 primers and a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). A control of amplification was performed by migration on a 1.5% agarose gel. If the amplification was correct, the amplified DNA was purified and mixed with primers (357f and 357r, 536f and 536r, 800f and 800r, 1050f and 1050r). New amplification, purification and sequencing were performed on an Abi Prism 3130xl Genetic Analyser (Applied Biosystems™, Foster City, USA). Sequencing data were analysed by ChromasPro® (Technelysium®, Brisbane, Australia) and compared to the NCBI database with Blast® software. A new species was defined by a 16S rRNA sequence homology inferior to 97.65% between the studied species and the nearest bacterial strain already described [7,8].

4.5 Implementation of the MALDI-TOF MS spectra

The MALDI-TOF MS spectra of the unidentified colonies were further associated with the bacterial species identified by 16S rDNA sequencing.

4.6 Strain preservation

Identified bacterial strains of each urine sample were conserved in Protect Microorganism Preservation System tubes (Technical Service Consultant Ltd, Lancashire, United Kingdom) at -80°C.

4.7 Classification of isolated prokaryotes

Motility was checked using the Guide to Medically Important Bacteria[9] or according to the formal species description. Additionally, tolerance to oxygen for each species or genus was determined using the "List of Prokaryotes according to their Aerotolerant or Obligate Anaerobic Metabolism" (https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/list-of-prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism/).

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Article III : original article

Culture and metagenomic urine bacterial exploration of urinary symptomatic and asymptomatic pediatric patients, a world to discover

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En cours d'écriture

Culture and metagenomic urine bacterial exploration of urinary symptomatic and asymptomatic pediatric patients, a world to discover

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Abstract

Introduction

Human urinary microbiota was recently rediscover. Indeed, until recent years, only bacterial pathogens growing fast in aerobic condition were considered, as there was a treshold of detection in order to identify urinary tract infection and not the carriage of different bacteria. Therefore urine microbiota was totally neglected.

Material and Methods

We collected the urine samples of patients aged less than 3 months-old suspected to have urinary tract infection, thanks to urinocol or bladder catheterization after perineal disinfection with chlorinated antiseptic (Amukine®) and the urine samples of patients aged from 2 to 18 years old with aquired urinary cleanliness who had systematic urine examination to check there renal function, their mid-stream urine were collected after careful perineal disinfection. These samples were grown on 4 solid culture media and 2 liquid culture media, aerobic and anaerobic for infant less than 3 month old and on 6 solid culture media and 4 liquid culture media, aerobic and anaerobic for children from 2 to 18 years old. The bacterial colonies were identified by mass spectrometry (MALDI-TOF) and if necessary the DNA coding for the 16S RNA was sequenced. A metagenomic analysis of urine samples was performed. We compare the bacteria we found in different samples with the human urinary tract repertoire of bacteria already published to determined if the bacteria had already been isolated in urine before our study.

Results

A total of 31 urine samples of infant less than 3 month old and 12 urine samples of children from 2 to 18 years old were collected. We identified an average of 11.16 bacteria per sample, a average of shanon index of 2.09 per sample and Total of 160 different bacterial

species, of which 58 were never identified in human urine before. The metabolism of these bacteria was anaerobic in 38.75 % of cases. The richness of urine sample was positively linked with the age and the weight of the children and was negatively linked with the previous use of antibiotic. Metagenomics has revealed some extremophilic bacteria not found in culture.

Discussion/Conclusion

There is a urinary microbiota in children that can be described by special culture techniques. Our culture techniques allow us to find more aerointolerant bacteria (38.75%) than previously described in urine in literature (17.8%). The standardization of these techniques and the use of more specific media for certain tedious or extremophilic bacterial species would allow a complementary approach to metagenomics to describe the urinary microbiota and understand its role in physiology and pathology.

Manuscript

1. Introduction

The microbiota was mainly described by high throughput sequencing techniques. These techniques are limited by biases related to DNA extraction techniques, primers used to amplify DNA, but also through depth because only the most abundant genomes in the sample can be detected. Finally, the metagenomic techniques do not make it possible to determine if the organism was alive at the time and place where it was found and do not make it possible to isolate the identified microorganisms in order to carry out in vitro or in vivo analyzes [1].

To better describe the digestive microbiota, Jean-Christophe Lagier et al. developed the Culturomics method, which consists of a set of techniques allowing the growth of microorganisms with slow and difficult growth, combined with rapid and reliable identification of microorganisms by MALDI-TOF mass spectrometry [2, 3]. These techniques include the use of culture in liquid media, the use of antioxidants, the use of enrichment with sheep rumen juice, previously used for the cultivation of spirochaetes [4-6]. This Culturomics method has allowed the discovery of new bacteria in the human intestine [7, 8] and has promoted the growth of Archaes [6, 9].

Urinary microbiota has been recently rediscover and described by metagenomic methods by Wolfe et al [10]. And since the rebirth of culture, Evann E. Hilt et al. have confirmed the existence of urinary microbiota with enhanced urine culture techniques [11]. In 2019 Morand A et al established the human urine repertoire and reported 562 bacterial species already described in the literature as belonging to the human urinary tract [12].

Only one study describing the pediatric human urinary microbiota is reported in the literature. It focused on the urinary microbiota of male adolescents using 16S RNA metagenomic techniques to identify urethral bacteria (urine sample from the first urinary

stream) and balano-prepucial sulcus to explore the link with sexual activity and bacterial vaginosis [13].

As the composition of the digestive flora changes with age [14-17] it is reasonable to assume that the pediatric urinary microbiota may differ from that of adults and change during the early childhood.

We report here the study of bacterial urinary tract microbiota of infant under 3 month old and children from 2 to 18 years old by culturomics techniques and by metagenomic techniques and implemented the bacterial urinary tract repertoire.

2. Material and methods

Material and methods are detailed in supplementary material.

3. Results

3.1 Global results

A total of 31 urine samples of infant less than 3 month old and 12 urine samples of children from 2 to 18 years old were collected and cultivated. By culture techniques 17364 colonies were tested, we identified an average of 11.16 bacteria per sample, a average of shanon index of 2.09 per sample and total of 160 different bacterial species, of which 58 were never identified in human urine before (Figure 1), and 6 were new bacterial species and genus. The metabolism of these bacteria was anaerobic in 38.75 % of cases (62/160) (Figure 2). Only 3/43 samples were sterile using this microbial Culturomics techniques whereas 18/43 samples were concluded sterile with standard culture methods, and on the 40/43 positive urine

samples with microbial Culturomics, only 3/43 were considered to be associated with clinical infection of the urinary tract (Figure 3).

The 31 urine samples of infant less than 3 month old and 7/12 urine samples of children from 2 to 18 years old were analysed by metagenomics techniques. Metagenomics allowed to reveal 428 OTUs at a subspecies level, 413 different species, of them 154 were common with literature and 259 were never described in urine in literature before and 328 were not cultivate by our microbial culturomics methods, 238 were only found by metagenomic technique in our study, whereas 36 were only found by culturomics in those samples. In these species figured some extremophilic bacteria not found in culture.

64 bacteria were commonly found in literature, by metagenomic technic and by microbial culturomics (Figure 4).

3.2 Subgroup of patient under 3 months-old

3.1.1 Samples and patients

Concerning the subgroup of patients under 3 months-old, 31 urine specimens were collected and analysed. (i) 20/31 (64.5%) samples were done in front of clinical symptoms that could make think of maternofoetal infection, (ii) 5/31 (16.1%) urine samples came from catheterization for retrograde ureterocystography, (iii) 4/31 (12.9%) urine specimens were sampled because of catheterization for sedation, (iiii) 2/31 (6.5%) were systematic urine sampling for controlling urine chlorine excretion on patients affected by pylorostenosis waiting for surgery. Samples came from 13 girls and 18 boys (sex ratio 0.7), mean age was 28 days (max=60-days-old, min=3-days-old), the mean weight was 4.0 kg (max=6 kg, min = 1.5 kg), 19/31 patients (61.3%) had good growth and 8/31 (25.8%) patients received neonatal antibiotherapy before the urine sample. The mean pH of the urine samples was 6.7 (max=8.5, min=5), the mean salinity of the urine samples was 18/1,000 (max=50/1,000, min=5/1,000).

3.1.2 Culture

The standard culture methods permitted to find 10/31 CBU sterile (32.3%), 11/31 CBU with non-significant bacterial development (35.5%), 10/31 CBU considered as positive (32.3%). Among the positive CBU, 2/31 had polymicrobial flora (6.5%), 8/31 (25.8%) had one germ identified (6 Escherichia coli, 1 Klebsiella pneumoniae, 1 Klebsiella oxytoca) and 3/31 (9.7%) were concluded to be associated with clinical pyelonephritis.

Via culturomics methods, 7152 colonies were tested, 124 different species were identified in which 43 were not identified in human urine before and 3 were new bacterial species (*Corynebacterium urinapleomorphum*, *Olsenella urinainfantis*, *Arcanobacterium urinomassiliense*). Those technics allowed us to find 28/31 positive samples (90,3%), 7/10 sterile samples with standard culture methods were positive with culturomics methods (70.0%). The mean number of different species found by culturomics in urinary samples was 9.3 (max=35, min=0).

3.1.3 Metagenomic

Via high throughput sequencing methods, 386 different OTUs were identified at a species level. 239 species were not identified in human urine before, 309 were not cultivate by microbil culturomics technique. All samples had positive results using this technic.

3.1.4 Principal component analysis and Pearson correlation coefficient

Concerning the analysis of the culturomics results, we found that a higher weight and to be older was statistically linked with a higher number of different species and with a higher proportion of aero-intolerant bacteria (Coeff Corrélation (CC) = ; $p<0.005$). Having a good growth was also linked with a higher proportion of aero-intolerant bacteria (CC= ; $p<0.005$) (Figure 5).

Concerning the analysis of the metagenomic results, it is noteworthy that a higher weight was statistically linked with a higher diversity index (CC = ; $p<0.005$). Having a good growth was linked with a higher number of different species (CC = ; $p<0.005$). On the contrary, having a good growth was negatively linked with antibiotic during maternal delivery labor, *Akkermansia municiphila* and *Corynebacterium durum* (CC= ; $p<0.005$). This was corroborated by the linear discriminant analysis: concerning metagenomic results infant with bad growth had an enrichment with *Corynebacterium durum* and *Akkermansia municiphila*.

For the culturomics and metagenomic results, there was no significative difference observed between female and male concerning the number of different species, the diversity index, the aero-intolerant proportion or specific species.

3.2 Subgroup 2-18-years-old

3.2.1 Samples and patients

Concerning the subgroup of patient from 2 to 18 years old, 12 urine specimens were collected and analyzed. (i) 6/12 patients had nephrotic syndrome (50.0%), (ii) 1/12 had non-dialysis renal insufficiency, (iii) 1/12 had kidney transplant, (iiii) 1/12 had glomerulonephritis, (iiiii) 3/12 were usually healthy control and benefited of acute surgical care. Samples came from 7 girls and 5 boys (sex ratio 1.4), mean age was 9.7-years-old (max=18-years-old, min=3-years-old), the mean weight was 41.2 kg (max=83 kg, min = 15 kg). The mean pH of the urine samples was 6.4 (max=8.1, min=5), the mean salinity of the urine samples was 96.5/1000 (max=350/1000, min=16/1000).

3.2.2 Culture

The standard culture methods allowed to find 8/12 CBU sterile (66.7%), 1/12 CBU with non-significant bacterial development (8.3%), 3 CBU considered as positive (25.0%). Among the positive CBU, 2 had polymicrobial flora (16.7%), 1 (8.3%) had one germ identified (Escherichia coli) and none was concluded to be associated with clinical pyelonephritis.

Via culturomics methods, 10212 colonies were tested, 95 different species were identified in which 30 were not identified in human urine before and 2 were new bacterial species (*Actinomyces urinae*, *Anaerococcus urinimassiliense*) and 1 was new bacterial genus (*Urinacoccus massiliensis*). Those technics allowed us to find 12/12 positive samples (10%), 8/8 sterile samples with standard culture methods were positive with culturomics methods (100%). The mean number of different species found by culturomics in urinary samples was 16.1 (max=25, min=6).

3.2.3 Metagenomic

Only 7 samples were analyzed via high throughput sequencing methods. 128 OTUs were identified at a species level, in which 51 were not identified in human urine before and 95 were not cultivate by bacterial culturomics in the samples. All samples had positive results using this technic.

3.2.4 Principal component analysis

Concerning culturomics and metagenomic results, no significative difference was observed between each group.

4. Discussion

We have shown that there is a microbiota in the urine of children (an average of 11.16 different bacterial species per urine sample) identifiable by special culture techniques. There was no significant difference in the number of different bacterial species between the urine considered "sterile" by the standard culture techniques in the clinical microbiology laboratory and those with a significant microbial development except for the population of patients who had antibiotics (8 samples, including 3 sterile in culturomics).

Of the 160 identified bacteria, 53 of them had so far not been described as isolated from human urine bedore.

This microbiota is composed of aerotolerant bacteria but also bacteria considered to have strictly anaerobic metabolism (38.75% of the total species). In contrast, none bacteria identified by standard ECBU in the clinical microbiology laboratory were aero-intolerant and only 17.8% of the bacteria identified in the literature were strictly anaerobes, 9.4% when considering only the bacterial species who has already been isolated in culture in literature.

Our special culture methodology applied to urine is derived from that described by Jean-Christophe Lagier et al. for stool entitled Culturomics [2]. In the Culturomics methodology, 18 culture conditions allowing the growth of the greatest possible diversity of bacterial species identifiable from stool samples were selected. We used a total of 10 different culture conditions. Of these, the preincubation condition in anaerobic blood culture flask in which microfiltered sheep rumen juice had been added and which was inoculated on Columbia agar + blood at 30 days of incubation was the one that allowed isolation of the greater bacterial diversity. The direct inoculation condition on Columbia agar + blood incubated in anaerobic medium was the 2nd most productive.

This work is an exploratory work to prove the usefulness of the concept of description of the urinary microbiota by special culture techniques. It is innovative in the study of

pediatric urine, the use of a liquid culture medium for the enrichment of tedious bacteria and the use of artisanal solid culture media containing antioxidants to improve bacterial culture. considered as anaerobic, rumen juice aims to promote the development of tedious bacteria by recreating the conditions of the digestive tract [4-9, 18]. The rumen juice preparation process involves the use of filters with a pore diameter of 0.2µm. The purpose of this treatment is to remove all bacteria whose diameter is greater than the pore diameter. Nevertheless, some bacterial species appear to be smaller in size than the pore diameter. These bacteria called ultramicrobacteria have an average size of 0.009 ± 0.002 µm3 [19]. In order to eliminate the risk of revealing bacteria from rumen juice, we used a negative control that did not show any microbial growth.

One of the limitations of using special culture methods on urine specimens of children under 3 month-old is the amount of biological material available. In fact, in some cases the urine collected did not exceed 3 ml, the diagnostic standard ECBU being a priority, there was sometimes too little sample to be able to inoculate the urine on a large number of different media and in particular no halophilic medium has been tested. We have repeatedly modified the protocol used for growing bacteria. Initially, preincubation conditions in blood culture flasks were not realized in our study for fear of not having enough material (urine of children under 3 months) to do all the conditions. However, in view of the very good results that this type of culture gave in another protocol of study of the service this protocol was put in place from the urine 10. One millilter of the urine of 1 to 9 was initially stored at -80 ° C, those 1mL were put in culture in preincubation in blood culture flask in a second time. Moreover, from the urine in direct inoculation and for all the seedings on agar after preincubation in a blood culture flask, the solid mediums homemade (R-Medium and Columbia agar + Blood + Rumen) were made with a doubling of the amount of agar to make the agar more rigid and thus slow the spread of Proteus mirabilis. Indeed, the Proteus mirabilis being a mobile

bacterium, it caused difficulties during the transplanting of the urine 1, 4, 7, 8, 20 and 24 during the direct inoculation on solid agar because it formed a biofilm of bacteria in less of 24 hours which impeded the transplanting and isolation of the other different bacterial species present in the sample.

Cultivation methods have the disadvantage of being operator dependent and it is possible that the technique developed between the beginning of the study and the end has improved. Moreover it is time consuming.

One of the possible biases explaining the lesser performance of our method of culturomics compared to metagenomics could also concern MALDI-TOF mass spectrometry identification which is sometimes not sufficiently precise for species diagnosis and concludes with a germ instead of another (often 2 very close species within the same bacterial genus), the 2 microorganisms being very close on the spectral plane. It would have been interesting to verify in 16S sequencing the strains obtained by culture and identified by mass spectrometry.

Urine samples of children from 2 to 18 years-old were midstream urine collections. Urine samples of children under 3 months, unable to urinary continence, were collected either by Urinocol (majority of cases) or by transurethral bladder catheterism (among the included patients, 11 (25.58%) had a urinary tract catheterism to perform a diagnostic examination or because they were in intensive care unit). These are the most commonly techniques used for collecting urine samples in young children who have not acquired "cleanliness" (average age of acquisition of daytime cleanliness is 30-36 months [20]). It should be noted that the method of collection by Urinocol (sterile pouch stuck on the perineum after perineal disinfection and left in place until the emission of urine) is considered to be at risk of perineal contamination [21], so the bag had to be changed at regular intervals in the absence of urine emission to minimize the risk of contamination. Conducting systematic by transurethral bladder catheterism or suprapubic punctures would not have been acceptable because of ethical

considerations. The selected patients would not have had any direct individual benefit from undergoing these painful procedures and exposing them to certain complications (urethral strictures, haemorrhages). It seems all the more difficult to impose these procedures on children in an exploratory study. Nevertheless, invasive urinary sampling procedures, while targeting the bladder microbiota and partially excluding bacteria colonizing the urethra or perineal contaminations, are far from clinical reality for most patients. They would describe a vesical and non-urinary microbiota in the same way that stool samples can describe a fecal and non-intestinal microbiota. Moreover, the anatomic continuity between the perineum, the urethra and the bladder does not prevent the ascending bacterial translocation despite the opposite urinary flow. Moreover, we wanted to be able to compare the number of bacterial species isolated in our study with that of all the bacterial species already described in the urine in the literature. The bibliographic search used did not allow to sort the results according to the type of samplings carried out. But in the absence of details, it is likely that most of the urinary specimens used to describe the bacterial species isolated in urine are midstream urine samples, made after perineal disinfection, or even sampling from urine collection by urinocol which makes possible the comparison with the bacterial species that we have isolated.

Our results are consistent with data from the literature. Hilt et al [11] showed bacterial growth in urinary bladder samples in 80% of cases using special culture techniques against 92% of urine without significant microbial growth under standard culture conditions. We have also isolated a wide variety of species considered strict anaerobes. Other studies have also isolated anaerobic bacteria [11, 22-24] in high proportion in the urine.

Our results open the possibility of a systematic study of the urinary microbiota by special culture techniques. By including a larger number of samples, standardizing and simplifying our protocol, we could describe and compare the microbiota in different physiological (age, sexe, weight, sexual activity, diet) and pathological (antibiotic,

immunosuppressive, urological, nephrological pathologies, hemodialysis, autoimmune diseases, pathological tumors, digestiv pathologies) or even bypass some of the processes that cause these pathologies, like for the bladder cancer for example [25, 26].

It would also be interesting to experiment the performance of new media or new culture conditions, targeted certain phyla or bacterial groups considered tedious, especially halophilic bacteria or archaea. These experiments have already began in our laboratory.

In the samples analyzed, metagenomics appeared more accurate and complete. In particular, this method allowed the identification of several halophilic bacteria and archaea.

In our study, the metagenomic analysis of the samples was done in a second time. However, there is interest in performing metagenomics before culture (although this is difficult to achieve because of the need to sow the urine within 6 hours). Indeed, this would guide the type of environment to be designed to better cultivate the species believed to be present in the sample and in particular some very tedious species (eg *Akkermansia muciniphila*), certain halophilic species (eg *Herminiimonas glaciei*) or archaea (example: *Methanobrevibacter smithii*).

However, it is highly likely that there are many false positives among the metagenomic results. Indeed, we do not know the clearance of bacterial DNA and other organisms in the urine (consisting largely of degraded materials more or less toxic to be eliminated by the body). A blatant example in our study is the metagenomic detection of *Pseudomallada prasinus*, which is an arthropod, and of which we can be almost certain of the absence in the urine of an infant.

In addition, there are also false negatives in the analysis of metagenomic samples due to depth bias. Only the organisms present in large quantities are identified.

For the principal component analyzes performed on culture results, there was a significant relationship between the number of different bacterial species found in a sample and the age and the weight of the child. Regarding the metagenomic results, the principal component analysis showed a statistically significant link at the $p < 0.05$ level between the number of different bacterial species in the same sample and the fact that the patient had good growth. This exploratory study does not allow us to draw any conclusions, however it could constitute axes for further studies.

On the other hand, it may be interesting to compare the results of several urine collection techniques in the same patient in order to understand which species are specific to which portion of the urinary tract.

And finally, it would be interesting to study for the same child several samples from different sites of the human body (meconium, stool, gastric fluid, urine, skin, mouth ...) but also its environment (breast milk [27], maternal and paternal skin , amniotic fluid, vaginal sampling, mouths of parents, maternal stools, maternal urine ...) in order to establish a map of the human microbiota and to establish links from one site to another [28, 29, 30, 31] (colonization of a site by bacterial translocation ? implication of lymphatic vessels ?) and from one individual to another (transmission by contact) and the role of exterial envents in the construction of the different microbiota during early childhood [14-17, 32].

5. Conclusion

The children urinary tract microbiota can be described by special culture techniques in association with metagenomic. Bacteria can be isolated from urinary samples from patients who do not meet the usual criteria for urinary tract infection, including when ECBU is considered sterile.

The special culture techniques allow to identify species that are not highlighted by the standard culture techniques but also new bacterial species not described in the literature. Those bacterial species were described in original paper using taxonogenomics [33-40]. These new species increase the number of known bacterial species in humans. It is notable that once discovered these bacteria are then better identified in clinical practice as can be seen for example for the bacterial species Corynebacterium urinapleomorphum which since its discovery was found in a clinical sample [41].

Moreover, our culture techniques allow us to find more aerointolerant bacteria (38.75%) than previously described in urine in literature (17.8%). However other species are not cultivated with the culture media that have been used (including some halophile bacteria, archaea, very anaerobic bacteria), so further studies using other culture conditions would be necessary in order to continue the description of the urinary microbiota.

We have drawn up a panorama of bacterial species isolated in urinary samples and extended the description of the composition of the urinary microbiota in physiological or pathological conditions. It should be supplemented by future studies of the urinary microbiota but also by publications reporting urinary infections with newly identified germs in urine.

And one of the key point is that the digestive origine of the human urine microbita should be explored.

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Figure 1: Representation of the bactérial species added to the global urinary tract repertoire of bacteria thanks to microbial culturomics

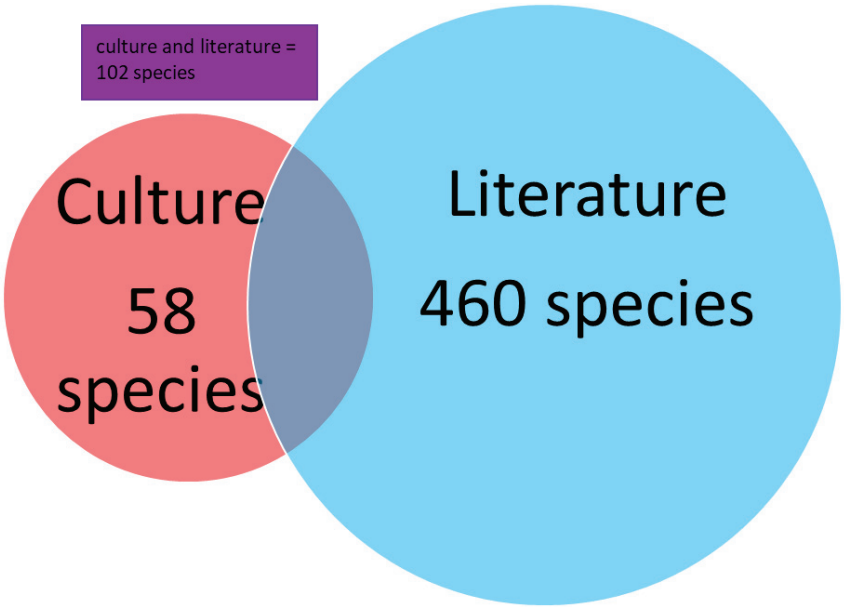


Figure 2: Proportion of aero-intolerant bacteria isolated in culture in this study and in literature

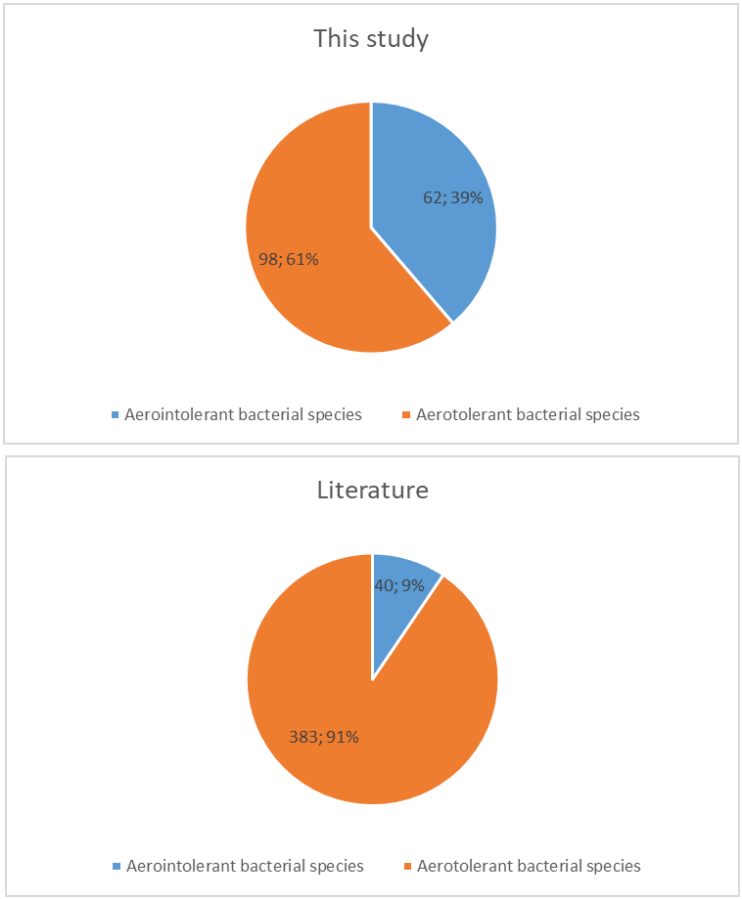


Figure 3: Representation of positive urine sample with microbial culturomics and the relation with clinical urinary tract infection

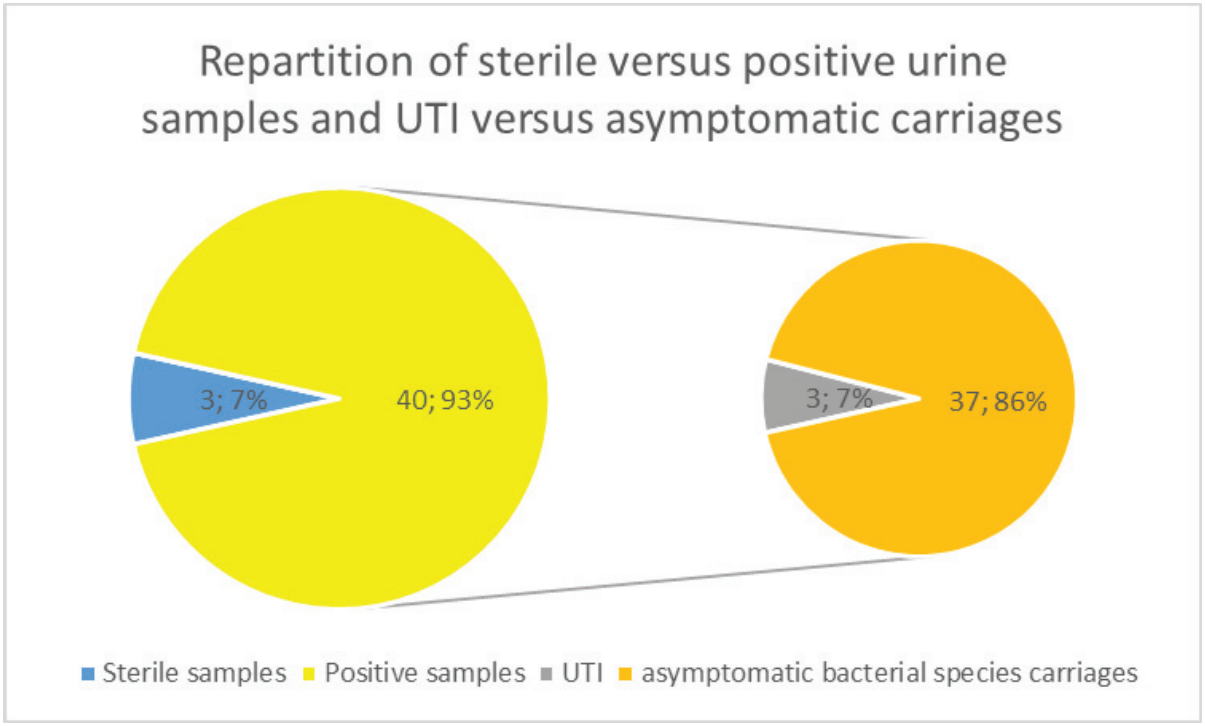


Figure 4: comparison of the bacterial species found in literature, by microbial culturomics and by metagenomic on the 38 urine sample analyzed by both technics

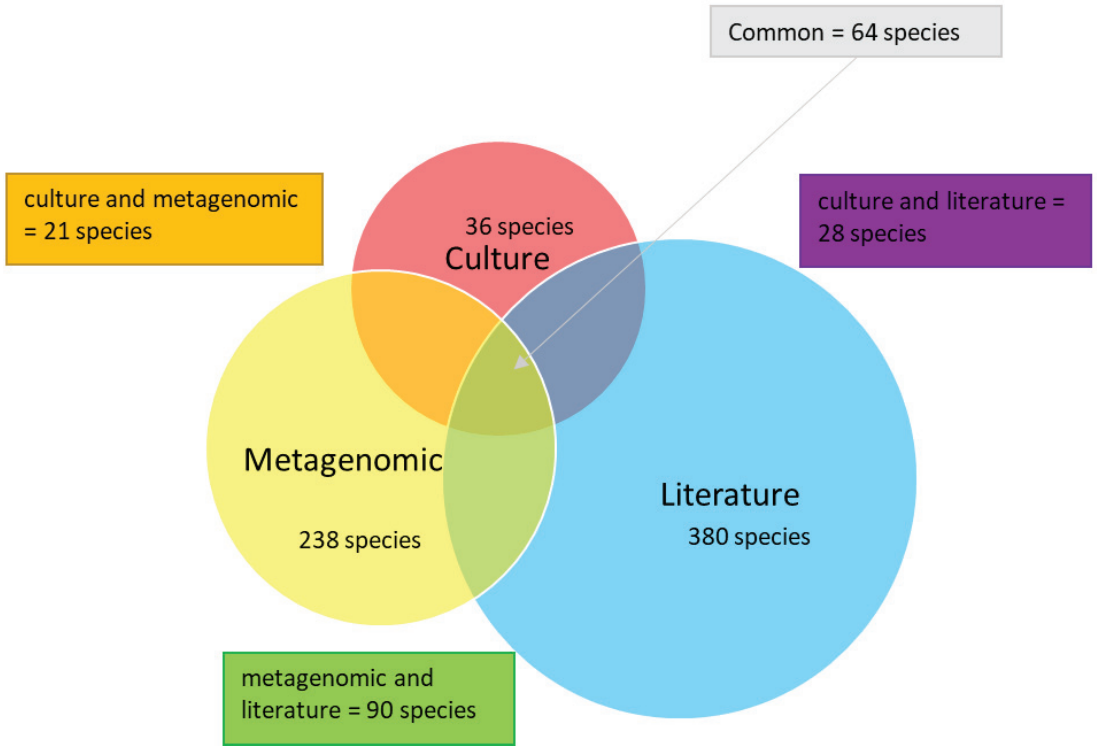
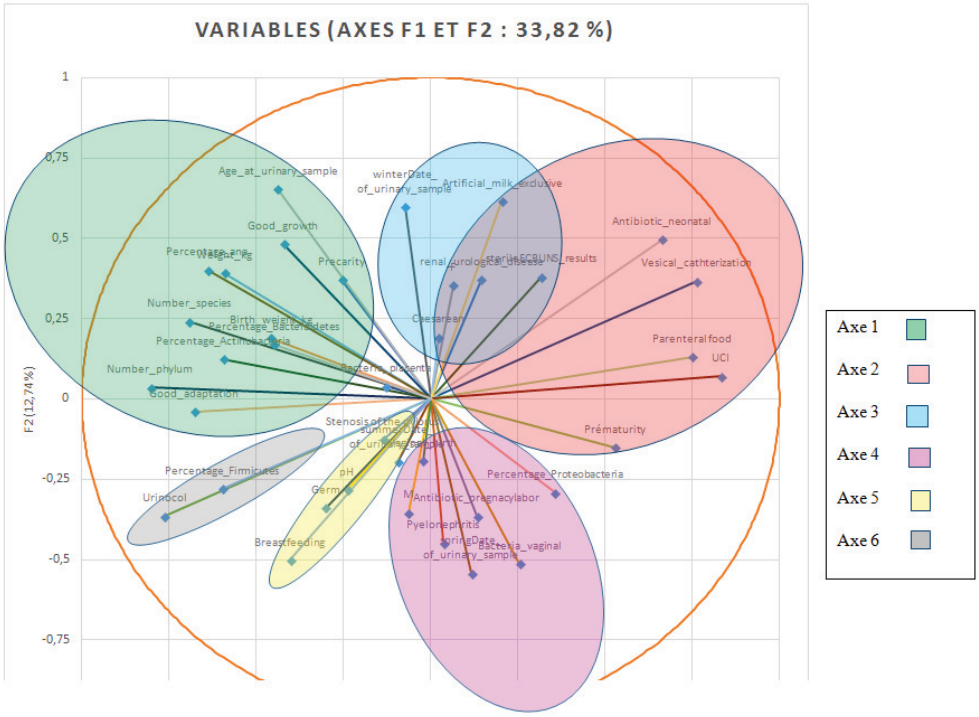
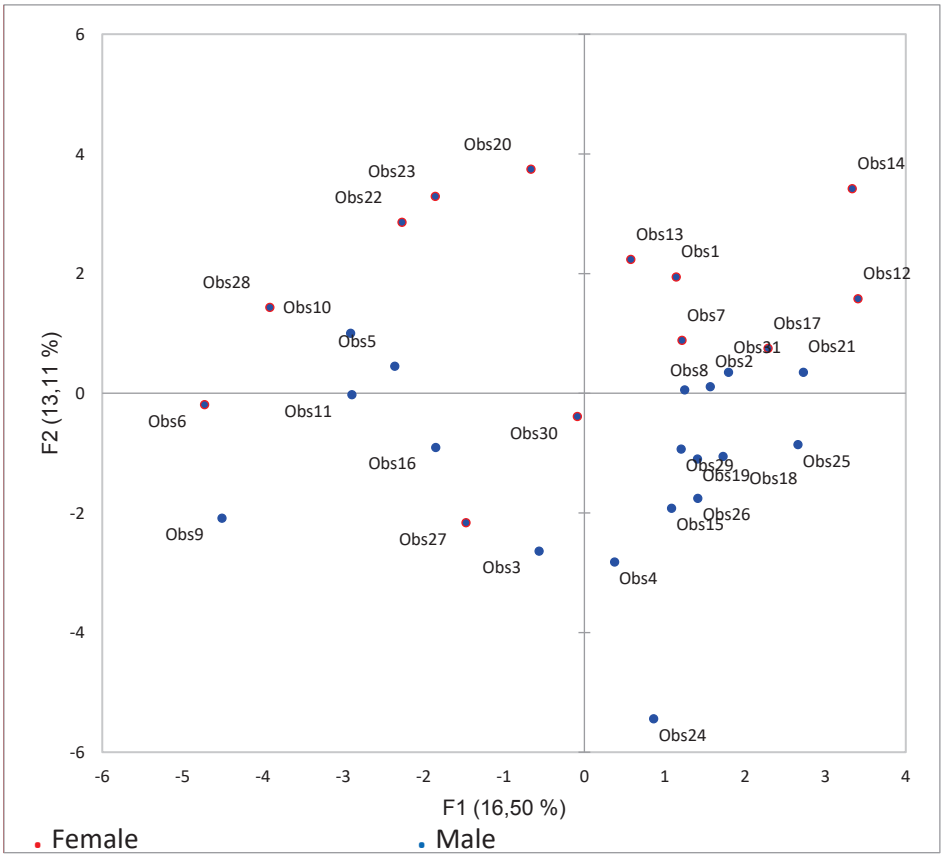


Figure 5: Principal component analysis representing the link between weight and age and the bacteria richness of the urine samples of infant under 3 months-old (Axe 1), and the repartition of patient depending of the sexes





Supplementary material :

2.1 Ethic:

Approval of the ethics committee of the IHU Méditerranée-Infection was obtained under the number 2016-01 and 2016-011. The study was explained to the parents. A consent form was given to the parents.

2.2 Samples and patients:

2.2.1 Urine sample of infant under 3-month-old

We analyzed urine sample obtained from children under three months old. Those children were hospitalized or consulted at the Timone universitary hospital, Marseille, France, from February to June 2016 and had sterile urine sample collected for one of the following reasons: (i) Fever or other clinical symptoms that could make think of maternofoetal infection (jaundice, poor weight curve, vomit, etc), (ii) urine catheterization for retrograde ureterocystography, (iii) urine catheterization for sedation, (iiii) systematic urine sampling for controlling urine chlorine excretion on patients affected by pylorostenosis waiting for surgery.

Urine samples were collected in a sterile by vesical catheterization, by suspubian punction, by midstream urine collection or by Urinocol® (B. Braun Medical SAS, Melsungen, Hessen, Germany) after a perineal wash with sterile compress sizzled with chloric antiseptic (Amukine®). The sample had to be collected by the medical staff.

Administrative data and clinical information were obtained from the parents, the medical staff and the patient medical documents: weight, sexe, pathology, informations about pregnancy, delivery, neonate history, breastfeeding or not, history of antibiotherapy, date and time of the urine miction.

2.2.2 Urine sample of infant from 2 to 18 years-old

We also analyzed voiding urine midstream sample obtained from children aged from 2 to 18 years old with good urinary continence were analyzed. Those children were hospitalized or consulted at the Timone universitary hospital, Marseille, France, from December 2014 to July 2015 and had one of the following pathology:

(i) Enuresis or other micturition disorders, (ii) nephritic syndrome, (iii) end-stage renal disease (glomerular filtration flow < 80 mL/min/1.73 m²) with dialysis (hemodialysis or peritoneal dialysis) or not, (iiii) repeated urinary tract infections with sterile urine culture between each infection. A control group was also analyzed. Those children had to be hospitalized in orthopedic pediatric surgery for an acute surgical problem and without any serious medical history.

2.3 Transport, repartition of the urine and urine qualitative analyses

All the urines samples were quickly transported in the microbiology laboratory universitar hospital institute (IHU) Méditerranée-Infection in order to be inoculated in the culture media in the 6 hours following the urine collection. All the samples had to be separated in three specimens, 1) one for the routine microbiology laboratory, 2) one for the special culture laboratory (where Culturomics was performed) and 3) one aliquot of 1 mL which was frozen at -80°C in order to be secondary ana lyzed by high-throughput sequencing methods.

The pH of the samples was measured thanks to reactive sticks (pH-Fix 4,5-10 reference 92120 (Macherey-Nagel®, Düren, Allemagne) and the salinity of the samples was measured with a refractometer PR-100SA (Atago®, Tokyo, Japon).

2.4 Technique of culture

All the manipulations were performed in a laboratory with level 2 biosafety. The bacterial manipulation were done under dometic microbiological safety cabinet with hood and laminar flow.

2.4.1 Standard culture in routine laboratory

One specimen of each sample was analyzed by standard culture method in the routine microbiology laboratory of the universitary hospital, la Timone, Marseille. A cytobacteriologic exam with culture and antibiogram (CBU) was performed. The number of leukocytes and red blood cells contain in the sample was quantified. A small volume of urine (10 µL) was seeded on 5% sheep blood-Columbia agar medium (COS agar) (bioMérieux, Marcy l'Etoile, France) and the culture of the sample was performed aerobically, between 35 and 37 °C for 48 hours. Each colony was identified by MALDI TOF MS (Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry) (Bruker, Billerica, USA).

Biologic validation was realized following expert recommendations [1]. The detection limit of this method was depending on the sexe of the patient and the bacterial specie:

- 10^3 colony forming units (CFU) / mL on men and women for Escherichia coli and Staphylococcus saprophyticus

- 10^3 CFU / mL on men for others Enterobacteria than Escherichia coli

- 10^4 CFU / mL on women for Enterococcus faecalis, Corynebacterium urealyticum, Pseudomonas aeruginosa, Staphylococcus aureus.

Four conclusions were available after laboratory analyses:

- Sterile urine

- No significant microbial developpement (<103 or <104CFU/mL)

- Polymicrobial developpement (>103 or >104CFU/mL)

- Bacterial identification and antibiogramm

2.4.3 Culturomics

2.4.3.1. The media's components were the following :

2.4.3.1.1. solid 5 % sheep blood-Colombia agar medium (bioMérieux)

2.4.3.1.2. solid R-medium

1st step. In autoclavable bottle

- 15 g Caseine hydrolysate
- 15 g proteose peptone
- 10 g yeast extract
- 250 mL sterile water
- Ajusted pH 7-7.5

2nd step. In autoclavable bottle

- 30 g bacterial agar
- 350 mL sterile water
- Ajusted pH 7-7.5

3rd step. In a bottle

- 0.83 g Dipotassium hydrogene phosphate (K2HPO4)
- 1.67 g chlorure de sodium (NaCl)
- 5.83 g glucose

- 0.4 g L-cystéine
- Antioxydants:
  - 1 g Ascorbic acid
  - 0.4 g uric acid
  - 0.1 g glutathion
- 350 mL sterile water
- Ajusted pH 7-7.5

4th step. Autoclave the bottle 1 and 2

5th step. Filter the bottle 3 with 0.2 µm pore

6th step. Add 50 mL defibrined sheep blood (bioMérieux) for 1000 mL (5%)

8th step. Mixing and then pouring the medium in 40 plates of 9 cm diameter

2.4.3.1.3. Solid medium Columbia + blood + rumen

1st step. In autoclavable bottle

- 10 g yeast extract
- 10 g Proteose peptone
- 10 g Brain Heart Infusion
- 15 g casein hydrolysate
- 300 mL sterile water
- Ajusted pH 7-7.5

2nd step. In a second autoclavable bottle

- 30 g Bacterial agar
- 300 mL sterile water
- Ajusted pH 7-7.5

3rd step. In a bottle

- 1 g NH4Cl
- 0.3 g K2HPO4
- 0.3 g KCl
- 300 mL sterile water
- Ajusted pH 7-7.5

4th step. Autoclave the bottle 1 and 2

5th step. Filter the bottle 3 with 0.2 µm pore

6th step. Mixed the 3 bottles

7th step. Add 50 mL defibrined sheep blood (bioMérieux) for 1000 mL (5%)

8th step. Add 50 mL 0,2 µm filtered rumen fluid for 1000 mL (5%)

9th step. Mixing and then pouring the medium in 40 plates of 9 cm diameter

2.4.3.1.4. Liquid media condition 1:

Anaerobic glass blood culture bottle (BACTEC™ Lytic/10 Anaerobic/F Culture Vials, Becton-Dickinson, Pont de Claix, France) enriched with 4 mL of rumen fluid sterilized by 0,2µm pore microfiltration and 5 mL defibrined sheep blood (bioMérieux).

2.4.3.1.5. Liquid media condition 2:

Aerobic glass blood culture bottle (BD BACTEC™ Plus Aerobic/F Culture Vials, Becton-Dickinson, Pont de Claix, France) enriched with 4 mL of rumen fluid sterilized by 0,2µm pore microfiltration and 5 mL defibrined sheep blood (bioMérieux).

2.4.3.1.6. Control liquid media:

Glass blood culture bottle with 4 mL of rumen fluid sterilized by 0,2µm pore microfiltration and 5 mL defibrined sheep blood (bioMérieux) wad incubated without any urine in it in order to be a negative control and to be sure that the sterilized rumen was really sterile.

2.4.3.2 Culture on media culture:

Concerning the sample from infant under 3-months-old, we seeded fresh urine samples in 3 different solid culture media. Two solid culture media were home-made. For each urine specimen, serial dilutions (from 1/1 up to 1/10) were performed in PBS (Dulbecco). A 100µL volume of each dilution was seeded on solid media and incubated in anaerobic atmospheric conditions at 37°C during 24 to 96 hours in the two-home-made media. Each dilution was plated two times on the commercial medium COS (bioMérieux) in order to be incubated at 37°C during 24 to 96 hours in aerobic and anaerobic atmospheric conditions.

Concerning the children from 3 to 18-years-old, we seeded fresh urines on six different solid culture media fresh urines. Five solid culture media were home-made. For each specimen, 1mL of fresh urine was seeded on the 5 home-made solid media and incubated in aerobic conditions at 37°C during 24 to 96 hours. The fresh urines were plated two times on the commercial medium COS (bioMérieux) in order to incubate it in aerobic and anaerobic conditions 24-96h.

2.4.3.3 Sub-culture in liquid media:

Concerning the sample of infant under 3-months-old, we sub-culture 1mL of fresh urine sample in one aerobic and one anaerobic liquid culture media. For each subculture specimen, at 30 days, liquid was sampled. We performed serial dilutions (from 1/1 up to 1/10) in PBS (Dulbecco). Then 100µL of each dilution was seeded on the different solid media previously described and incubated 24-96 hours at 37°C in the same atmospheric conditions described before.

Concerning the sample of children aged from 2 to 18-years-old, they were preincubated in 4 liquid media. In each bottle 1 mL of fresh urine was inoculated. At day 1, day 3, day 7, day 10, day 14, day 21 and day 30, 1 mL of the suspension was sampled and diluated from 1/1 up to 1/10 in PBS (Dulbecco). Then 1 ml of each dilutions was seeded on commercial COS agar (BD Diagnostics Becton Dickinson GmbH®, Heidelberg, Germany) for the conditions 1 and 2 and on home-made medium with Columbia agar and enrichment with 5 % rumen fluid sterilized by 0,2µm pore microfiltration and 5 % defibrinated sheep blood for the liquid condition 3 and 4. Those cultures were incubated at 37°C during 24-96 hours in aerobic atmosphere for the liquid condition 1 and 3 and 72-120 hours in anaerobic atmosphere for the liquid condition 2 and 4.

2.5 Isolation by quadrant picking and Identification by MALDI-TOF MS

Colonies were isolated by quadrant picking on COS agar (bioMérieux) and incubated at 37°C in the same atmospheric conditions than before and identified after 24-96 hours of growth by MALDI TOF MS (Bruker) [2]. Only qualitative analyze was performed. Each deposit was covered with 2 mL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % aceto-nitrile and 2.5 % trifluoroacetic acid). A Microflex spectrometer (Bruker Daltonics) was used to perform this analysis according to the manufacturer's recommendations. A maximum of 100 peaks were used for each spectrum, and those peaks were compared with the computer database at the Bruker base and base-specific lab at the

Timone universitary hospital. Our database was previously incremented with the spectra of the new bacterial species cultured during previous studies.

An isolate was considered as correctly identified at the species level when at least one spectrum had a score≥1.9 and rest of the spectra had a score≥1.7 [2].

2.6 16S identification of species not identified by MALDI TOF MS

If identification of the bacterial specie wasn't allowed by MALDI TOF MS, a pure culture of the species was obtained on COS agar. New attempt of identification by MALDI TOF MS was performed. If the identification score was inferior to 1.9, and if the quality of the spectra was good on flexAnalysis software (Bruker), the pure culture of the bacterial specie was analyzed for the 16SrRNA ribosomal gene using the robot EZ1 advanced XL (Qiagen®, Venlo, Holand) and the DNA Extraction kit EZ1 DNA Tissue Kit (Qiagen®).

The 16SrRNA gene was sequenced using the fD1-rP2 primers, using a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). A control of amplification was done by migration on 1,5 % agarose gel, if this amplification was good, amplificated DNA was purified and mixed with primers (357 f et r, 536 f et r, 800 f et r, 1050 f et r), new amplification, purification and sequencing were performed on Abi Prism 3130xl Genetic Analyzer (Applied Biosystems™, Foster City, USA).

Sequencing data were analyzed by ChromasPro® (Technelysium®, Brisbane, Australia) and compared to NCBI database by Blast® software.

New specie was defined by a 16S rRNA sequence homology inferior to the cut-off with the nearer bacterial strain already described.

2.7 Implementation of the MALDI TOF MS spectra

When bacterial species were identified by 16SrRNA technique the MALDI TOF MS spectra were associated with the bacterial specie name and the data of Bruker were implemented in our laboratory.

2.8 Conservation of the strains

Identified bacteria strain of each urine sample were conserved in Protect Microorganism Preservation System tube (Technical Service Consultant Ltd, Lancashire, United-Kingdom) at -80 °C.

2.9 Metagenomic

2.9.1 Metagenomic sequencing

Urine samples were amplified, barcoded, pooled and then sequenced for 16SrRNA sequencing on MiSeq technology (Illumina, Inc, San Diego CA 92121, USA) with paired end strategy, constructed according the Nextera XT library kit (Illumina).

For 16SrRNA sequencing, DNA was amplified for the 16S “V3-V4” regions by PCR, using the Phusion Taq (Thermo Fisher Scientific Inc, Waltham, MA U.S.A.) and the surrounding conserved region V3_V4 primers with overhang adapters (FwOvAd_341F TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG ; RevOvAd_785R GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). After purification on AMPure beads (Beckman Coulter Inc, Fullerton, CA, USA), concentration was measured using High sensitivity Qubit technology (Beckman Coulter Inc, Fullerton,CA,USA) and dilution to 0.2 ng/µl was performed. Using a subsequent limited cycle PCR on 1 ng of cleaned PCR product, Illumina sequencing adapters and dual-index barcodes were added to the amplicon. After purification on AMPure beads (Beckman Coulter Inc, Fullerton,CA,USA), the library was then normalized by beads according to the Nextera XT

protocol (Illumina Inc, San Diego, CA, USA). Each sample was pooled with other multiplexed samples into a single library for sequencing on MiSeq (Illumina Inc, San Diego, CA, USA). Automated cluster generation and paired-end sequencing with dual index reads was performed in a single 39-hour run in a 2x251bp.

Total information of 4.4 Gb was obtained from a 1130 K/mm2 cluster density with a cluster passing quality control filters of 37.1 % (22,849,000 clusters). Within this run, the index representation was determined with an average of 0.8 %. The raw data were configured in fastaq files for R1 and R2 reads.

2.9.2 Metagenomic bioanalysis

The corresponding paired-end sequences from the Illumina Miseq raw fastq files were merged into longer sequences using FLASH [3] by choosing a quality score cut off of 33. These longer sequences were then filtered in QIIME [4] by removing the primers, and eliminating the sequences containing N. Sequences with the length shorter than 200 nts and longer than 1000 nts were also removed. Chimeric sequences were removed using chimeraslayer of QIIME [4]. These filtered sequences were clustered into OTUs by UCLUST [5] in QIIME with de novo method at 97% similarity, without considering the singletons. OTUs were then searched against Silva SSU and LSU database [6] using BLASTN [7]. The best matches with each of OTUs greater than 80% identity were extracted from the reference database and taxonomy was assigned with a majority voting [8-10].

2.10 Analyse of data between groups

The comparative analysis of medians and quartiles between groups was performed using the software XLStat (Addinsoft, Paris, France) using the Mann Whitney test for nonparametric variables. The principal component analysis and the factor analysis were

performed using the XL Stat software (Addinsoft, Paris, France) using the Pearson correlation test.

2.11 Comparison of the results obtain by Culturomics and metagenomic and with the human urinary tract repertoire of bacteria

Results of the species and OTUs diversity obtained by Culturomics and metagenomic were compared using the Fisher exact test.

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4.Partie III : La culturomics comme outil pour faire progresser la taxonomie bactérienne :

Avant-propos :

La culturomics appliquée aux prélèvements d'urine nous a permis d'isoler 18 souches d'espèces bactériennes encore non identifiées jusqu'alors. Nous avons actuellement pu faire la description de 6 d'entre elles : *Actinomyces urinae* sp. nov., *Anaerococcus urinomassiliensis* sp. nov., *Urinacoccus massiliensis* gen.nov. sp.nov., *Corynebacterium urinapleomorphum* sp. nov., *Olsenella urininfantis* sp. nov., *Arcanobacterium: urinimassiliense*, sp. nov.. Nous avons utilisé la taxono-génomique afin de décrire ces nouvelles espèces bacteriennes par une approche polyphasique intégrant à la fois les données phénotypiques et génotypiques [15]. La culturomics, couplée à la taxonogénomique, appliquée à la culture des urines a ainsi permis d'augmenter le répertoire bactérien associé à l'homme. Ces nouvelles espèces bactériennes viennent s'ajouter aux plus de 500 nouvelles espèces bactériennes ayant été découvertes grâce à la méthode culturomics [17].

Article IV :

"*Corynebacterium urinapleomorphum*" sp. nov., isolated from a urine sample of a 2-month-old boy affected by rotavirus gastroenteritidis.

Aurélie MORAND, Brigitte CHABROL, Frédéric CADORET, Pierre-Edouard FOURNIER and Didier RAOULT

Publié dans le journal New Microbes New Infections

"*Corynebacterium urinapleomorphum*" sp. nov., isolated from a urine sample of a 2-month-old boy affected by rotavirus gastroenteritis

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Abstract

We report the main characteristics of "*Corynebacterium urinapleomorphum*" strain Marseille-P2799^T (CSURP2799), isolated from a urine sample from a 2-month-old boy with rotavirus gastroenteritis.

Keywords: "*Corynebacterium urinapleomorphum*", culturomics, genomics, taxonogenomics, taxonomy
Original Submission: 25 August 2016; **Revised Submission:** 17 September 2016; **Accepted:** 21 September 2016
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In 2016, as a part of culturomics study [1,2] of the human microbiome, a bacterial strain that could not be identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) screening using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [3] was isolated from the urine of a 2-month-old boy with rotavirus gastroenteritis. The patient's parents provided signed informed consent, and the ethics committee of the Institut Fédératif de Recherche IFR48 approved the study under number 09-022.

A pure culture of strain Marseille-P2799 was obtained after 72 hours of incubation at 37°C on 5% sheep's blood–antioxidant agar homemade R-medium (Hôpital de la Timone, Marseille, France) in anaerobic atmosphere generated using the GENbag anaer system (bioMérieux, Marcy l'Étoile, France). Agar-grown colonies were pale grey and had a mean diameter of 500 µm. Bacterial cells were nonmotile, Gram-positive, pleomorphic bacilli with a length ranging from 700 to 2000 nm and width ranging from 400 to 600 nm. Strain Marseille-P2799 exhibited catalase activity but no oxydase activity. The 16S rRNA gene was sequenced using the fD1-rP2 primers as previously described [4], using a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). Strain Marseille-P2799 exhibited 98% sequence similarities to *Corynebacterium appendicis* strain IMMIB R-3491^T (GenBank accession no. AJ314919), its phylogenetically closest species with standing in nomenclature (Fig. 1) [5]. *Corynebacterium appendicis* strain IMMIB R-3491^T was isolated from an abdominal swab of a patient with appendicitis accompanied with abscess formation. Strain IMMIB R-3491^T stained Gram positive and consisted of nonmotile, thin, pleomorphic, coryneform cells. On Columbia blood agar, colonies were very small, dry and slightly greyish in color. The strain IMMIB R-3491^T was growing in facultative anaerobic atmosphere and was catalase positive. Because the nucleotide sequence of strain identity Marseille-P2799 was lower than the 98.63% cutoff recommended to delineate bacterial species [6], we consider strain Marseille-P2799^T to be the type strain of a novel *Corynebacterium* species, "*Corynebacterium urinapleomorphum*" sp. nov. (u.ri.na.pleo.morph.um composed of u.ri.na L.N. gen. fem. *urina*, the Latin word for "urine," as strain Marseille-P2799 was first found in a paediatric urine sample, and pleo.morph.um. L. neutral. adj. *pleomorphum* of *pleo*, "several" or "different," and *morph*, "shape," as cells were bacilli with cytoplasmic inclusion that could make us think that the bacterium was catenary Gram-positive cocci).

http://dx.doi.org/10.1016/j.nmni.2016.09.010

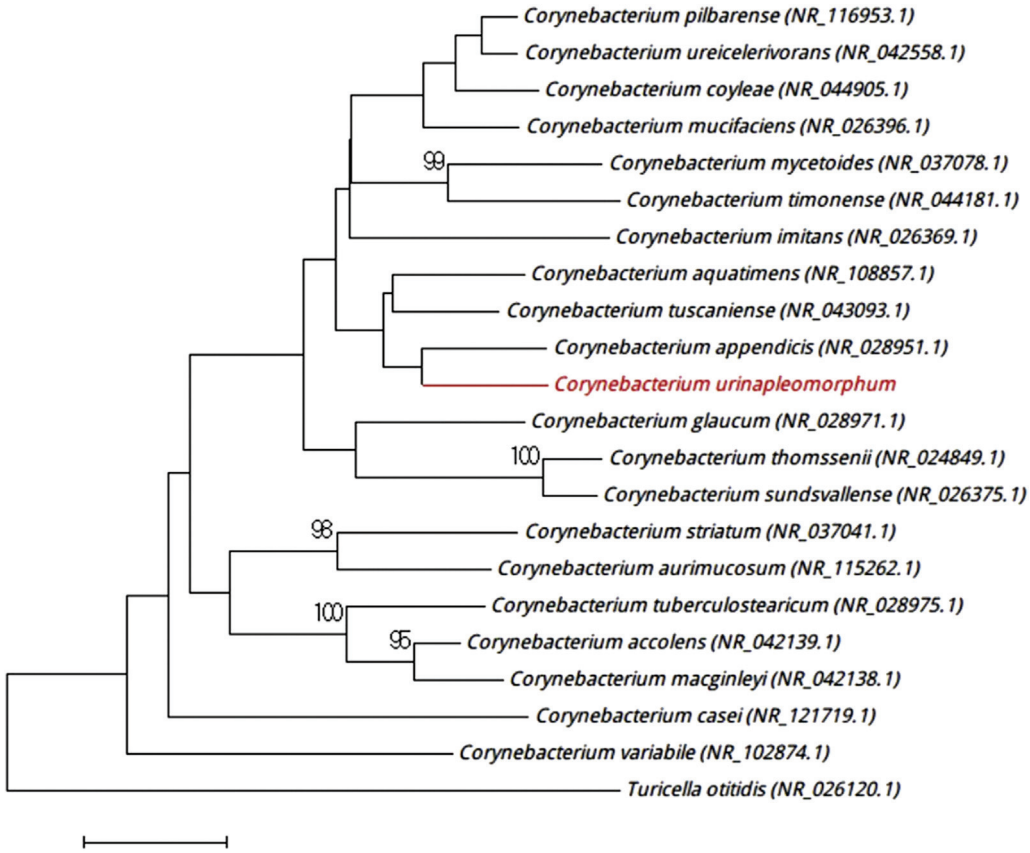

FIG. 1. Phylogenetic tree showing position of "*Corynebacterium urinapleomorphum*" strain Marseille-P2799 relative to other phylogenetically close neighbours. 16S rRNA sequences were aligned using CLUSTALW and phylogenetic inferences obtained using maximum-likelihood method within MEGA software. Numbers at nodes are percentages of bootstrap values (≥95%) obtained by repeating analysis 500 times to generate majority consensus tree. GenBank accession numbers are indicated in parentheses. Scale bar indicates a 1% nucleotide sequence divergence.

The MALDI-TOF MS spectrum of "*Corynebacterium urinapleomorphum*" strain Marseille-P2799^T is available at http://mediterranee-infection.com/article.php?laref=256&titre=urms-database.

Nucleotide sequence accession number

The 16S rRNA gene sequence was deposited in GenBank under accession number LT576404.

Deposit in a culture collection

Strain Marseille-P2799^T was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) under number P2799.

Acknowledgement

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Conflict of Interest

None declared.

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Article V :

Corynebacterium urinapleomorphum sp. nov., a new bacterial species isolated from human urine sample.

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Publié dans le journal New Microbes New Infections

Corynebacterium urinapleomorphum sp. nov., a new bacterial species isolated from human urine sample

E. H. A. Niang[1,2], C. I. Lo[2,3], A. Morand[1,2], S. Ndongo[1,2], D. Raoult[1,2], P.-E. Fournier[2,3] and F. Fenollar[2,3]

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Abstract

Corynebacterium urinapleomorphum sp. nov. stain Marseille-P2799^T (= CSURP2799; = DSM103272) is a new species from the order *Corynebacteriales* that was isolated from urine of a 2-month-old child with gastroenteritis.

Keywords: *Corynebacteriales*, *Corynebacterium urinapleomorphum*sp. nov., culturomics, human microbiota, urinary microbiota
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Introduction

Currently, the implication of bacterial diversity for normal physiological functions and disease must be understood [1]. To explore the diversity of human intestinal bacteria, the culturomics approach, based on diversified culture conditions, was designed to isolate species never cultivated before and also to complete the metagenomics of 16S rRNAs [2–4]. This culturomics approach has also been extended for the characterization of other human bacterial systems such as those of the vaginal and urinary tract [5,6]. Recently, a new taxonomic method called taxonogenomics has been developed for a description associating the analysis of complete sequences of the genome and the phenotypic characteristics of new bacterial species [7]. Herein, we give a short description, based on taxonogenomics, of a new species within the genus *Corynebacterium*, isolated from a young boy's urine.

Isolation and growth conditions

In 2016, the strain Marseille-P2799^T was isolated from a urine sample from a 2-month-old child with rotavirus gastroenteritis. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the strain has not been identified. The screening was performed on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany), as previously reported [8]. Spectra obtained (Fig. 1) were imported and analysed using the BIOTYPER 3.0 software against the Bruker database, which was constantly updating from the MEPHI database http://www.mediterranee-infection.com/article.php?larub=280&titre=umrs-database [1]. The strain Marseille-P2799^T was isolated after 72 hours of incubation at 37°C on 5% sheep's blood–antioxidant agar homemade R-medium (Hôpital de la Timone, Marseille, France) in anaerobic atmosphere generated using the GENbag anaer system (bioMérieux, Marcy-l'Étoile, France) [9].

Phenotypic characteristics

Colonies of the Strain Marseille-P2799^T were pale grey and had a mean diameter of 0.5 mm. Bacterial cells were non-

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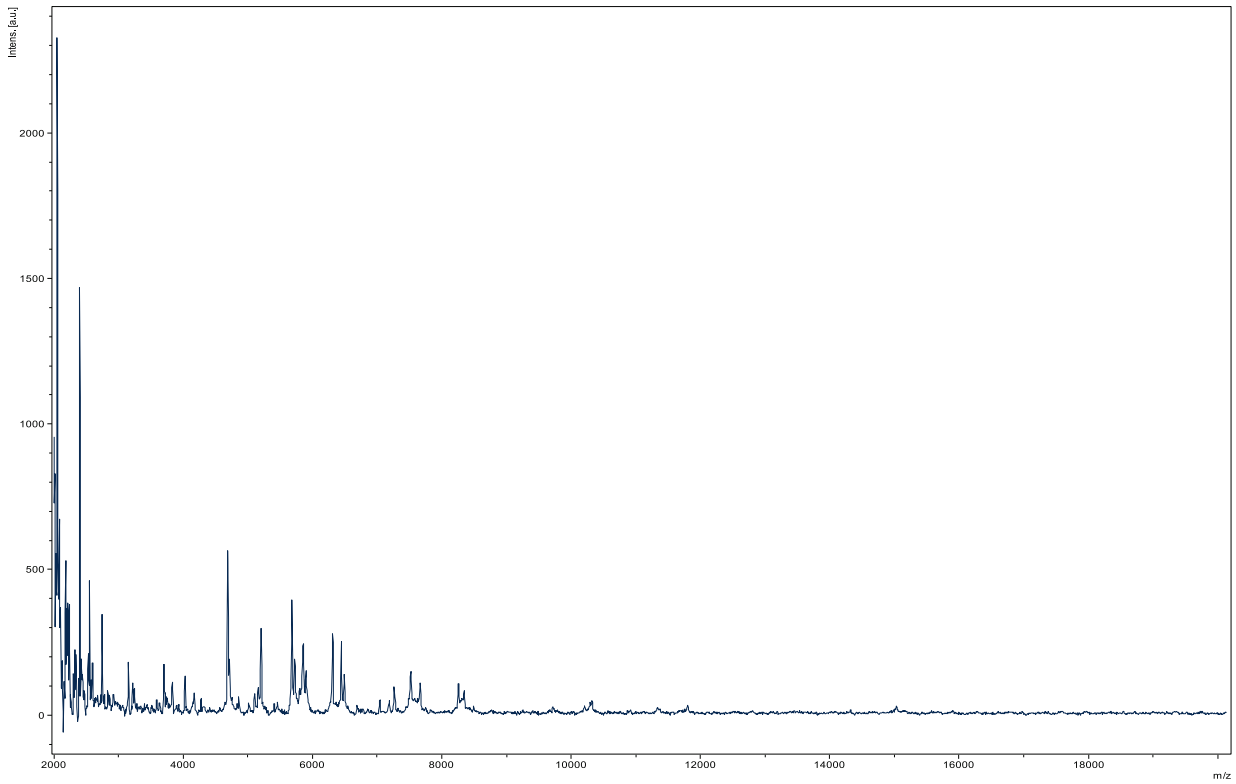

FIG. 1. MALDI-TOF MS reference spectrum of *Corynebacterium urinapleomorphum* sp. nov. The reference spectrum was generated by comparison of spectra from 12 individual colonies.

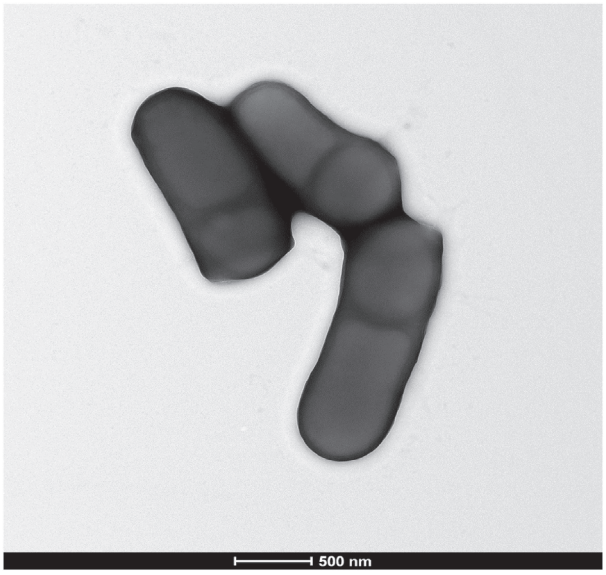

FIG. 2. Scanning electron microscopy (SEM) of stained *Corynebacterium urinapleomorphum* sp. nov. A colony was collected from agar and immersed in a 2.5% glutaraldehyde fixative solution. Then a drop of the suspension was directly deposited on a poly-L-lysine-coated microscope slide for 5 minutes and treated with 1% phosphotungstic acid aqueous solution (pH 2.0) for 2 minutes to increase SEM image contrast. The slide was gently washed in water; air-dried and examined in a tabletop SEM (Hitachi TM4000) approximately 60 cm in height and 33 cm in width to evaluate bacteria structure. The scale is shown on the figure.

TABLE 1. Phenotypic characterization of *Corynebacterium urinapleomorphum* sp. nov., based on analytical profile index (API) tests

Biochemical characteristics	*Corynebacterium urinapleomorphum* sp. nov.
Alkaline phosphatase	+
Esterase (C-4)	+
Esterase lipase (C-8)	+
Lipase (C-14)	−
Leucine arylamidase	+
Valine arylamidase	−
Cystine arylamidase	−
Trypsine	−
α-chymotrypsine	−
Acid phosphatase	+
Naphthalo-AS-BI-phosphohydrolase	+
α-galactosidase	−
β-galactosidase	−
β-glucuronidase	−
α-glucosidase	−
β-glucosidase	−
N-acetyl-β-glucosaminidase	−
α-mannosidase	−
α-fucosidase	−
Nitrates to nitrites	−
Indole	−
Glucose fermentation	−
Arginine dihydrolase	+
Urease	+
Protease	+
Glucose assimilation	−
Arabinose	−
Mannose	−
Mannitol	−
N-acetyl-glucosamine	−
Maltose	−
Potassium gluconate	−
Capric acid	−
Adipic acid	−
Malate	−
Trisodium citrate	−
Phenylacetic acid	−

motile, Gram-positive, pleomorphic bacilli with a length ranging from 0.7 to 2 µm and width ranging from 0.4 to 0.6 µm (Fig. 2). Strain Marseille-P2799^T was catalase positive and oxidase negative. API ZYM and API 20NE tests were performed at 37°C under aerobic conditions (Table 1). Table 2 compares the main biochemical characteristics of the closest *Corynebacterium* species with standing in nomenclature.

Strain identification

In order to classify this bacterium, the 16S rRNA gene was amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary sequencer (Thermofisher, Saint-Aubin, France), as previously described [10]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com).

Strain Marseille-P2799^T exhibited 98% sequence similarities to *Corynebacterium appendicis* strain IMMIB R-3491^T (GenBank Accession no. AJ314919), the phylogenetically closest species with standing in nomenclature (Fig. 3). We consequently proposed classifying strain Marseille-P2799^T as a new species within the genus *Corynebacterium* belonging to the phylum *Actinobacteria*.

TABLE 2. Differential phenotypic characteristics of *Corynebacterium urinapleomorphum* sp. nov. (1), *Corynebacterium phoceense* (2), *Corynebacterium freiburgense* (3), *Corynebacterium aurimucosum* (4) and *Corynebacterium appendicis* (5)

Properties	1	2	3	4	5
Cell diameter (µm)	0.2	0.5	0.5	0.5	0.3
Oxygen requirement	+	+	+	+	−
Gram stain	+	+	+	+	+
Salt requirement	−	−	−	−	−
Motility	−	−	−	−	−
Endospore formation	−	+	−	−	−
Alkaline phosphatase	+	+	−	+	+
Catalase	+	+	+	+	+
Oxidase	−	−	na	na	na
Nitrate reductase		+	+	−	−
Urease	+	−	−	−	+
β-galactosidase	−	−	+	−	−
N-acetyl-glucosamine	−	−	−	−	−
Arabinose	−	na	−	+	−
Lipase (C8)	+	+	+	−	−
Pyrrolidonyl arylamidase	−	+	−	−	−
Mannose	−	+	+	−	+
Mannitol	−	−	+	−	−
Sucrose		−	+	+	−
D-glucose	−	+	+	+	+
D-fructose	−	+	+	+	na
D-maltose	−	+	+	+	+
Habitat	Human	Human	Human	Human	Human

+, positive result; −, negative result; na, data not available.

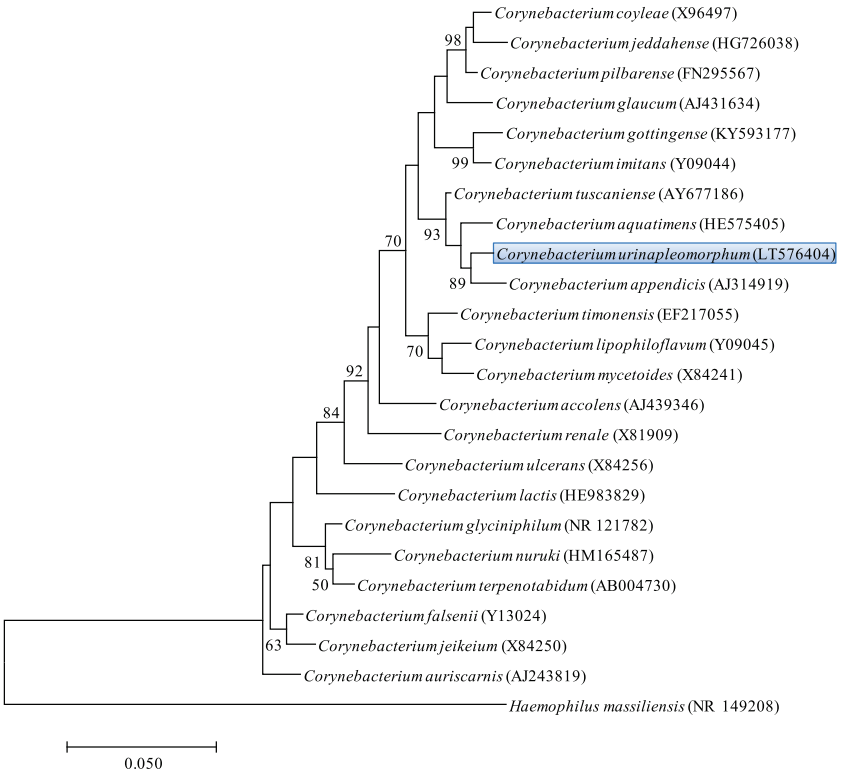

FIG. 3. Phylogenetic tree highlighting the position of *Corynebacterium urinapleomorphum* sp. nov., relative to the most closely related type strains within the genus *Corynebacterium*. GenBank Accession numbers of 16S rRNA are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference was obtained using the maximum likelihood method and MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 5% nucleotide sequence divergence. *Haemophilus massiliensis* was used as an outgroup.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [11]. The assembly was performed using a pipeline containing several softwares (Velvet [12], Spades [13] and Soap Denovo [14]), and trimmed (MiSeq and Trimmomatic [15] softwares) or untrimmed (only MiSeq software) data. GapCloser was used to reduce assembly gaps. Scaffolds <800 bp and scaffolds with a depth value < 25% of the mean depth were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). The genome of Strain Marseille-P2799^T was 2.26 Mb with 63.4% G + C content. The degree of genomic similarity of the strain with closely related species was calculated using OrthoANI software [16]. OrthoANI values among closely related species (Fig. 4) ranged from 60.04% between *Corynebacterium gottingense* and *Corynebacterium lipophiloflavum* to 84.98% between *Corynebacterium imitans* and *Corynebacterium gottingense*. When *Corynebacterium urinapleomorphum* was compared with these closely related species, values ranged from 62.43% with *Corynebacterium gottingense* to 74.85% with *Corynebacterium mycetoides*.

Conclusion

On the basis of unique phenotypic features, including MALDI-TOF spectrum, a 16S rRNA sequence divergence >1.3% and an OrthoANI value < 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P2799^T as the type strain of *Corynebacterium urinapleomorphum* sp. nov, which is a new species in the genus *Corynebacterium*.

Description of *Corynebacterium urinapleomorphum* strain Marseille-P2799^T sp. nov.

Corynebacterium urinapleomorphum (u.ri.na.pleo.morph.um composed of u.ri.na L.N. gen. fem. *urina*, the Latin word for 'urine', as strain Marseille-P2799 was first found in a paediatric urine sample, and pleo. morph.um. L. neutral. adj. *pleomorphum* of pleo, 'several' or 'different', and morph, 'shape', as cells were bacilli with cytoplasmic inclusions that could make us think that the bacterium was a catenary Gram-positive coccus). The strain grows at temperatures ranging between 37°C and 45°C in anaerobic conditions (at an optimum temperature of 37°C). This is a facultative aero-

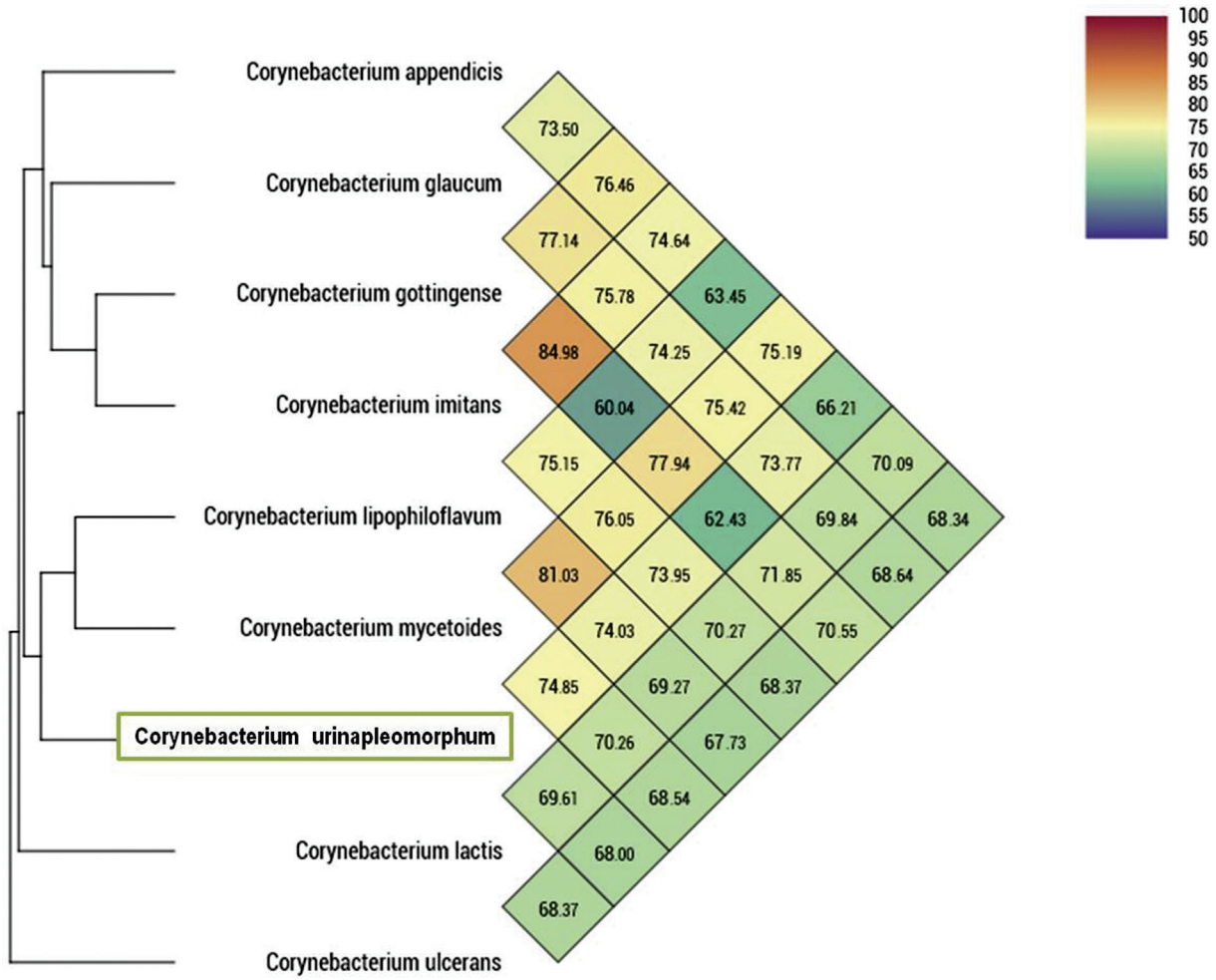

FIG. 4. Heatmap generated with OrthoANI values calculated using the OAT software between *Corynebacterium urinapleomorphum* sp. nov. and other closely related species with standing in nomenclature.

anaerobic bacterium. Salinity range growth was tested between 10% and 20% (no growth was observed), and pH growth occurred between pH 5 and 8 (with an optimum of pH 7). The potential pathogenicity of the type strain Marseille-P2799^T (= CSURP2799; = DSM103272) is unknown. However, this bacterium, as well as *Staphylococcus saprophyticus* and *Helicobacter pylori*, has recently been isolated from the gallbladder of a patient with acute cholecystitis [17]. It was isolated from the urine sample of a 2-month-old child who came into our hospital with gastroenteritis. This strain exhibited a G + C content of 63.4%.

Nucleotide sequence accession number

The 16S rRNA gene and genome sequences were deposited in GenBank under Accession number LT576404 and FTLL00000000, respectively.

Deposit in culture collections

Strain Marseille-P2799^T was deposited in two different strain collections under following numbers (= CSURP2799; = DSM103272).

Ethics and consent

The study was **approved** by the ethics committee of the Institut Federatif de Recherche 48 under reference 2016-010. The patient's guardian gave an approved and signed consent for participating in this study.

Conflicts of interest

None to declare.

Funding sources

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the programme *Investissements d'avenir*, reference ANR-10-IAHU-03, the Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI.

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Article IV :

Olsenella urininfantis", a new bacterial species isolated from a urine sample of a 26-day-old boy suffering from gastroesophageal reflux.

Aurélie MORAND, Brigitte CHABROL, Pierre-Edouard FOURNIER.

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"*Olsenella urininfantis*", a new bacterial species isolated from a urine sample of a 26-day-old boy suffering from gastroesophageal reflux

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ABSTRACT

We report here the main characteristics of "*Olsenella urininfantis*" strain Marseille-P3197^T (CSUR P3197). That strain was isolated from a urine sample of a 26-day-old boy suffering from gastroesophageal reflux.

We conducted a culturomics study [1,2]. We cultivated a bacterial strain, strain Marseille-P3197^T, that could not be identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [3] of the urine from a 26-day-old boy affected by gastroesophageal reflux. The patient's parents gave a signed informed consent and the ethics committee of the Institut Federatif de Recherche IFR48 approved the study under number 09-022.

The initial growth was obtained after 30 days of incubation in an anaerobic blood culture vial (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 4 mL of 0.2 µm-filtered rumen fluid and 5 mL of defibrined sheep blood (bioMérieux, Marcy l'Etoile, France). A pure culture of strain Marseille-P3197^T was then obtained after 72 h of incubation at 37 °C on 5% sheep blood-enriched Columbia agar medium (bioMérieux) in anaerobic atmosphere generated using the GENbag Anaer system (bioMérieux). Agar-grown colonies were small, with a mean diameter of 300 µm, and translucent white. Bacterial cells were rod-shaped and Gram-positive with a length ranging from 0.6 to 2.0 µm and a width ranging from 0.4 to 1.0 µm. Strain Marseille-P3197 did not exhibit catalase or oxidase activities. The 16S rRNA gene was sequenced using the fD1-rP2 primers as previously described [4], using a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). Strain Marseille-P3197 exhibited a 97.43% sequence similarity with *Olsenella profusa* strain D315A-29^T (Genbank accession number NR036821) [5], its phylogenetically closest species with standing nomenclature (Fig. 1). *Olsenella profusa* was first isolated from the oral cavity and cells were described as short, Gram-positive, strictly anaerobic, rod-shaped bacteria.

Strain Marseille-P3197^T presenting a 16S rRNA gene sequence divergence >1.3% with *Olsenella profusa* [6]. We considered strain Marseille-P3197^T as a potential new species of the genus *Olsenella* (family *Coriobacteriaceae*, phylum *Actinobacteria*). We propose the creation of the new species "*Olsenella urininfantis*" sp. nov. strain Marseille-P3197^T as the type strain of "*Olsenella urininfantis*" (u.ri.n. L. fem. n. *urina*, the latin name of urine; and in.fant'is. L. n. infans the latin name of an infant; u.rin.in.fan'tis. N.L. gen. n. *urininfantis* of the urine of an infant).

MALDI-TOF Spectrum of "*Olsenella urininfantis*" strain Marseille-P3197^T is available in: (http://mediterranee-infection.com/article.php?laref=256&titre=urms-database).

Nucleotide sequence accession number. The 16S r RNA gene sequence was deposited in Genbank under accession number LT598594.

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http://dx.doi.org/10.1016/j.humic.2016.11.003
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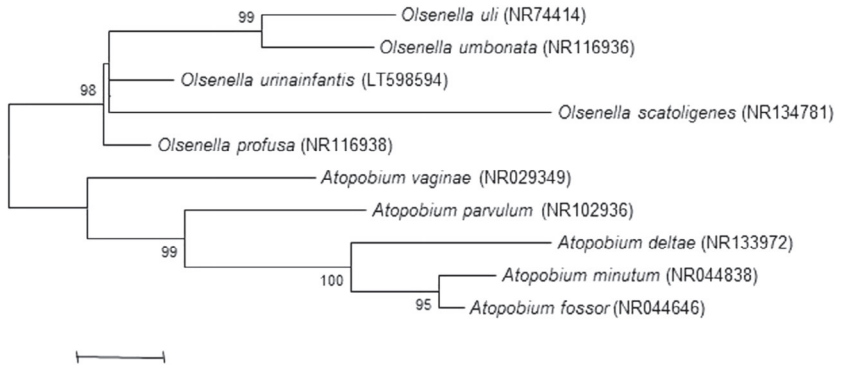

Fig. 1. Phylogenetic tree showing the position of "*Olsenella urininfantis*" strain Marseille-P3197^T relative to other phylogenetically-closest neighbours. The 16S rRNA gene sequences were aligned using CLUSTALW, and phylogenetic inferences obtained using the maximum-likelihood method within the MEGA software. Numbers at the nodes are percentages of bootstrap values ($\geqslant$95%) obtained by repeating the analysis 500 times to generate a majority consensus tree. Genbank accession numbers are indicated in parentheses. The scale bar indicates a 1% nucleotide sequence divergence.

Deposit in a culture collection. Strain Marseille-P3197^T was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) under number P3197.

Funding sources: This work was funded by Mediterrannée-Infection Foundation.

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Article VII :

"*Arcanobacterium: urinimassiliense*", sp. nov, a new bacterium isolated from the urogenital tract.

Khoudia DIOP, Aurélie MORAND, Jean-Christophe DUBUS, Pierre-Edouard FOURNIER, Didier RAOULT and Florence FENOLLAR.

Article publié dans le journal New Microbes New Infections

'*Arcanobacterium urinimassiliense*' sp. nov., a new bacterium isolated from the urogenital tract

K. Diop[1], **A. Morand**[1,2], **J. C. Dubus**[2], **P.-E. Fournier**[1], **D. Raoult**[1,3] **and F. Fenollar**[1]

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Abstract

Herein we report the main characteristics of '*Arcanobacterium urinimassiliense*' strain Marseille-P3248^T (=CSUR P3248) isolated from a urine sample of a 54-day-old girl with rotavirus gastroenteritis.

Keywords: '*Arcanobacterium urinimassiliense*', culturomics, human microbiota, rotavirus gastroenteritis, taxonomy
Original Submission: 8 February 2017; **Revised Submission:** 18 March 2017; **Accepted:** 28 March 2017
Article published online: 31 March 2017

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In 2016, as a part of the culturomics study [1,2] of the human microbiome, a bacterial strain that could not be identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [3] was isolated from a urine sample of a 54-day-old girl with rotavirus gastroenteritis. The study was approved by the local ethics committee of the Institut Federatif de Recherche IFR48 (Marseille, France) under the agreement number 09-022. The parents provided written informed consent.

The sample was preincubated in an anaerobic blood culture bottle (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 4 mL rumen that was filter-sterilized through a 0.2 µm pore filter (Thermo Fisher Scientific, Villebon-sur-Yvette, France) and 5 mL of defibrined sheep's blood (bioMérieux, Marcy l'Etoile, France). After 30 days of preincubation, the supernatant was cultured on homemade R medium (Timone Hospital, Marseille, France) and then incubated in anaerobic atmosphere generated using the GENbag Anaer system (bioMérieux). After 3 days of incubation, strain Marseille-P3248^T was isolated. On agar, colonies were small and beige with a mean diameter of 200 µm. Bacterial cells were Gram variable and rod shaped, with length ranging from 400 to 600 nm and width ranging from 300 to 400 nm. Strain Marseille-P3248^T was nonmobile. Catalase and oxidase reactions were negative.

The 16S rRNA gene was sequenced using the fD1-rP2 primers as previously described [4], using a 3130-XL sequencer (Applied Biosciences, Saint-Aubin, France). Strain Marseille-P3248^T exhibited 94.7% sequence similarity with *Arcanobacterium phocae* strain DSM 10002 (GenBank accession no. NR-117159) [5], its phylogenetically closest species with standing in nomenclature (Fig. 1). Because this sequence was smaller than the 98.65% threshold set defined by Kim *et al.* [6] to support a new species, strain Marseille-P3248^T can be classified as a new species of *Arcanobacterium* genus belonging to the family *Actinomycetaceae* classified within the *Actinobacteria* phylum.

Because strain Marseille-P3248^T exhibited a 16S rRNA gene sequence divergence of 3.95% with its phylogenetically closest species with standing in nomenclature [6], we propose that strain Marseille-P3248 may be the representative strain of the new species called '*Arcanobacterium urinimassiliense*' (u.ri.ni.mas.sil.ien′se, N.L. u.ri.no, N.L. gen. fem. *urina*, 'urine,' from which this bacterium was first cultivated; and mas.si.li.en′sis, L. gen. adj. *massiliensis*, from 'Massilia,' the Latin name of

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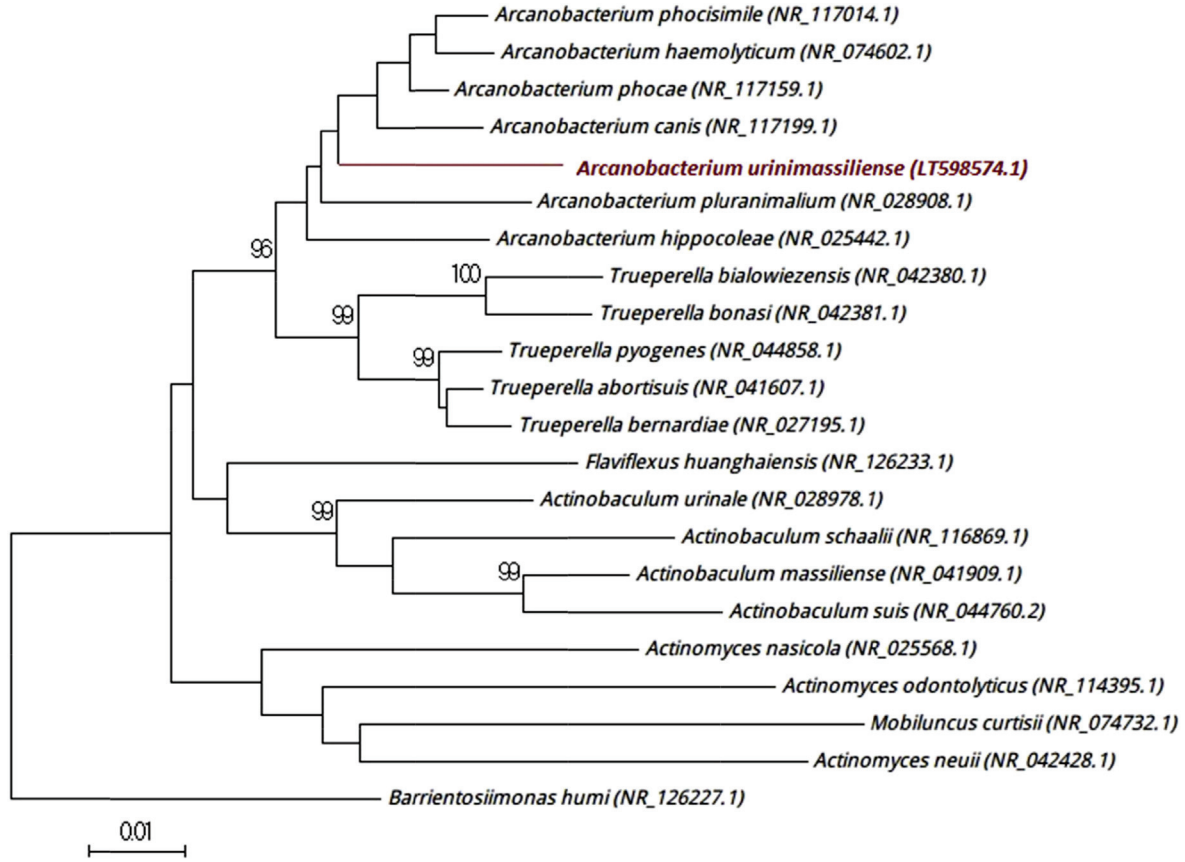

FIG. 1. Phylogenetic tree showing position of *'Arcanobacterium urinimassiliense'* strain Marseille-P3248^T relative to other phylogenetically close neighbours. Sequences were aligned by CLUSTALW, and phylogenetic inferences were obtained by maximum-likelihood method within MEGA software. Number at node is percentages of bootstrap value (≥95%) obtained by repeating analysis 500 times to generate majority consensus tree. GenBank accession numbers are indicated in parentheses. Scale bar indicates 1% nucleotide sequence divergence.

Marseille, France, where the microorganism was first isolated). Strain Marseille-P3248^T is the type strain.

MALDI-TOF MS spectrum

The MALDI-TOF MS spectrum of *'Arcanobacterium urinimassiliense'* strain Marseille-P3248^T is available online (http://mediterranee-infection.com/article.php?laref=256&titre=urms-database).

Nucleotide sequence accession number

The 16S rRNA gene sequence of the strain Marseille-P3248^T was deposited in GenBank under accession number LT598574.

Deposit in a culture collection

Strain Marseille-P3248^T was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR) under number P3248.

Acknowledgement

This study was funded by the Fondation Méditerranée Infection.

Conflict of Interest

None declared.

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Article VIII :

Urinacoccus massiliensis gen.nov. sp.nov., identified in urine sample of a 7-year-old boy hospitalized for dental care under general anesthesia.

Aurélie MORAND, Florent CORNU, Michel TSIMARATOS, Frédéric CADORET, Jean-Christophe LAGIER, Pierre-Edouard FOURNIER and Didier RAOULT.

Publié dans le journal New Microbes New Infections

'*Urinacoccus massiliensis*' gen. nov. sp. nov., identified in urine sample of a 7-year-old boy hospitalized for dental care under general anaesthesia

A. Morand[1,2], F. Cornu[3], M. Tsimaratos[3], F. Cadoret[1,2], J.-C. Lagier[1,2], P. E. Fournier[1,2] and D. Raoult[1,2]

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Abstract

We report here the main characteristics of '*Urinacoccus massiliensis*' gen. nov. sp. nov., strain FC2 (CSURP1992). This strain was isolated from the urine of an asymptomatic 7-year-old boy.

Keywords: Culturomics, genomics, taxonomy, *Urinacoccus massiliensis*, urinary microbiome
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As part of a culturomics study [1,2] of the human microbiome, in 2015 we isolated from the urine of a 7-year-old boy hospitalized for dental care under general anaesthesia a bacterial strain that was not identified by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) screening using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [3]. The patient's parents provided signed informed consent and the ethics committee of the Institut Federatif de Recherche IFR48 validated the study under number 09-022.

The initial growth was obtained after 10 days of incubation in an anaerobic blood culture vial (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 5 mL of 0.2-µm filtered rumen fluid. A subculture of strain FC2 was then obtained after 48 h of incubation at 37°C on 5% sheep blood–Columbia agar medium (bioMérieux, Marcy l'Etoile, France) in anaerobic atmosphere generated using the GENbag Anaer systems (bioMérieux). Agar-grown colonies were microscopic and translucent with a mean diameter of 1 µm. Bacterial cells were Gram-positive cocci, ranging in length from 300 to 500 nm. Strain FC2 was catalase and oxidase negative. The 16S rRNA gene was sequenced using the fD1-rP2 primers as previously described [4], using a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). Strain FC2 exhibited a 91% sequence identity with *Peptoniphilus coxii* strain RMA 16757^T (GenBank Accession number GU938836) [5], the phylogenetically closest species with standing in nomenclature (Fig. 1), which putatively classifies it as a new genus member of the family *Peptoniphilaceae* in the phylum *Firmicutes*.

Strain FC2 presents a 16S rRNA gene sequence divergence >5% with its phylogenetically closest species with standing in nomenclature [6], so we propose the creation of the new genus '*Urinacoccus*' gen. nov. (u.ri.na.coc'cus, N. L. masc. n. composed of u.ri.na, L. N. gen. fem. *urina*, from *urina*, the Latin name of urine and coc'cus. L. N. gen. masc. *coccus*, from *coccus*, the Latin name of bacterium having a spherical or spheroidal shape) as strain FC2 is a coccus and was first isolated from a paediatric urine sample. We propose the new species '*Urinacoccus massiliensis*' sp. nov. (mas.sil.ien'sis. L. Adj. gen. fem. *massiliensis*, of *massilia*, the Latin name of Marseille) because strain FC2 was first found in the city of Marseille.

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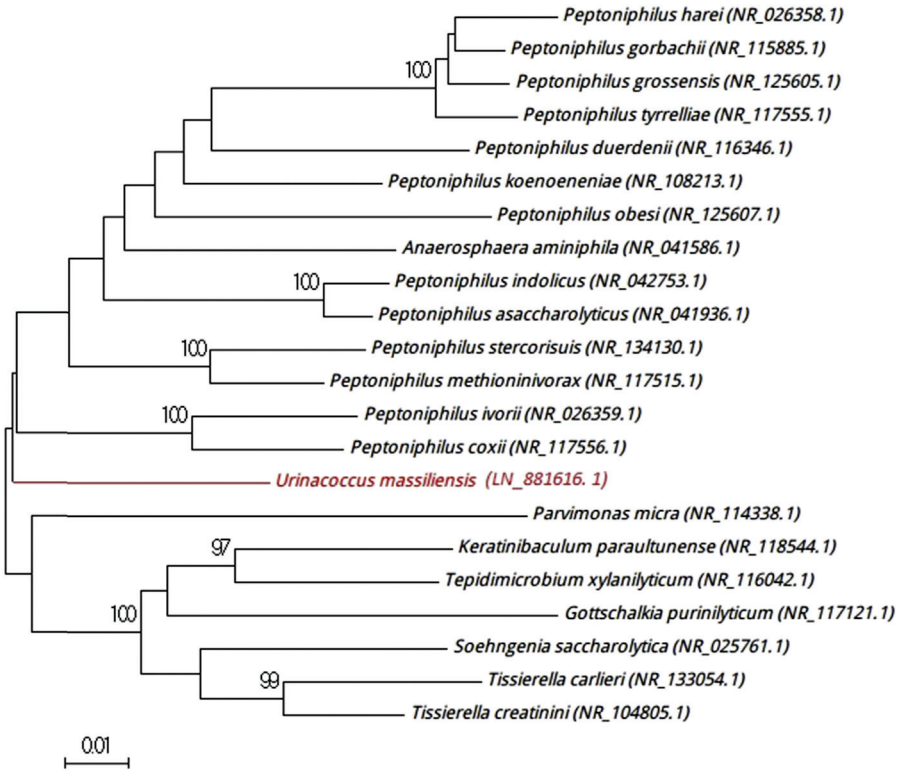

FIG. 1. Phylogenetic tree showing the position of '*Urinacoccus massiliensis*' strain FC2 relative to other phylogenetically close neighbours. Sequences were aligned using CLUSTALW, and phylogenetic inferences were obtained using the maximum-likelihood method within the MEGA software. GenBank Accession numbers are indicated in parentheses. Numbers at the nodes are percentages of bootstrap values (≥95%) obtained by repeating the analysis 500 times to generate a majority consensus tree. The scale bar indicates a 1% nucleotide sequence divergence.

MALDI-TOF spectrum accession number. The MALDI-TOF spectrum of '*Urinacoccus massiliensis*' strain FC2^T is available at: http://mediterranee-infection.com/article.php?laref=256&titre=urms-database.

Nucleotide sequence accession number. The 16S rRNA gene sequence was deposited in GenBank under Accession number LN881616.

Deposit in a culture collection. Strain FC2 was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) under number P1992.

Funding

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Conflict of Interest

None declared.

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Article IX :

Anaerococcus urinomassiliensis sp. nov., isolated from a urine sample of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis.

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Anaerococcus urinomassiliensis sp. nov., isolated from a urine sample of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis

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Abstract

We report the main characteristics of '*Anaerococcus urinomassiliensis*' strain FC4^T (CSURP2143) that was isolated from a urine sample of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis.

Keywords: *Anaerococcus urinomassiliensis*, culturomics, genomics, taxono-genomics, taxonomy
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In 2015, as part of a culturomics study [1,2] of the human microbiome, a bacterial strain that could not be identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) screening using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [3] was isolated from the urine of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis. The patient's parents gave signed informed consent and the ethics committee of the Institut Federatif de Recherche IFR48 approved the study under number 09-022.

The initial growth was obtained after 10 days of incubation in an anaerobic blood culture vial (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 5 mL of 0.2-µm filtered rumen fluid. A pure culture of strain FC4 was then obtained after 48 h of incubation at 37°C on 5% sheep blood–Columbia agar medium (bioMérieux, Marcy l'Etoile, France) in anaerobic atmosphere generated using the GENbag Anaer system (bioMérieux). Agar-grown colonies were small with a mean diameter of 50 µm and were translucent white. Bacterial cells were Gram-positive cocci with a diameter ranging from 400 to 600 nm. Strain FC4 did not exhibit catalase or oxidase activities. The 16S rRNA gene was sequenced using the fD1-rP2 primers as previously described [4], using a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France). Strain FC4 exhibited sequence similarities with *Anaerococcus octavius* strain NCTC 98 IOT (GenBank accession number NR-026360) [5], its phylogenetically 94.73% closest species with standing nomenclature in the range of 92.3% to 97.2% observed among *Anaerococcus* species (Fig. 1). This putatively classifies strain FC4 as a member of the genus *Anaerococcus* in the family *Peptostreptococcaceae* within the phylum *Firmicutes*.

Strain FC4 presents a 16S rRNA gene sequence divergence with its phylogenetically closest species with standing in nomenclature [6], so we propose the creation of the new species '*Anaerococcus urinomassiliensis*' sp. nov. strain FC4^T as the type strain of '*Anaerococcus urinomassiliensis*' (u.ri.no.mas.sil.ien'sis composed of u.ri.no L. V. intransitive. of *urino*, the Latin name for the verb to swim, closed of u.ri.na. L. N. gen. fem. *urina*, of *urina*, the Latin name of urine as this strain FC4 was first found in a paediatric urine sample and mas.si.li.en'sis. L. fem. adj. *massiliensis*, of Massilia, the ancient Greek and Roman name for Marseille, France, where the type strain was isolated).

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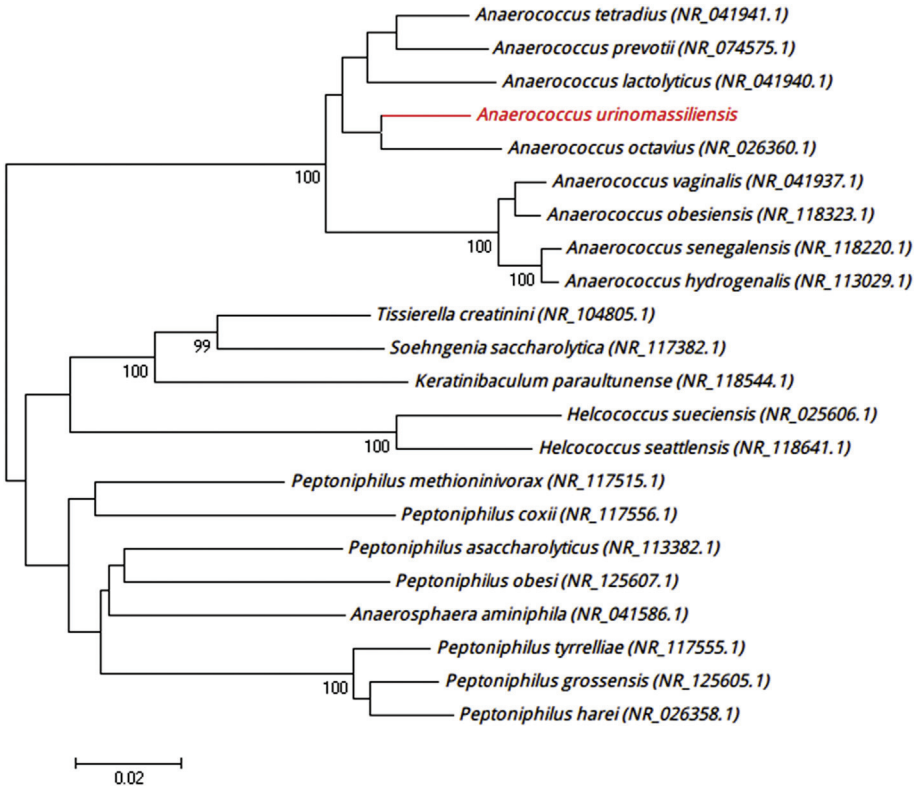

FIG. 1. Phylogenetic tree showing the position of '*Anaerococcus urinomassiliensis*' strain FC4 relative to other phylogenetically close neighbours. Sequences were aligned using CLUSTALW, and phylogenetic inferences were obtained using the maximum-likelihood method within the MEGA software. Numbers at the nodes are percentages of bootstrap values (≥ 95%) obtained by repeating the analysis 500 times to generate a majority consensus tree. GenBank accession numbers are indicated in parentheses. The scale bar indicates a 2% nucleotide sequence divergence.

MALDI-TOF Spectrum

MALDI-TOF spectrum of '*Anaerococcus urinomassiliensis*' strain FC4^T is available at http://mediterranee-infection.com/article.php?laref=256&titre=urms-database.

Nucleotide Sequence Accession Number

The 16S r RNA gene sequence was deposited in GenBank under Accession number LN898272.

Deposit in a Culture Collection

Strain FC4^T was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) under number P2143.

Funding

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Article X :

Anaerococcus urinomassiliensis sp. nov., a new bacterium isolated from urine

Mamadou Lamine TALL, Aurélie Morand, Edmond KUETE YIMAGOU, Issa Isaac NGOM, Cheikh Ibrahima LO, Didier RAOULT, Pierre-Edouard FOURNIER, Anthony LEVASSEUR.

En cours d'écriture

Anaerococcus urinomassiliensis sp. nov., a new bacterium isolated from urine

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Running title: *Anaerococcus urinomassiliensis* sp. nov.

Keywords: *Anaerococcus urinomassiliensis* sp. nov., culturomics, urine microbiota

Abstract

Anaerococucus urinomassiliensis sp. nov., strain Marseille-P2143^{T} (= CSUR P2143) is a new species from the family *Peptoniphilaceae* that was isolated from the urine of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis. The strain Marseille-P2143^{T} is gram positive cocci with translucent colonies on blood agar. Its genome was 2,895,509 bp-long with a 30.4 mol% G+C content and exhibited 98.48 % 16S rRNA similarity with *Anaerococcus provencensis* strain 9402080.

1. Introduction

The genus *Anaerococcus* belonging to the phylum *Firmicutes*, was first described in 2001 [1]. Members of this bacterial genus are mainly anaerobic gram positive cocci [2]. They are mostly encountered in human vagina, and can also be detected in nostrils or skin [3]. *Anaerococcus* sp. were involved in human infectious and were isolated from different site of human body such as peritoneal, ovarian, and cervical abscesses, an arthritic knee, bacteremias, foot ulcers, a sternal wound, and vaginoses [4-6]. Actually, the genus *Anaerococcus* contains 13 species validly described with standing in nomenclature [7].

The culturomics concept has recently been developed in our laboratory as an alternative method to expand the human gut repertoire through the multiplication of culture conditions with a rapid identification method by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [8-11]. Furthermore, the taxono-genomics strategy including proteomic information obtained by MALDI-TOF MS, the complete genomic analysis and phenotypic characteristics, was developed to describe new taxon [12]. Using this technique, we present here a description of *Anaerococcus urinomassiliensis* strain Marseille-P2143 (= CSUR P2143), a bacterium isolated from the urine of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis and classified into *Peptoniphilaceae* family.

2. MATERIAL AND METHODS

2.1 Sample collection

In 2015, we isolated from the urine of a 17-year-old boy affected by autoimmune hepatitis and membranoproliferative glomerulonephritis, a bacterial strain that could not be identified.

Signed and informed consent was collected from the patient and parents and the study obtained approval from ethics committee of the Institut Fédératif de Recherche IFR48 under number 09-022.

2.2 Strain isolation and identification by MALDI-TOF MS

The initial growth was obtained after 10 days of incubation in an anaerobic blood culture vial (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 5 mL of 0.2-µm filtered rumen fluid. A pure culture of strain P2143^T was then obtained after 48 h of incubation at 37°C on 5% sheep blood–Columbia agar medium (bioMérieux, Marcy l'Etoile, France) in anaerobic atmosphere generated using the GENbag Anaer system (bioMérieux).

A bacterial strain Marseille-P2143^T was not be identified by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The screening was performed on a Microflex LT spectrometer (Bruker, Daltonics, Bremen, Germany) as previously reported [13]. Spectra obtained (Figure 1) were imported and analyzed using the Biotyper 3.0 software against the Bruker database that was continually incremented with MEPHI database.

2.3 Strain identification and Phylogenetic tree

In order to classify this bacterium, the 16S rRNA gene was amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary sequencer (Thermofisher, Saint-Aubin, France) as previously described [14]. The 16S rRNA nucleotide sequence was assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). For phylogenetic analysis, sequences of the phylogenetically closest species were obtained after performing a BLASTn search within the 16S rRNA database of “The All-Species Living Tree" Project of Silva [15]. The alignment was performed using CLUSTALW [15] and MEGA 7 software [17] was used for phylogenetic inferences group using the maximum likelihood method.

2.4 Phenotypic characteristics and biochemical features

Phenotypic and biochemical characteristics were tested for strain Marseille-P2143. Utilization of carbon sources was essayed with API 50 CH strips, which were scored after incubation at 37°C for 24 hours. Antibiotic susceptibility was determined by disc diffusion plate method as according the instructions of the CA-SFM / EUCAST (Edition 2018). Used discs were : erythromycin (15 µg/ml), penicillin G (10 UI), doxycyclin (30 µg/ml), rifampicin (30 µg/ml), vancomycin (30 µg/ml), clindamycin (15 µg/ml), fosfomicin (50 µg/ml), amoxicillin (25 µg/ml), colistin (15 µg/ml), gentamycin (500 µg/ml), amoxicillin-clavulanic acid (30 µg/ml), ceftriaxon (30 µg/ml), colistin (50 µg/ml), trimethoprim-sulfamethoxazole (25 µg/ml), oxacillin (5 µg/ml), imipenem (10 µg/ml), tobramycin (10 µg/ml), and metronidazole (4 µg/ml). The strain was incubated at 37°C for 24 hours.

2.6 Genome sequencing and assembly

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [18]. The assembly was performed using a pipeline containing several softwares (Velvet [19], Spades [20] and Soap Denovo [21]), and trimmed (MiSeq and Trimmomatic [22] softwares) or untrimmed data (only MiSeq software). GapCloser was used to reduce assembly gaps. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value lower than 25% of the mean depth were removed. The best assembly was selected using different criteria (number of scaffolds, N50, number of N). The genome of strain Marseille-P2143^{T} was 2,190,108 bp bp-long with 33.47 %G+C content. The degree of genomic similarity of strain Marseille-P2143^{T} with closely related species was estimated using the OrthoANI software [23]. OrthoANI values among closely related species (Figure 4) ranged from 65.27% between

Anaerococcus phacaensis and Peptoniphilus phoceensis to 90.64 % between Anaerococcus provencensis and Anaerococcus urinomassiliensis. When Anaerococcus urinomassiliensis was compared to these closely species, values ranged from 72.87% with Anaerococcus mediterraneensis to 90.64% with Anaerococcus provencensis.

2.7 Genome annotation and analysis

The prediction was performed using prodigal in the open reading frame (ORF) [25] with default settings. Planned ORFs covering a sequencing gap region (containing N) have been excluded. The bacterial proteome was predicted with BLASTP (E-value of 1e03, coverage of 0.7 and identity percentage of 30) against the database of orthologic group clusters (COG). If no matches are found, we will search the nr database [26] using BLASTP with an E value of 1e03, a coverage of 0.7 and an identity percentage of 30. An E-value of 1e05 is used if it is only if the sequence length was less than 80 amino acids. The domains that are maintained by the PFAM (PFAM-A and PFAM-B domains) were searched on each protein using the hhmscan analysis tools. RNAmmer [27] and the tRNAScanSE tool [28] were used to find ribosomal RNA genes and tRNA genes. This is during if all BLASTP tests performed had negative results in this case the ORFan will be identified (value E less than 1e03 for ORFs with sequence size greater than 80 aa or value E less than 1e05 for ORFs with sequence length less than 80 aa). Artemis [29] was used for data management and visualization of genomic characteristics, and to analyze the average level of similarity of nucleotide sequences at the genome level, the in-house MAGI software was used. It calculated the average genomic identity of gene sequences (AGIOS) among the compared genomes [30]. This software combines Proteinortho software [31] to detect orthologic proteins in pairwise genomic comparisons. Then, the corresponding genes were recovered and the average percentage of nucleotide sequence identity among orthologic ORFs was determined using the Needleman - Wunsch global alignment algorithm.

We also used the Genome-to-Genome Distance Calculator Web service to calculate DNA: Digital DNA hybridization estimates (dDDH) with confidence intervals according to recommended parameters (Formula 2, BLAST) [32].

3. Results

3.1 Strain identification

Strain Marseille-P2143^T exhibited a 95.7 % 16S rRNA similarity with *Anaerococcus octavius* NCTC 9810 (Genbank accession number NR_026360.1), the phylogenetically closest species with standing in nomenclature (Figure 3). We consequently proposed to classify strain Marseille-P2143^T as a new species within the genus *Anaerococcus* in the phylum *Firmicutes.*

3.2 Phenotypic characteristics and biochemical features

The optimum growth of strain Marseille-P2143^T was obtained after 5 day of culture at 37°C in anaerobic conditions (anaeroGEN, Oxoid Ltd, Dardilly, France). Agar-grown colonies were small with a mean diameter of 50 µm and were translucent white. Bacterial cells were Gram-positive bacilli ranging in length from 0.4 to 0.6µm and in width (Figure 2). Strain Marseille-P2143^T did not exhibit catalase or oxidase activities. All characteristics of the strain are summarized in Table 1. Using API ZYM strip, positive reactions were observed for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase and β-galactosidase. But negative reactions were noted with lipase, valine arylamidase, α-chymotrypsin, acid phosphatase, and β-glucuronidase. Using API 50 CH strip (bioMérieux), strain Marseille-P2143was able to metabolize glycerol, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucopyranoside, N-acetylglucosamine, D-maltose, D-lactose, D-saccharose, D-trehalose, D-turanose, D-tagatose and potassium 5-ketogluconate. However, negative reactions were obtained with erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol,

D-galactose, D-glucose, D-mannose, L-sorbose, dulcitol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-maltose, D-lactose D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, gentiobiose, D-turanose , D-tagatose, D-arabitol, L-arabitol, potassium gluconate and Potassium 5-cetogluconate. The Antimicrobial susceptibility test according to the EUCAST, showed that strain Marseille-P2143 was susceptible to rifampin, vancomycin, clindamycin, fosfomicin, amoxicillin, penicillin, amoxicillin-clavulanic acid, oxacillin, imipenem, tobramycin but resistant to Erythromycin , Doxycyclin ,Colistin, Gentamicin, Ceftriaxone, Trimethoprim-sulfamethoxazole,

3.3 Fatty acid methyl ester (FAME) analysis by Gas Chromatography/ Mass Spectrometry (GC/MS)

Cellular fatty acid methyl ester (FAME) analysis was performed by GC/MS as described by Sasser et al, 2006 (21). GC/MS analyses were carried out as described before (22). Spectral database search was performed using MS Search 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg, USA) and the FAMEs mass spectral database (Wiley, Chichester, UK). The major fatty acid found for this strain by far was Hexadecanoic acid (57%) followed by 9-Octadecenoic acid (14%) and 9,12-Octadecadienoic acid (14%). Minor amounts of unsaturated, branched and other saturated fatty acids were also described (Table1).

3.5 Genome properties

The genome is 2,190,108 bp long with 33.47 %mol G+C content. It is composed of 10 scaffolds (composed of 11 contigs). Of the 2,076 predicted genes, 2,021 were protein-coding genes and 55 were RNAs (5 genes are 5S rRNA, 2 genes are 16S rRNA, 2 genes are 23S rRNA, 46 genes are tRNA genes). A total of 1,517 genes (75.06 %) were assigned as putative function (by cogs

or by NR blast). 44 genes were identified as ORFans (2.18%). The remaining genes were annotated as hypothetical proteins (387 genes => 19.15%).

3.4 Genome comparison

The draft genome sequence of *Anaerococcus urinomassiliensis* (2.19 Mb) is smaller than those of *Anaerococcus lactolyticus* (2.20 Mb), but larger than those of *Anaerococcus vaginalis*, *Anaerococcus hydrogenalis*, *Anaerococcus tetradius*, *Peptoniphilus duerdenii*, *Peptoniphilus lacrimalis* and *Peptoniphilus harei* (1.89, 1.88, 2.14, 2.12, 1.84 and 1.83 MB respectively). The G+C content of *Anaerococcus urinomassiliensis* (33.47mol%) is smaller than those of *Anaerococcus tetradius*, *Peptoniphilus duerdenii*, *Anaerococcus lactolyticus* and *Peptoniphilus harei* (34.15, 34.24, 34.94 and 34.44 mol%, respectively), but larger than those of *Anaerococcus vaginalis*, *Anaerococcus hydrogenalis* and *Peptoniphilus lacrimalis* (28.87, 29.64 and 30.22 % respectively). The gene content of *Anaerococcus urinomassiliensis* (2,021genes) is smaller than those of *Anaerococcus hydrogenalis*, *Anaerococcus tetradius* and *Anaerococcus lactolyticus* (2,069, 2,079 and 2,253, respectively), but larger than those of *Anaerococcus vaginalis*, *Peptoniphilus duerdenii*, *Peptoniphilus lacrimalis* and *Peptoniphilus harei* (1,764, 1,988, 1,745 and 1,724, respectively).

Conclusion

On the basis of unique phenotypic features, including the MALDI-TOF spectrum, a 16S rRNA sequence divergence greater than > 1.3 % and, an OrthoANI value lower than 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P2143^{T} as the type strain of *Anaerococcus urinomassiliensis* sp. nov., a new species within the genus *Anaerococcus.*

Description of *Anaerococcus urinomassiliensis* **sp. nov.**

Anaerococcus urinomassiliensis From popular Latin "aurina ", crossed from the latin urina and aurum because of its golden color and "massiliensis", of the roma name of Marseille , France with represent respectively the sample and the place where the strain Marseille-P2143 *was isolated* (The characteristics of the species are detailed in Table 1. The type strain is P2143^{T} (= CSUR P2143)

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278 **Table 1**: Description and characteristics of *Anaerococcus urinomassiliensis* Strain-Marseille-

279 P2143.

Property	Terms
Taxonumber	
Genus name	*Anaerococcus*
Species name	*Anaerococcus urinamassiliensis*
Specific epithet	*urinamassiliensis*
Species status	sp. nov.
Designation of the type strain	Strain-Marseille-P2143^{T}
Strain collection numbers	CSUR P2143
16S rRNA gene accession number	LN898272
Genome accession number	FQRX00000000
Genome size	2,190,108 bp
G+C (mol %)	33.47
Origin	Niamey, Niger
Date of isolation	2016-04
Source of isolation	Human stool sample
Conditions used for standard cultivation	Columbia agar + with 5% sheep blood for 48h of incubation
Gram stain	Positive
Cell shape	Rod shaped
Cell size (length X diameter)	1.5-2.1 X 0.5-0.7 (µm)
Motility	nonmotile
Colony morphology	white and smooth
Temperature optimum	37°C
pH range	5.5-8
Relationship to O_2	Anaerobe
Oxidase	Negative
Catalase	Positive

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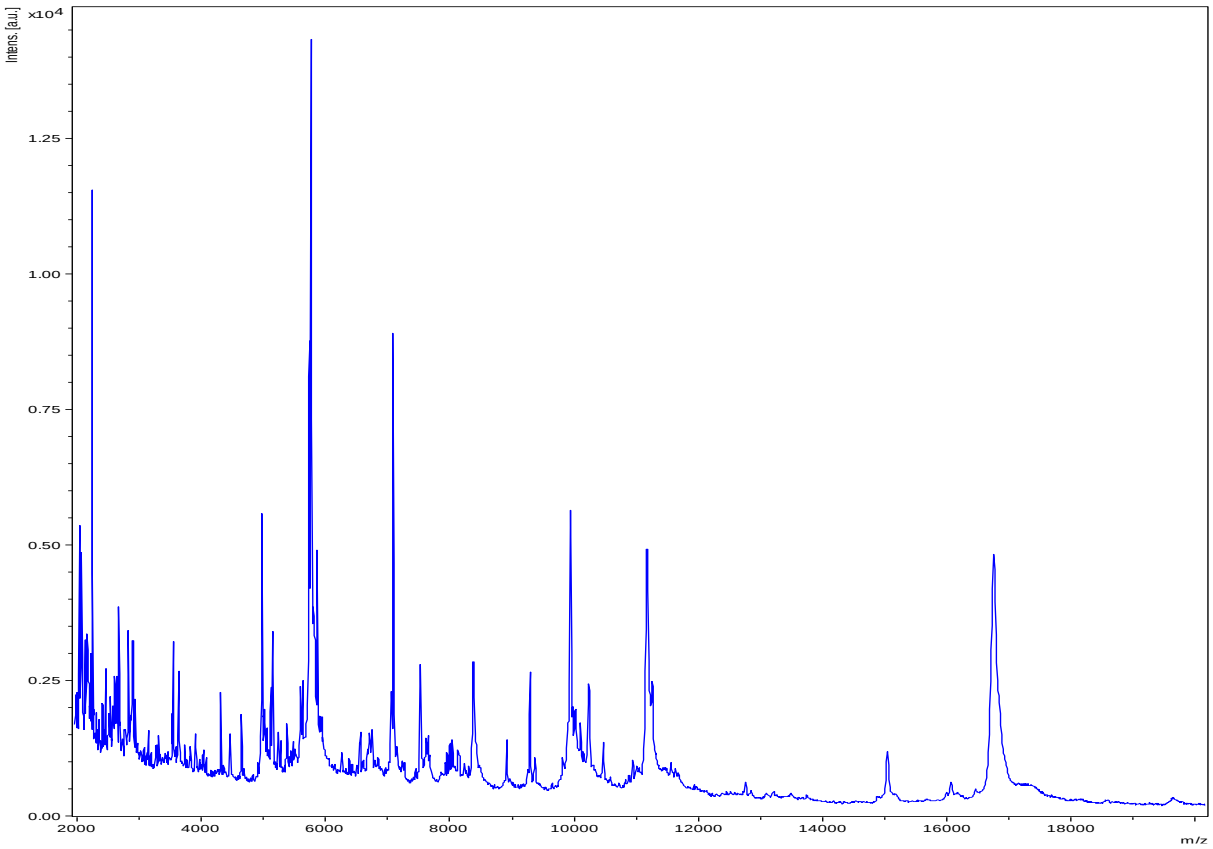

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290 **Figure 1:** MALDI-TOF MS reference spectrum of *Anaerococcus urinomassiliensis* sp. nov.

291 The reference spectrum was generated by comparison of spectra from 12 individual colonies.

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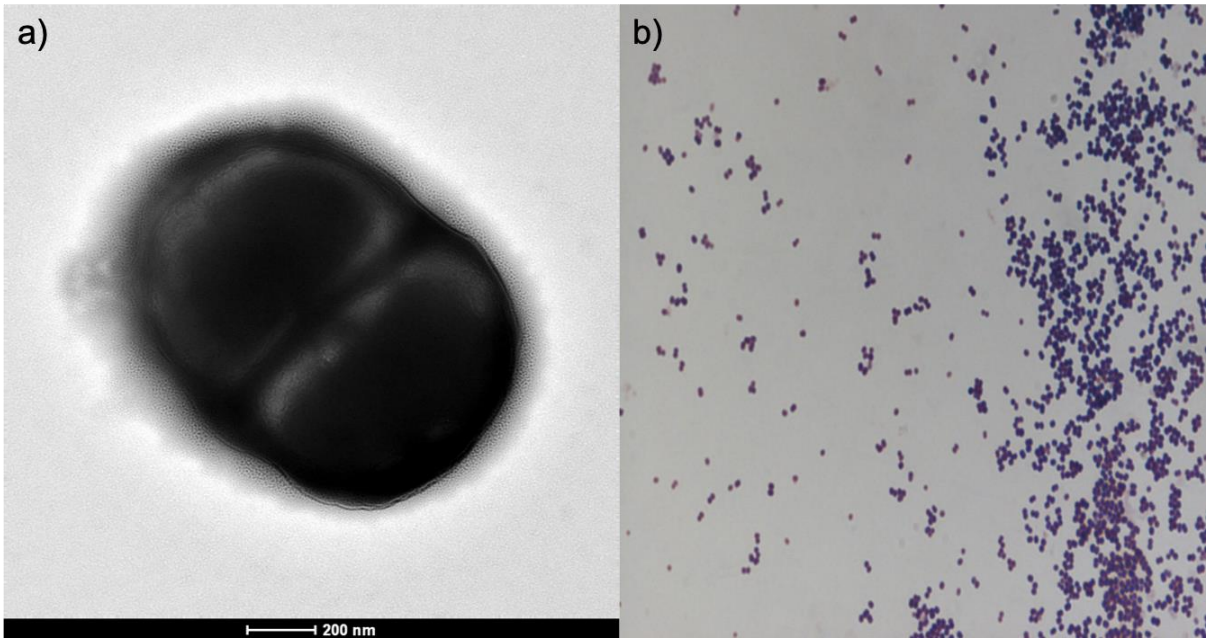

Figure 2: a) Scanning electron microscopy of stained *Anaerococcus urinomassiliensis* sp. nov. A colony was collected from agar and immersed into a 2.5 % glutaraldehyde fixative solution. Then a drop of the suspension was directly deposited on a poly-L-lysine coated microscope slide for 5 minutes and treated with 1 % phosphotungstic acid (PTA) aqueous solution (pH 2.0) for 2 minutes to increase SEM image contrast. The slide was gently washed in water; air dried and examined in a tabletop SEM (Hitachi TM4000) with approximately 60 centimeters in height and 33 cm in width to evaluate bacteria structure. Scales and acquisition settings are shown of figures b) Gram staining of *Anaerococcus urinomassiliensis sp.* sp. nov. strain Marseille-P2143^{T}.

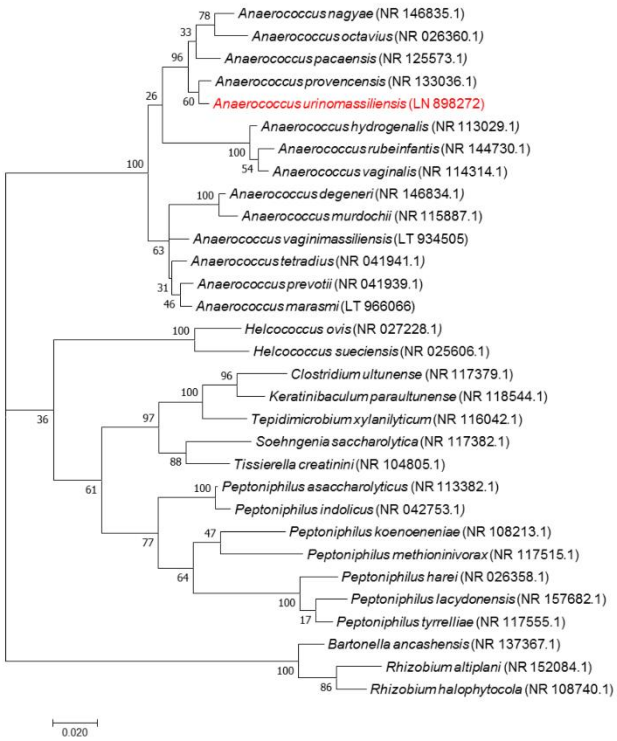

Figure 3: Phylogenetic tree highlighting the position of *Anaerococcus urinomassiliensis* sp. nov. with regard to others closely related species. Genbank accession numbers of 16S rRNA are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference were obtained using the Maximum likelihood method and the MEGA 7 software. Bootstrap values obtained by repeating the analysis 1,000 times to generate a majority consensus tree are indicated at the nodes. The scale bar indicates a 2 % nucleotide sequence divergence.

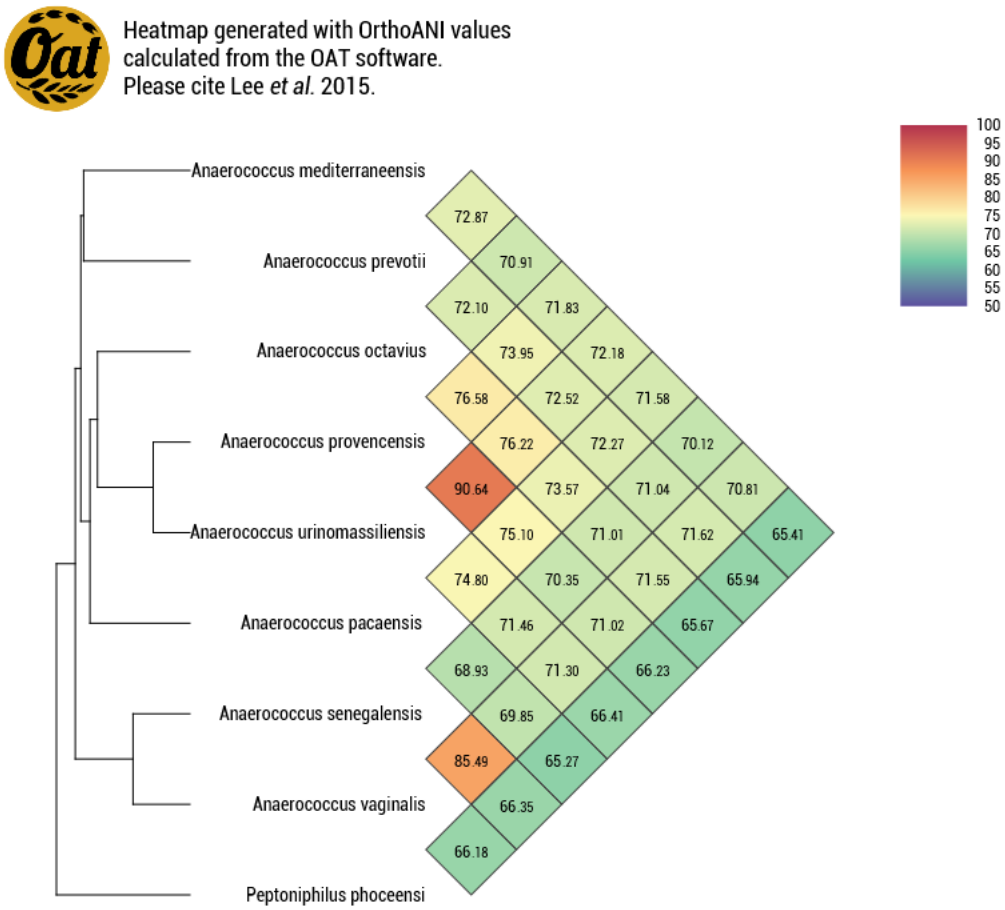

Figure 3: Heatmap generated with OrthoANI values calculated using the OAT software between *Anaerococcus urinomassiliensis* sp. nov. and other closely related species with standing in nomenclature.

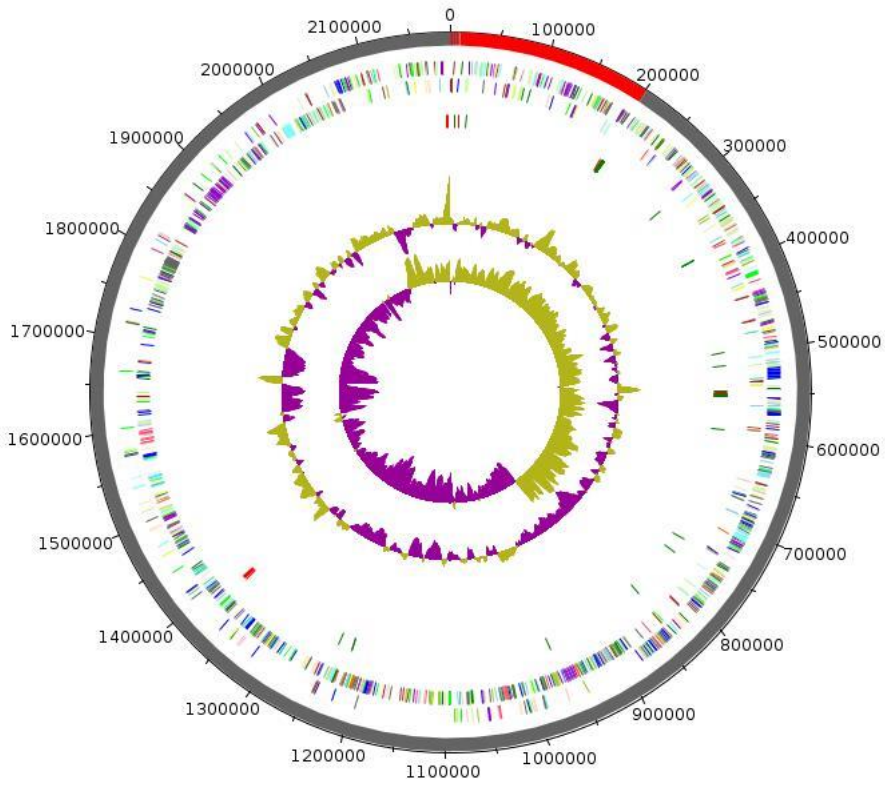

Figure 4: Graphical circular map of the chromosome. From outside to the center: Genes on the forward strand colored by COG categories (only genes assigned to COG), genes on the reverse strand colored by COG categories (only gene assigned to COG), RNA genes (tRNAs green, rRNAs red), GC content and GC skew

Table: Number of orthologous proteins shared between genomes (upper right) and AGIOS values (%) obtained (lower left)

	(Ah)	(At)	(Al)	(Au)	(Pl)	(Av)	(Ph)	(Pd)
A. hydrogenalis **(Ah)**	**2069**	946	905	887	651	977	673	681
*A. tetradius***(At)**	61.8	**2079**	1098	1021	733	1056	757	771
A. lactolyticus **(Al)**	56.98	59.26	**2253**	1038	763	1014	765	816
A.urinomassiliensis **(Au)**	61.06	63.78	62	**2021**	735	985	748	773
P. lacrimalis **(Pl)**	58.84	61.05	61.08	66.43	**1745**	726	917	889
A. vaginalis **(Av)**	62.66	62.20	62.63	62.08	61.98	**1764**	751	758
P. harei **(Ph)**	57.82	55.56	57.74	53.16	54.76	56.88	**1724**	900
P. duerdenii **(Pd)**	56.78	58.41	59.75	58.26	60.98	59.23	58.17	**1988**

342 **Table** :Cellular acid composition

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Fatty acids	Name	Mean relative % (a)
16:0	Hexadecanoic acid	56.8 ± 1.1
18:1n9	9-Octadecenoic acid	13.6 ± 0.4
18:2n6	9,12-Octadecadienoic acid	13.5 ± 0.6
14:0	Tetradecanoic acid	5.4 ± 0.4
18:0	Octadecanoic acid	4.7 ± 0.3
18:1n7	11-Octadecenoic acid	2.4 ± 1.0
15:0	Pentadecanoic acid	TR
16:1n7	9-Hexadecenoic acid	TR
17:0 anteiso	14-methyl-Hexadecanoic acid	TR
20:4n6	5,8,11,14-Eicosatetraenoic acid	TR
12:0	Dodecanoic acid	TR
17:0	Heptadecanoic acid	TR
10:0	Decanoic acid	TR

344 [a] Mean peak area percentage ; TR = trace amounts < 1 %

Table : Genome GC Percent and size comparaison

Species	Size (Mb)	G+C (%mol)	Total of genes
Anaerococcus vaginalis strain ATCC 51170	1.89	28.87	1764
Anaerococcus hydrogenalis strain DSM 7454	1.88	29.63	2069
Anaerococcus tetradius strain CCUG 46590	2.14	34.14	2079
Peptoniphilus duerdenii strain ATCC BAA-1640	2.12	34.23	1988
Anaerococcus urinomassiliensis strain Marseille-P2143	2.19	33.47	2021
Peptoniphilus lacrimalis strain CCUG 31350	1.84	30.21	1745
Anaerococcus lactolyticus strain CCUG 31351	2.20	34.93	2253
Peptoniphilus harei strain DSM 10020	1.83	34.43	1724

Article XI :

Actinomyces urinae sp. nov., isolated from 13-year-old girl affected by nephritic syndrome.

Aurélie MORAND, Florent CORNU, Michel TSIMARATOS, Jean-Christophe LAGIER, Saber KHELAIFIA and Didier RAOULT.

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Actinomyces urinae sp. nov., isolated from 13-year-old girl affected by nephritic syndrome

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Abstract

Here, we report the main characteristics of *Actinomyces urinae* strain Marseille-P2225^T (CSURP2225) isolated from a human urine sample.

Keywords: *Actinomyces urinae*, culturomics, genomics, taxono-genomics, taxonomy
Original Submission: 2 May 2016; **Revised Submission:** 13 May 2016; **Accepted:** 23 May 2016
Article published online: 26 May 2016

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A bacterial strain that could not be identified by our systematic matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) screening on a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) [1] was isolated in our search unit in 2015 using culturomics methodology [2,3] to study the human urinary microbiome. This strain was isolated from the urine sample of a 13-year-old girl affected by nephritic syndrome. The urine sample was collected in April 2015. The patient's relatives gave a signed informed consent and the study was validated by the ethics committee of the Institut Federatif de Recherche IFR48 under number 09-022.

Strain Marseille-P2225 initially grew after a 10-day incubation in an anaerobic blood culture (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 5 mL of 0.2-µm filtered rumen fluid. A pure culture of the strain Marseille-P2225 was then isolated after 48 h of incubation at 37°C spread on 5% sheep blood–Columbia agar medium (bioMérieux, Marcy l'Etoile, France) in an anaerobic atmosphere generated using the GENbag Anaer systems (bioMérieux). Strain Marseille-P2225 has translucent, beige micro-colonies with a mean diameter of 100 µm. Bacterial cells were Gram-positive, elongated bacillus-shaped, ranging in length from 400 to 600 nm and width from 200 to 400 nm. Strain Marseille-P2225 was catalase-negative and oxidase-negative. We used fD1-rP2 primers as previously described with a 3130-XL sequencer (Applied Biosciences, Saint Aubin, France) to sequence the 16S rRNA gene [4]. Strain Marseille-P2225 exhibited a 98.4% sequence identity with *Actinomyces europaeus* strain CCUG 32789A (GenBank Accession number NR114971) [5], the phylogenetically closest species with standing nomenclature (Fig. 1), which putatively classifies it as a member of the genus *Actinomyces* within the family *Actinomycetaceae* in the phylum *Actinobacteria*.

As the strain Marseille-P2225 exhibited a 16S rRNA sequence divergence >1.3% from its phylogenetically closest species with standing in nomenclature [6,7], we propose the creation of the new species *Actinomyces urinae* sp. nov., because this bacteria is close to other *Actinomyces* species [5] and was first described in a human urine sample. Strain Marseille-P2225^T is the type strain of the new species *Actinomyces urinae* sp. nov. (u.ri.na'e. L. N. gen. fem. *urinae*, of *urina*, the Latin name of urine).

MALDI-TOF spectrum. The spectrum of *Actinomyces urinae* strain Marseille-P2225^T is available at: http://mediterranee-infection.com/article.php?laref=256&titre=urms-database.

Nucleotide sequence accession number. The 16S rRNA gene sequence was deposited in GenBank under Accession number LN870295.

http://dx.doi.org/10.1016/j.nmni.2016.05.013

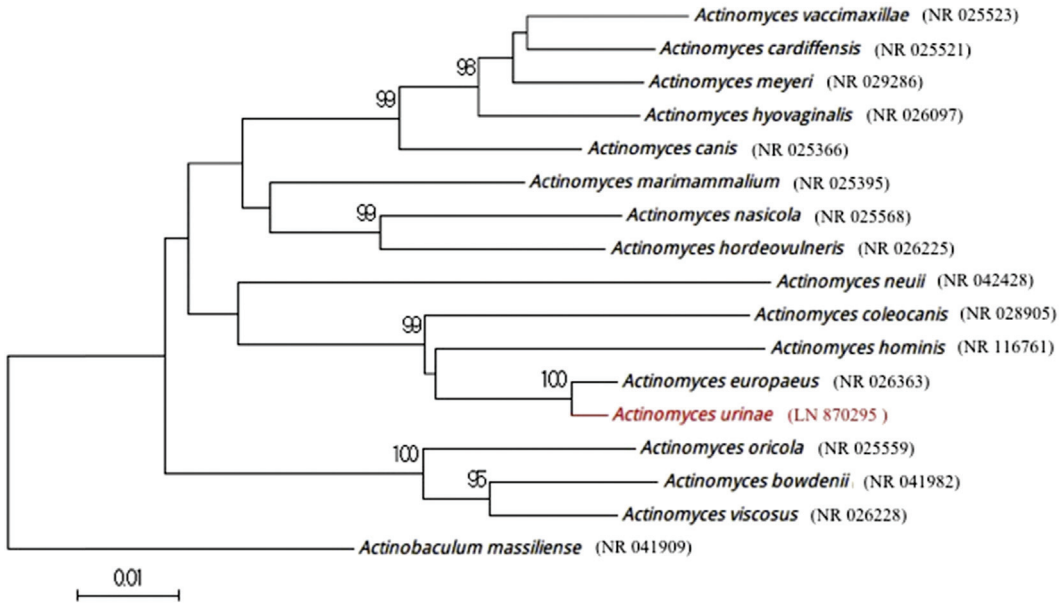

FIG. 1. Phylogenetic tree showing the position of *Actinomyces urinae* strain Marseille-P2225 relative to other phylogenetically close neighbours. Sequences were aligned using CLUSTAL W, and phylogenetic inferences were obtained using the maximum likelihood method within the MEGA software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 500 times to generate a majority consensus tree. Only the bootstraps score of at least 90% were retained. The scale bar indicates a 1% nucleotide sequence divergence.

Deposit in a culture collection. Strain Marseille-P2225 was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) under number P2225.

Funding

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Transparency Declaration

The authors have no conflicts of interest.

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Article XII :

Non-contiguous finished genome sequences and descriptions of Actinomyces ihuae, Actinomyces bouchesdurhonensis, Actinomyces urinae, Actinomyces marseillensis, Actinomyces mediterranea and Actinomyces oralis sp.nov. identified by Culturomics.

Maxime Descartes Maxime FONKOU, Morgane MAILHE, Sokhna NDONGO, Davide RICABONI, Aurélie MORAND, Florent CORNU, Marianne Tidjani ALOU, Melhem BILEN, Claudia ANDRIEU, Anthony LEVASSEUR, Frédéric CADORET and Didier RAOULT.

Publié dans le journal New Microbes New Infections

ORIGINAL ARTICLE

Noncontiguous finished genome sequences and descriptions of *Actinomyces ihuae, Actinomyces bouchesdurhonensis, Actinomyces urinae, Actinomyces marseillensis, Actinomyces mediterranea* and *Actinomyces oralis* sp. nov. identified by culturomics

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Abstract

The taxonogenomic approach, including the culturomics techniques, is now currently used to isolate and characterize new bacteria. These approaches notably allowed us to discover six new species of the *Actinomyces* genus: *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. Each is the type strain of the corresponding bacterial species. 16S ribosomal RNA gene sequence comparison was used to classify these strains among the *Actinomyces* genus. These strains are all Gram positive, rod shaped and facultative aerobic. We describe the main characteristics of each bacterium and present their complete genome sequence and annotation.

Keywords: Culturomics, Human gut microbiota, Human lung microbiota, New species, Taxonogenomics
Original Submission: 19 April 2018; **Revised Submission:** 5 June 2018; **Accepted:** 10 June 2018
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Introduction

Known bacteria of the genus *Actinomyces* are all Gram positive. The majority are facultative anaerobes, but some are strict anaerobes. Members of the genus *Actinomyces* have a high DNA G+C content [1]. The genus *Actinomyces* was first established in 1877 by Harz and colleagues as a member of the *Actinobacteria* phylum [2]. The first isolated species of this genus was *Actinomyces bovis* [3]. Today 47 species have been identified with validly published names with standing in nomenclature. The number of discovered *Actinomyces* species increased considerably from the 1980s, corresponding with the start of utilization of PCR (Fig. 1). This date was a turning point in the characterization of *Actinomyces* species, which now is not just only based on phenotypic observation but also on genetic analysis. Now, the development of quick and low-price genome sequencing and annotating allow us to go further in the characterization of bacterial species. *Actinomyces* species are known to be ubiquitous, colonizing soil, animals or humans. In humans, they are particularly present in the oral mucosa or urogenital and intestinal tracts [4]. Several of them, such as *Actinomyces israeli* [5–7], are present in the ground and can play an important role in the decomposition of organic matter and biotechnologic studies. However, bacteria of the genus *Actinomyces* are also part of the normal flora of the oral cavity and respiratory tract, and can be implicated in blood and organ infections [8,9].

In this study, we used a new approach, including matrix-assisted desorption ionization–time of flight mass spectrometry

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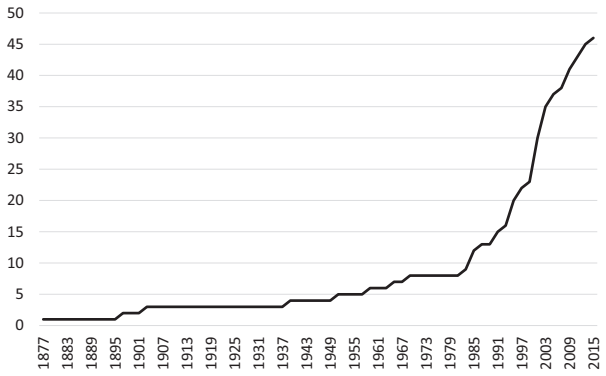

FIG. 1. Identification of *Actinomyces* new species since first one, *Actionmyces bovis,* was described by Harz and collaborators in 1877 [3].

(MALDI-TOF MS), phenotypic description and genome sequencing [10–14] to describe six new *Actinomyces* species: *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. These are all Gram positive, rod shaped and facultative aerobic. They were respectively isolated from sputum sample of healthy persons living in Marseille (France) (*A. marseillensis* and *A. oralis*), stool sample of an HIV-infected man (*A. ihuae*), urine sample of a girl with nephrotic syndrome (*A. urinae*), duodenum wash sample of a woman with oesophagitis (*A. mediterranea*) and stomach wash sample of a man with iron-deficiency anaemia (*A. bouchesdurhonensis*) (Table 1). These new bacterial species were part of a culturomics study which aimed to explore the diversity in the whole human microbiota using multiple culture conditions [15,16].

On the basis of the results of phenotypic, genomic and phylogenetic analyses, these strains are considered to represent new species of the *Actinomyces* genus. However, our study uses a new concept of bacterial description combining a proteomics analysis with the MALDI-TOF MS profile [17] associated with phenotypic and genomic descriptions of these six new species.

Here we present a summary of classification, main features and complete genomic sequencing and annotation of the present type strains of these six *Actinomyces* new species: *Actinomyces ihuae* strain SD1 (= CSUR P2006 = DSM 100538), *Actinomyces bouchesdurhonensis* strain Marseille-P2825 (= CSUR P2825 = DSM 103075), *Actinomyces urinae* strain Marseille-P2225 (= CSUR P2225 = DSM 100700), *Actinomyces marseillensis* strain Marseille-P2818 (= CSUR P2818 = CCUG 71898), *Actinomyces mediterranea* strain Marseille-P3257 (= CSUR P3257 = CCUG 70143) and *Actinomyces oralis* strain Marseille-P3109 (= CSUR P3109 = DSM 103942). These characteristics support the creation of these six new species.

TABLE 1. Sample information

Characteristic	A	B	C	D	E	F
Sample origin Patient information	Human stomach wash 60-year-old man with iron-deficiency anaemia	Human stool 50-year-old HIV-infected man	Human sputum Healthy Marseille woman	Human duodenum wash 76-year-old woman with oesophagitis	Human sputum Healthy Marseille man	Human urine 13-year-old girl with nephritic syndrome
Authorization/consent	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille) consent of girl's parents
Storage Isolation conditions	−80°C COS medium day 3 anaerobic 37°C	−80°C COS medium day 2 microaerobic 37°C	−80°C Haemoculture + rumen day 30 anaerobic 30°C	−80°C Haemoculture + rumen + sang day 7 anaerobic 37°C	−80°C Haemoculture + rumen day 15 aerobic 37°C	−80°C Haemoculture + rumen day 10 anaerobic 37°C

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^{T}; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.

Materials and methods

Strain identification and phylogenetic analysis. Culturomics methodology, previously described by Lagier *et al.* [18], doubles the number of bacteria species isolated at least once from the human gut [16]. We used this methodology to isolate these strains from several diverse human samples. Samples, conditions of isolation and origins are summarized in Table 1. All patients provided informed consent, and the study was validated by the ethics committee of the Institut Fédératif de Recherche 48, Faculty of Medicine, Marseille, France, under agreement 09-022.

Purified colonies were identified by MALDI-TOF MS using a Microflex LT spectrometer and a MSP 96 MALDI-TOF target plate (Bruker Daltonics, Bremen, Germany), as previously described [17]. The obtained spectra were imported into MALDI Biotyper 3.0 software (Bruker) and analysed by standard pattern matching (with default parameter settings) against the main spectra of the 7537 bacteria included in the databases (constantly updated Bruker and Unité des Maladies Infectieuses et Tropicales Emergentes (URMITE) databases). The resulting score enabled the identification (or not) of tested species: a score of $\geq$2 with a validly published species enabled identification at the species level; a score of $\geq$1.7 but <2 enabled identification at the genus level; and a score of <1.7 did not enable any identification. Any significant score has been obtained for our six strains, suggesting that the isolates were not members of known species.

We thus realized sequencing of 16S rRNA genes in order to identify these strains. DNA was previously extracted by EZ1 DNA Tissue Kit using BioRobot EZ1 Advanced XL (Qiagen, Courtaboeuf, France). The amplification and purification of the 16S rRNA gene was done as previously described by using the universal primer pair fD1 and rP2 (Eurogentec, Angers, France). Sequencing was then done using the Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA), as previously described [19]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com), and BLASTn searches were performed against the GenBank National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi) to determine the percentage of similarity with the closest bacteria. A similarity threshold <98.7% allows the definition of a new species, whereas a threshold <95% allows the definition of a new genus without performing DNA-DNA hybridization [20]. A custom Python script was used to automatically retrieve all species from the same family of the new species and to download 16S sequences from NCBI by parsing NCBI eUtils results and the NCBI taxonomy page, which only keeps sequences from type strains. In cases of multiple sequences for one type strain, it selects the sequence obtaining the best identity rate from the BLASTn alignment with our sequence. The script then separates 16S gene sequences in two groups: one containing the sequences of strains from the same genus (group A) and one containing the others (group B). It finally only keeps the 48 closest strains from group A and the closest three strains from group B. Different species are selected because they are the closest species of each five studied strains.

All the spectra were integrated into the URMITE database (http://www.mediterranee-infection.com/article.php?laref=256&titre=urms-database). We compared the proteomic profiles between our strains and their closest species.

Phenotypic features. Optimal growth conditions of our strains were determined by testing five growth temperatures (20, 25, 30, 37 and 45°C) in an aerobic atmosphere with or without 5% CO_2 and under anaerobic and microaerophilic conditions using the GENbag anaer and GENbag microaer systems, respectively (bioMerieux, Marcy l'Etoile, France). Phenotypic characteristics such as Gram staining, motility, sporulation, and catalase and oxidase activities were tested as previously described [14]. Negative staining was done in order to observe cell morphology. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1 hour at 4°C. A drop of cell suspension was deposited for approximately 5 minutes on glow-discharged formvar carbon film on 400 mesh nickel grids (FCF400-Ni; Electron Microscopy Sciences (EMS), Hatfield, PA, USA). The grids were dried on blotting paper, and the cells were negatively stained for 10 seconds with 1% ammonium molybdate solution in filtered water at room temperature. Electron micrographs were acquired with a Tecnai G20 Cryo (FEI Company, Limeil-Brévannes, France) transmission electron microscope operated at 200 keV.

Biochemical analysis of SD1, Marseille-P2825, Marseille-P2225, Marseille-P2818, Marseille-P3257 and Marseille-P3109 was carried out using API 50CH, API 20A and API ZYM strips according to manufacturer's instructions (bioMérieux). Table 2 lists data of our six new species compared to published data of type strains of close species: *Actinomyces oricola* strain CIP 107639 [21], *A. gerencseriae* strain CIP 105418 [22], *A. naeslundii* strain CIP 103128 [22,23], *A. timonensis* strain 7400942 [24] and *A. massiliensis* strain 4401292 [25].

Cellular fatty acid methyl ester (FAME) analysis was performed by gas chromatography/mass spectrometry (GC/MS). FAMEs were prepared as described by Sasser [26]. GC/MS analyses were carried out as previously described [27]. Briefly, FAMEs were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500-SQ 8 S; PerkinElmer, Courtaboeuf, France). A spectral database search was

TABLE 2. Comparison of phenotypic characteristics

Characteristic	A	B	C	D	E	F	G	H	I	J	K
Optimal temperature	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C
Atmosphere	Anaerobic	Aerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
pH range	5.0–8.0	5.0–8.0	6.0–8.5	5.0–8.0	6.0–8.5	6.0–7.5	NA	5.7 - 6.6	NA	NA	NA
Colony aspect	Smooth and grey	Bright grey	Smooth and shiny	Smooth and white colour	Smooth and grey	Translucent, beige micro colonies	Pin point, breadcrumb like, white and nonhaemolytic	Circular, peaked to pulvinate, lumpy, opaque and white	NA	Circular, white, dry, embedded in the agar and pin point	Circular, white, shiny and pinpoint
Cell shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Cell size (μm)	1.5–1.9	0.7–1	2.0–2.2	2.3–2.6	1.6–1.8	0.4–0.6	NA	NA	NA	1.0–3.2	0.5–1.7
Cell diameter (μm)	0.5–0.6	0.5–0.7	0.4–0.5	0.5–0.6	0.6–0.7	0.2–0.4	NA	NA	NA	0.3–0.5	0.35–0.74
Gram stain	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Salt tolerance (g.L-1)	<10%	<10%	5	<10%	5	10–15%	NA	NA	NA	NA	NA
Motility	No	No	No	No	No	No	No	No	No	NA	NA
Endospore formation	No	No	No	No	No	No	No	No	No	No	No
Major cellular fatty acid	18:1n9	16:0	18:1n9	18:1n9	16:0	16:0	16:0	NA	NA	NA	18:1v9c
Production of:											
Alkaline phosphatase	−	−	−	−	−	−	−	−	−	−	−
Catalase	−	−	−	−	−	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−	NA	−	NA	−	−
Nitrate reductase	+	−	+	−	+	−	−	+	+	−	+
Urease	−	−	+	+	+	+	−	−	−	−	−
β-Galactosidase	+	+	+	+	−	−	−	+	+	−	+
N-Acetyl-glucosamine	+	−	+	−	+	+	−	−	−	−	−
Acid from:											
L-Arabinose	−	−	−	+	−	+	−	−	−	−	−
Ribose	−	−	+	+	+	+	−	−	−	w/+	−/w
Mannose	−	+	+	+	+	+	−	−/+	+	w/+	−/w
Mannitol	−	−	−	−	−	+	−	−	−	+	−
D-Saccharose	+	+	+	+	+	+	NA		NA	NA	NA
D-Glucose	+	−	+	+	+	+	−	−/+	+	+	+
D-Fructose	+	−	+	+	+	+	−	−	+	+	+
D-Maltose	+	−	+	−	+	+	−	−/+	+	+	+
D-Lactose	+	+	−	+	+	+	−	+	−	+	+
Habitat	Human stomach	Human gut	Human lung	Human duodenum	Human lung	Human bladder	Human dental abscess	Human parotid abscess	Human sinus	Human clinical osteo-articular	Human blood

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^{T}; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225; G, *A. oricola* strain CIP 107639T; H, *A. gerencseriae* strain CIP 105418T; I, *A. naeslundii* strain CIP 103128T; J, *A. timonensis* strain 7400942T; K, *A. massiliensis* strain 4401292T.
+, positive result; −, negative result; w, weakly positive result; NA, data not available.

performed using MS Search 2.0 operated with the Standard Reference Database 1A (National Institute of Standards and Technology, Gaithersburg, MD, USA) and the FAME mass spectral database (Wiley, Chichester, UK). Antibiotic susceptibility was tested using the disc diffusion method [28] and according to European Committee on Antimicrobial Susceptibility Testing 2015 recommendations.

Genome description and comparison. Genomic DNA (gDNA) of *Actinomyces urinae, Actinomyces mediterranea, Actinomyces oralis* and *Actinomyces marseillensis* were first extracted by a mechanical treatment by acid-washed glass beads (G4649-500g; Sigma, St Louis, MO, USA) using a FastPrep BIO 101 instrument (Qbiogene, Strasbourg, France) at maximum speed (6.5) for 3 × 30 seconds. Then, for all the *Actinomyces* strains, successive pretreatments by a lysozyme incubation at 37°C for 2 hours (3 hours for *Actinomyces oralis*) were done, followed by proteinase K for 3 hours for *Actinomyces ihuae* only. gDNA was then extracted on the EZ1 biorobot (Qiagen) with the EZ1 DNA tissues kit. The elution volume was 50 µL. gDNA was quantified by a Qubit assay with a high-sensitivity kit (Life Technologies, Carlsbad, CA, USA) (Supplementary Table S1).

The genome's assembly was performed with a pipeline that enabled us to create an assembly with different software (Velvet [29], Spades [30] and Soap Denovo [31]) on untrimmed data. gDNA was sequenced by MiSeq Technology (Illumina, San Diego, CA, USA) with the mate-pair strategy for all the *Actinomyces* strains, and paired end for *Actinomyces urinae, Actinomyces mediterrane* and *Actinomyces oralis.* The gDNA was barcoded in order to be mixed with 11 other projects with the Nextera Mate Pair sample prep kit (Illumina).

The mate-pair library was prepared with 1.5 µg of gDNA using the Nextera mate-pair Illumina guide. The gDNA sample was simultaneously fragmented and tagged with a mate-pair junction adapter. The pattern of the fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with a DNA 7500 labchip. The DNA fragments ranged in size from 1 to 11 kb (with an optimal size at 2.458, 2.458, 6.284, 5.830, 9.683 and 6.518kb for *A. urinae, A. mediterranea, A. oralis, A. ihuae, A. bouchedurhonensis* and *A. marseillensis,* respectively). No size selection was performed, and 600 ng (for *A. marseillensis and A. ihuae*), 284.7 ng (for *A. bouchedurhonensis*), 96.1 ng (for *Actinomyces urinae*), 112 ng (for *A. mediterranea*) and 250.7 ng (for *Actinomyces oralis*) of tagmented fragments were circularized. The circularized DNA was mechanically sheared to small fragments, with an optimum at 1163, 1041, 406, 736, 1033 and 949 bp for *A. urinae, A. mediterranea, A. oralis, A. ihuae, A. bouchedurhonensis* and *A. marseillensis,* respectively, on the Covaris device S2 in T6 tubes (microtubes for *A. oralis* and *A. marseillensis*) (Covaris, Woburn, MA, USA). The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies), and the final concentration library was measured (Supplementary Table S1). The libraries were normalized at 2 nM (4 nM for *A. ihuae*) and pooled. After a denaturation step and dilution at 15 pM, the pool of libraries was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and a sequencing run were performed in a single 39-hour run in a 2 × 251 bp read length (2 × 250 bp read length for *A. oralis, A. marseillensis* and *A. marseillensis*). The paired reads were finally trimmed and assembled. For each assembly performed, GapCloser [31] was used to reduce gaps. Complementary information is provided in Supplementary Table S1.

To prepare the paired end library, dilution was performed to require 1 ng of each genome as input. The tagmentation step fragmented and tagged the DNA. Then limited-cycle PCR amplification (12 cycles) completed the tag adapters and introduced dual-index barcodes. After purification on AMPure XP beads (Beckman Coulter, Brea, CA, USA), the libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina). Normalized libraries were pooled for sequencing on the MiSeq device. Automated cluster generation and paired-end sequencing with dual index reads were performed in a single 39-hour run in 2 × 250 bp read length. Complementary information is available in Supplementary Table S1.

Open reading frames (ORFs) were predicted using Prodigal [32] with default parameters, but the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank [33] and the Clusters of Orthologous Groups database (COGs) using BLASTP (*E* value 1e-03, coverage 0.7 and identity percentage of 30%). If no hit was found, it was searched against the NR database using BLASTP with an *E* value of 1e-03, coverage of 0.7 and an identity percentage of 30%, and if the sequence length was smaller than 80 aa, we used an *E* value of 1e-05. The tRNAScanSE tool [34] was used to find tRNA genes, while ribosomal RNAs were found using RNAmmer [35]. Lipoprotein signal peptides and the number of transmembrane helices were predicted using Phobius [36]. Mobile genetic elements were predicted using PHAST [37] and RAST [38]. ORFans were identified if all the BLASTP performed did not give positive results (*E* value smaller than 1e-03 for ORFs with sequence size larger than 80 aa or *E* value smaller than 1e-05 for ORFs with sequence length smaller than 80 aa). Such parameter thresholds have already been used in previous studies to define ORFans. Artemis [39] and DNA Plotter [40] were used for data management and the visualization of genomic features, respectively. The Mauve alignment tool

(version 2.3.1) was used for multiple genomic sequence alignment [41]. PFAM-A conserved domains were searched on each protein with the HMMscan of the HMMER3 suite [42]. PKS and NRPS were searched against the ClusterMine360 [43] database. Resistome was analysed by using the ARG-ANNOT database [44]. The closest species for genomic comparison were identified in the 16S RNA tree using Phylopattern software [45]. For each selected genome, the complete genome sequence, proteome genome sequence and ORFeome genome sequence were retrieved from the FTP of NCBI.

An annotation of the entire proteome was performed to define the distribution of functional classes of predicted genes according to the COGs of proteins (using the same method as for the genome annotation). Annotation and comparison processes were performed in the multiagent software system DAGOBAH [46], which includes Figenix [47] libraries that provide pipeline analysis. To evaluate the genomic similarity between studied genomes, we determined two parameters, digital DNA-DNA hybridization (dDDH), which exhibits a high correlation with DNA-DNA hybridization (DDH) [20,48], and average genomic identity of orthologous gene sequences (AGIOS), which was designed to be independent from DDH. The AGIOS score is the mean value of nucleotide similarity between all couples of orthologous proteins between the two studied genomes [49].

Results

Strain identification and phylogenetic analysis

A phylogenetic tree of our strains is provided in Fig. 2. Strain SD1 (accession no. LN866997) exhibited a 98.6% 16S rRNA gene sequence identity with *Actinomyces radingae* strain APL1 (accession no. NR_026169), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces ihuae* strain SD1 (= CSUR P2006 = DSM 100538). Strain Marseille-P2825 (accession no. LT576385) revealed a 98.33% sequence similarity with the 16S rRNA of *Actinomyces odontolyticus* strain JCM 14871 (accession no. AJ234040), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces bouchesdurhonensis* strain Marseille-P2825 (= CSUR P2825 = DSM 103075). Strain Marseille-P2225 (accession no. LN870295) revealed an exhibited 98.45% sequence identity with *Actinomyces europaeus* strain CCUG 32789A (accession no. NR_026363), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces urinae* strain Marseille-P2225 (= CSUR P2225 = DSM 100700). Strain Marseille-P2818 (accession no. LT576400) exhibited a 98.1% 16S rRNA gene sequence identity with *Actinomyces odontolyticus* strain JCM 14871 (accession no. AJ234040), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces marseillensis* strain Marseille-P2818 (= CSUR P2818 = CCUG 71898). Furthermore, *Actinomyces bouchesdurhonensis* strain Marseille-P2825 and *Actinomyces marseillensis* strain Marseille-P2818 showed only 97.55% of 16S gene sequence identity, indicating that there are two distinct species of *Actinomyces odontolyticus.* Strain Marseille-P3257 (accession no. LT598588) exhibited 93.94% 16S rRNA gene sequence identity with *Actinomyces hyovaginalis* strain BM 1192/5 (accession no. X69616), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces mediterranea* strain Marseille-P3257 (= CSUR P3257 = CCUG 70143). Strain Marseille-P3109 (accession no. LT627670) revealed a 98.5% of sequence similarity with the 16S rRNA of *Actinomyces naeslundii* strain JCM 8349 (accession no. NR_113326), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces,* for which we suggest the name *Actinomyces oralis* strain Marseille-P3109 (= CSUR P3109 = DSM 103942).

The analysis of the gel view shows that all the profiles of our studied strains have similar general characteristics with the other *Actinomyces* species used for the comparison (Fig. 3). Furthermore, the outsider species *Actinobaculum urinale* strain DSM 15805 profile shows several unique differences.

Phenotypic features

The main phenotypic results of each studied strains are presented in Table 2. Results show that these six new species are all Gram positive and rod shaped. These observations have been permitted by electronic microscopy (Fig. 4), which reveals similar morphologies characteristic of the *Actinomyces* genus.

The cellular fatty acid composition of our strains is provided in Table 3, and the antibiotic analyses are presented in Table 4. As Table 3 indicates, 9-octadecenoic acid and hexadecanoic acid were the two most abundant cellular fatty acid of all the bacteria. 9-Octadecenoic acid was the most abundant for the strains Marseille-P2825, Marseille-P2818 and Marseille-P3257; and hexadecanoic acid was the second most abundant. Hexadecanoic acid was the most abundant for the strains Marseille-P2225, SD1 and Marseille-P3109; and 9-octadecenoic acid was

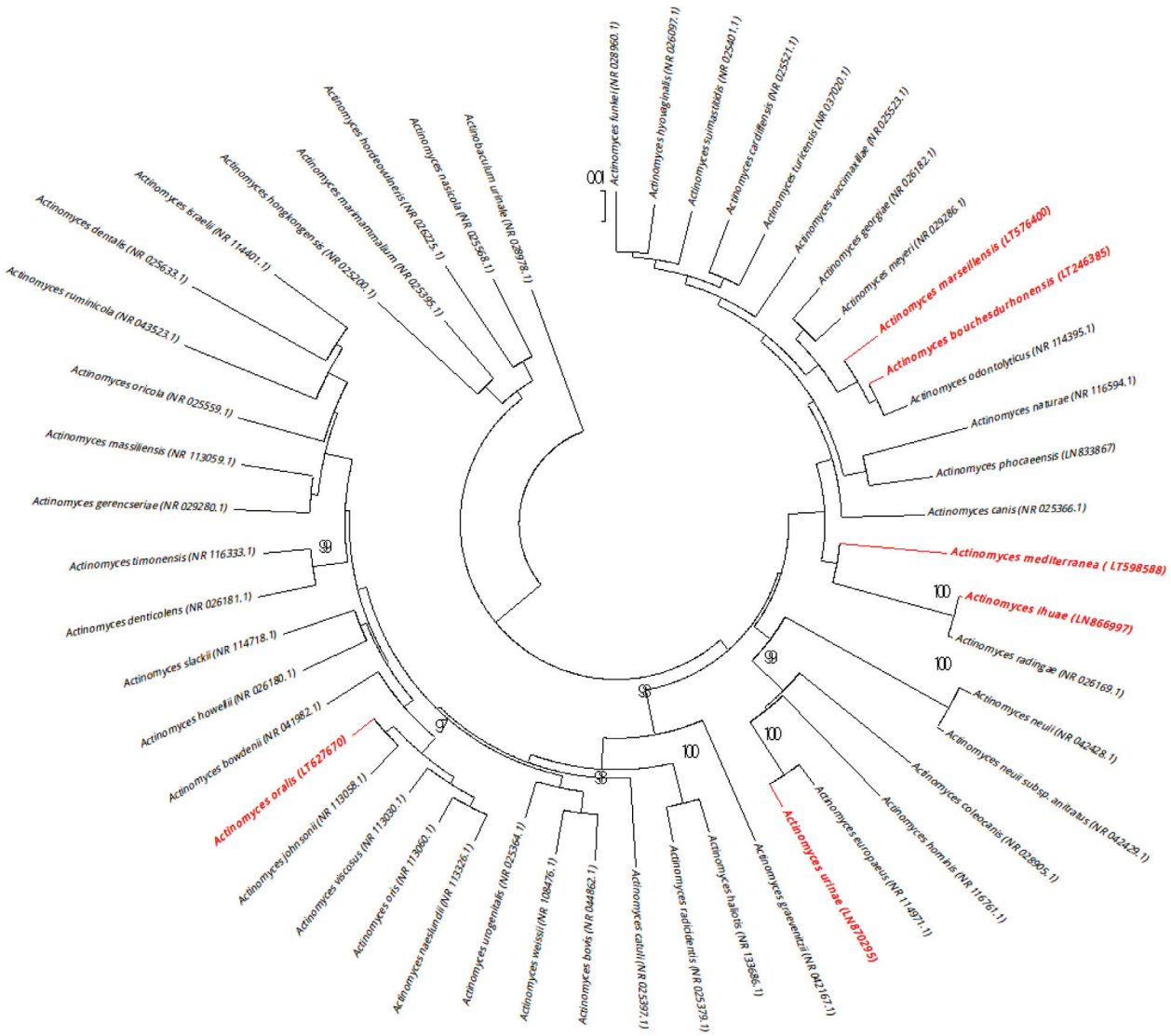

FIG. 2. Phylogenetic tree highlighting position of *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 relative to other type strains within *Actinomyces* genus. Strains and their corresponding GenBank accession numbers for 16S rRNA genes sequences are indicated in brackets. Sequences were aligned using CLUSTALW (http://www.clustal.org/clustal2/), and phylogenetic inferences were obtained using maximum-likelihood method within MEGA 6 (http://www.megasoftware.net/mega.php). Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. *Actinobaculum urinale* (NR 028978.1) was used as outgroup. Scale bar = 1% nucleotide sequence divergence.

the second most abundant. Table 4 shows the results of antibiotic susceptibility tests. The strains were all susceptible to amoxicillin 25 µg, rifampicin 30 µg and gentamicin 500 µg and were resistant to colistin 50 µg and metronidazole 4 µg. These results support the notion that these strains are all members of the same *Actinomyces* genus.

Genome description and comparison

Maps of genomes of our different strains are presented in Supplementary Fig. S1. The properties and statistics of the genomes are summarized in Table 5, and the distribution of predicted genes of our strains according to COGs categories are shown in Table 6. We can observe that for all the 25 general COGs functional categories, values of our six new *Actinomyces* species are in the same range. Genomic characteristics of our strains are compared to those of closely related species with an available genome in Table 7. Although the genome *A. urinae* is smaller than the other species, it has the same GC percentage range, between 49.60% and 72.58%, as the other characterized genomes of known *Actinomyces* species

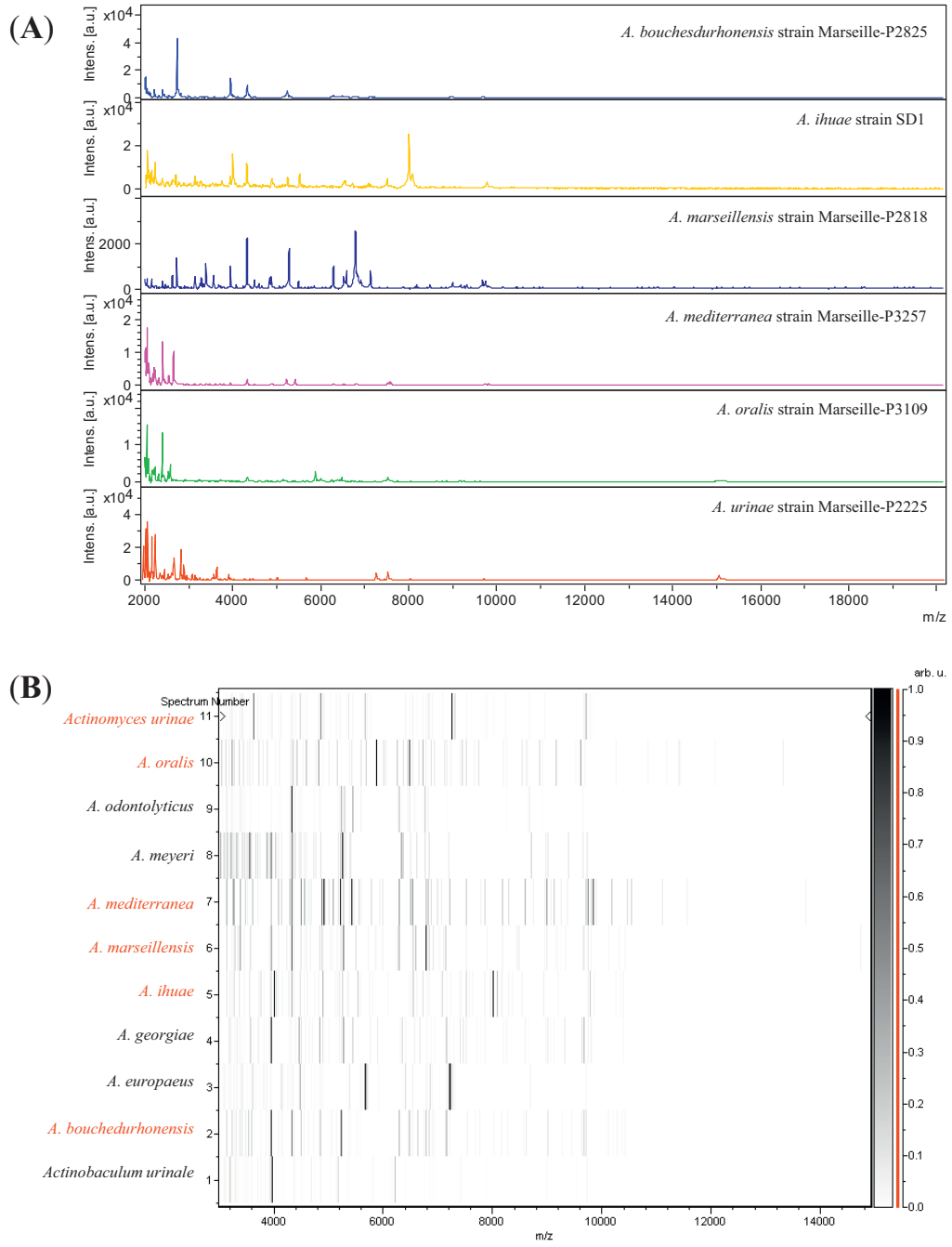

FIG. 3. Reference mass spectra from *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. Spectra from 12 individual colonies were compared and each reference spectrum generated (A). Gel view comparing *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 to other species within genus *Actinomyces*. Gel view displays raw spectra of loaded spectrum files arranged in pseudo-gel–like look; *x*-axis indicates *m*/*z* value and left *y*-axis running spectrum number originating from subsequent spectra loading. Peak intensity expressed by greyscale scheme code. Colour bar and right *y*-axis indicate relation between colour peak, with peak intensity in arbitrary units. Displayed species are indicated at left (B).

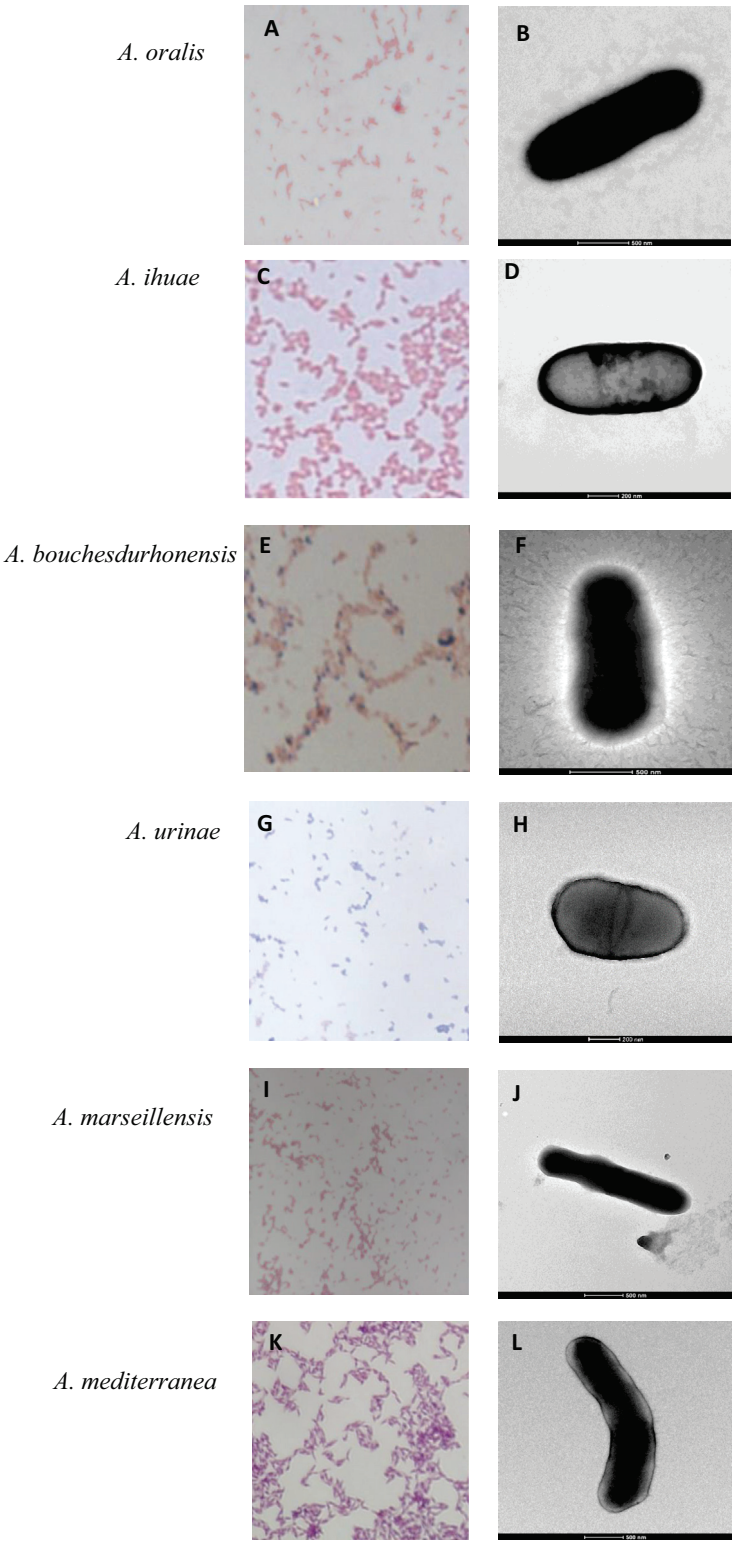

FIG. 4. Gram staining and electron micrographs, respectively, of *Actinomyces oralis* strain Marseille-P3109 (A, B), *Actinomyces ihuae* strain SD1 (C, D), *Actinomyces bouchesdurhonensis* strain Marseille-P2825 (E, F), *Actinomyces urinae* strain Marseille-P2225 (G, H), *Actinomyces marseillensis* strain Marseille-P2818 (I, J) and *Actinomyces mediterranea* strain Marseille-P3257 (K, L).

TABLE 3. Cellular fatty acid composition (in %[a])

Fatty acid	IUPAC name	A	B	C	D	E	F
12:0	Dodecanoic acid	1.0 ± 0.2	TR	TR	TR	1.6 ± 0.3	TR
14:0	Tetradecanoic acid	8.4 ± 1.5	1.2 ± 0.1	3.8 ± 0.3	2.3 ± 0.3	3.9 ± 0.2	1.9 ± 0.1
16:0	Hexadecanoic acid	30.1 ± 1.9	32.3 ± 0.1	27.9 ± 3.5	32.2 ± 0.5	55.0 ± 0.2	44.9 ± 0.3
16:1n7	9-Hexadecenoic acid	TR	TR	No	TR	2.1 ± 0.2	No
17:0	Heptadecanoic acid	TR	1.2 ± 0.1	TR	TR	TR	TR
18:0	Octadecanoic acid	14.3 ± 2.5	18.0 ± 1.2	13.2 ± 0.6	20.1 ± 0.3	3.7 ± 0.5	18.1 ± 0.8
18:1n5	13-Octadecenoic acid	No	No	No	TR	No	2.5 ± 0.1
18:1n6	12-Octadecenoic acid	No	No	1.1 ± 0.1	No	No	No
18:1n7	11-Octadecenoic acid	3.0 ± 0.2	3.1 ± 0.3	1.4 ± 0.2	1.4 ± 0.1	No	1.5 ± 0.2
18:1n9	9-Octadecenoic acid	32.4 ± 1.7	23.8 ± 0.6	44.9 ± 3.8	35.1 ± 1.0	31.4 ± 0.2	21.4 ± 0.8
18:2n6	9,12-Octadecadienoic acid	8.7 ± 0.4	16.8 ± 0.3	5.8 ± 1.0	6.8 ± 0.1	2.1 ± 0.3	7.8 ± 0.3

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
TR, trace amounts <1%.
[a]Mean peak area percentage ± standard deviation.

[50,51]. Furthermore, dDDH values (Table 8) are less than 70% and confirm that all the studied species are distinct species [20,48]. These results are supported by AGIOS values (Supplementary Table S2), which demonstrated that comparison between our strains and other species within *Actinomyces* genus resulted in a similar range of values compared to the comparison between the same species except our strains, thus confirming their new species status.

Conclusion

In this study, we used the polyphasic approach developed in our laboratory to describe six new species of the *Actinomyces* genus. This concept is based on genome sequences, MALDI-TOF MS identification and main phenotypic characteristics of the studied new species. As previously observed, the presented strains, which have been isolated from diverse origin, possess close morphologic properties. Their cellular fatty acid composition and their profile of resistance to antibiotics support that these five strains belong to the same genus. Their 16S rRNA gene sequencing, supported by genome analysis compared to other characterized strains of the *Actinomyces* genus, indicated that *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 are all members of the *Actinomyces* genus.

Description of *'Actinomyces ihuae'* sp. nov.

Cells are Gram positive and rod shaped, with a length of 0.7 to 1 µm and a width of 0.5 to 0.7 µm. This strain exhibited no catalase or oxidase activity. *'Actinomyces ihuae'* is nonmotile and non–spore forming. Colonies are bright grey, with a diameter of 1 to 2 mm. Optimum growth occurs at 37°C in an aerobic

TABLE 4. Antibiotic resistance tests

	A		B		C		D		E		F	
Characteristic	IMC	Status	IMC	Status	IMC	Status	IMC	Status	IMC	Status	IMC	Status
Gentamicin 15 µg	3	R	9	R	11.1	I	7	R	24.6	S	0	R
Gentamicin 500 µg	16	I	25	S	30.6	S	35	S	34.3	S	21.5	S
Amoxicillin/clavulanic acid 30 µg	20	I	26	S	30.0	S	30	S	41.9	S	9.8	R
Ceftriaxone 30 µg	21	R	20	R	14.4	R	>30	S	40.4	S	19.1	R
Colistin 50 µg	0	R	0	R	0	R	0	R	0	R	0	R
Penicillin G 10 IU	21	I	27	I	20.2	I	28	I	33.9	S	16.1	R
Imipenem 10 µg	26	S	30	S	31.1	S	>30	S	22.6	I	22.4	I
Trimethoprim/ sulfamethoxazole 25 µg	6	R	5	R	30.7	S	11	I	0	R	0	R
Clindamycin 15 µg	30	S	24.1	S	23.9	S	25	S	7.4	R	10.9	R
Metronidazole 4 µg	6	R	10	R	19.8	R	5	R	6.5	R	0	R
Tobramycin 10 µg	7	R	10.9	R	23.7	S	12	R	0	R	0	R
Rifampicin 30 µg	25	S	28	S	19.4	S	>30	S	40.0	S	14.4	I
Fosfomycin 50 µg	7	R	12	R	6.5	R	>30	S	13.5	R	9.4	R
Amoxicillin 25 µg	22	I	29.4	S	31.5	S	>30	S	36.3	S	27.0	S
Vancomycin 30 µg	13	R	20	S	27.6	S	22	S	32.4	S	17.2	S
Doxycycline 30 IU	3	R	29.2	S	28.3	S	>30	S	8.9	R	27.2	S
Erythromycin 15 IU	24	S	10	R	30.6	S	>30	S	19.4	I	9.4	R

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
I, indeterminate; R, resistant; S, susceptible; IMC, inhibitory minimal concentration.

TABLE 5. Nucleotide content and gene counts levels of genome

Characteristic	A		B		C		D		E		F	
Scaffolds; contigs	31; 114		5; 5		2; 18		4; 4		8; 8		3; 4	
	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]
Size (bp)	2 304 249	100.0	2 458 350	100.0	2 007 553	100.0	2 395 621	100.0	3 149 233	100	1 946 897	100.0
G+C content (%)	1 436 338	63.3	1 447 601	58.9	1 131 738	57.1	1 528 162	63.8	2 154 177	68.4	1 092 830	56.1
Coding region (bp)	1 612 522	70.0	2 240 376	91.1	1692	100.0	2 151 624	89.8	2 750 321	87.3	1 787 920	91.8
Total genes	1821	100.0	2.200	100.0	1754	100.0	2133	100.0	2639	100.0	1756	100.0
Protein-coding genes	1766	100.0	2135	100.0	1692	100.0	2081	100.0	2579	100.0	1701	100.0
RNA genes	55	3.0	65	3.0	62	3.5	52	2.4	60	2.3	55	3.1
Proteins with function prediction	1257	71.2	1533	71.8	1279	75.6	1571	75.5	2013	78.1	1257	73.9
Proteins assigned to COGs	1016	57.5	1324	62.0	1091	64.5	1312	63.0	1615	62.6	1166	68.5
Proteins with peptide signals	181	10.2	205	9.6	203	12.0	239	11.5	287	11.1	179	10.5
No. of proteins associated with ORFan	89	5.0	142	6.7	33	2.0	100	4.8	55	2.1	37	2.2
Genes associated with PKS or NRPS	2	0.1	3	0.1	4	0.2	8	0.4	12	0.5	4	0.2
No. of antibiotic resistance genes	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0
No. of genes associated with Pfam-A domains	1519	83.0	1936	88.0	1549	88.0	1.89	88.0	2381	90.0	1583	90.0

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
COGs, Clusters of Orthologous Groups database.
[a]Total is based on either size of genome (bp) or total number of protein-coding genes in annotated genome.

TABLE 6. Number of genes associated with 25 general COGs functional categories

		A		B		C		D		E		F	
Code	Description	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]
J	Translation	145	8.21	156	7.31	163	9.63	166	7.98	165	6.40	158	9.29
A	RNA processing and modification	1	0.07	1	0.04	1	0.06	1	0.05	1	0.04	1	0.06
K	Transcription	65	3.69	91	4.26	70	4.14	98	4.71	145	5.62	76	4.47
L	Replication, recombination and repair	48	2.72	71	3.33	64	3.78	68	3.27	72	2.79	65	3.82
B	Chromatin structure and dynamics	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
D	Cell cycle control, mitosis and meiosis	19	1.08	22	1.03	18	1.06	19	0.91	20	0.78	21	1.23
Y	Nuclear structure	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
V	Defense mechanisms	37	2.10	41	1.92	36	2.13	43	2.07	68	2.64	34	2.0
T	Signal transduction mechanisms	32	1.81	41	1.92	31	1.83	42	2.02	67	2.60	32	1.88
M	Cell wall/membrane biogenesis	55	3.11	59	2.76	49	2.90	72	3.46	88	3.41	60	3.53
N	Cell motility	6	0.34	6	0.28	6	0.35	8	0.38	8	0.31	7	0.41
Z	Cytoskeleton	0	0.0	0	0.0	0	0.0	0	0.0	1	0.04	0	0.0
W	Extracellular structures	3	0.17	4	0.19	1	0.06	4	0.19	4	0.16	4	0.24
U	Intracellular trafficking and secretion	15	0.85	17	0.80	17	1.00	16	0.77	19	0.74	19	1.12
O	Posttranslational modification, protein turnover, chaperones	56	3.17	61	2.86	59	3.49	64	3.08	64	2.48	60	3.53
X	Mobilome: prophages, transposons	51	2.89	21	0.98	2	0.12	19	0.91	27	1.05	13	0.77
C	Energy production and conversion	63	3.57	81	3.79	73	4.31	91	4.37	92	3.57	81	4.77
G	Carbohydrate transport and metabolism	89	5.04	218	10.21	118	6.97	186	8.94	205	7.95	151	8.88
E	Amino acid transport and metabolism	98	5.55	126	5.90	113	6.68	131	6.30	169	6.55	111	6.53
F	Nucleotide transport and metabolism	50	2.83	73	3.42	51	3.01	67	3.22	71	2.75	67	3.94
H	Coenzyme transport and metabolism	62	3.51	74	3.47	59	3.49	63	3.03	102	3.96	66	3.89
I	Lipid transport and metabolism	34	1.93	38	1.78	51	3.01	49	2.35	65	2.52	37	2.18
P	Inorganic ion transport and metabolism	51	2.89	72	3.37	67	3.96	60	2.88	91	3.53	70	4.12
Q	Secondary metabolites biosynthesis, transport and catabolism	16	0.91	17	0.80	14	0.83	18	0.86	35	1.36	19	1.12
R	General function prediction only	74	4.19	99	4.64	84	4.96	112	5.38	149	5.78	77	4.53
S	Function unknown	53	3.00	67	3.14	54	3.19	51	2.45	79	3.06	53	3.12
—	Not in COGs	750	42.47	811	38.0	601	35.52	769	36.95	964	37.39	535	31.45

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
COGs, Clusters of Orthologous Groups database.
[a]Total is based on either size of genome (bp) or total number of protein-coding genes in annotated genome.

TABLE 7. Genome comparison of closely related species.

Organism	Strain	INSDC	Size (Mb)	G+C%	Total genes
Actinotignum massiliense	FC3	CYUL00000000	2.067	60.17	1771
Actinobaculum suis	C1-22-1	MASX00000000	2.23	57.75	1611
Actinomyces bouchedurhonensis	Marseille-P2825	FQSA00000000	2.3	63.28	1766
Actinomyces cardiffensis	F0333	AQHZ00000000	2.19	61.49	1983
Actinomyces coleocanis	DSM 15436	ACFG00000000	1.72	49.60	1546
Actinomyces europaeus	ACS-120-V-Col10b	AGWN00000000	1.911	56.65	1670
Actinomyces georgiae	DSM 6843	AUBM00000000	2.5	69.87	2103
Actinomyces graevenitzii	C83	ACRN00000000	2.21	57.80	1853
Actinomyces ihuae	SD1	CZPX00000000	2.45	58.89	2135
Actinomyces israelii	DSM 43320	JONS00000000	4.03	71.44	3387
Actinomyces johnsonii	F0542	AWSE00000000	3.33	67.45	3324
Actinomyces marseillensis	Marseille-P2818	FTLP00000000	2.01	57.09	1692
Actinomyces mediterranea	Marseille-P3257	FTPB00000000	2.4	63.79	2081
Actinomyces meyeri	W712	CP012072	2.05	65.51	1667
Actinomyces naeslundii	Howell 279	ALJK00000000	3.11	67.85	2930
Actinomyces neuii subsp. *neuii*	DSM 8576	ATUW00000000	2.27	56.18	2013
Actinomyces odontolyticus	F0309	ACYT00000000	2.43	65.25	2372
Actinomyces oralis	Marseille-P3109	OOHN00000000	3.15	68.40	2579
Actinomyces oris	K20	BABV00000000	2.87	67.80	3006
Actinomyces radicidentis	CCUG 36733	CP014228	3.05	72.58	2342
Actinomyces slackii	ATCC 49928	AUAK00000000	3.17	70.14	2611
Actinomyces suimastitidis	DSM 15538	AUBF00000000	2.29	56.43	1943
Actinomyces turicensis	ACS 279 V Col4	AGWQ00000000	1.95	57.18	1717
Actinomyces urinae	Marseille-P2225	FPKP01000000	1.95	56.13	1701
Actinomyces urogenitalis	DORA 12	AZLV00000000	2.6	68.66	3034
Actinomyces viscosus	C505	ACRE00000000	3.13	68.60	2587
Isoptericola dokdonensis	DS-3	CP014209	3.83	73.81	3403
Isoptericola variabilis	JZ7	LWGM00000000	3.48	74.58	3529
Mobiluncus curtisii subsp. *curtisii*	ATCC 43063	CP001992	2.15	55.41	1909
Mobiluncus mulieris	28-1	ADBR00000000	2.45	55.07	2321
Varibaculum cambriense	DORA 20	AZMI00000000	2.28	52.76	2025

INSDC, International Nucleotide Sequence Database Collaboration.

atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain SD1 was susceptible to gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL), rifampicin (30 µg/mL), amoxicillin (25 µg/mL), doxycycline (30 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid. The genome of strain SD1^T is 2 458 350 bp long with 58.89% G+C content. The 16S rRNA gene and genome sequences are available in the European Molecular Biology Laboratory (EMBL)-EBI (European Bioinformatics Institute) database under accession numbers LN866997 and CZPX00000000, respectively.

We propose the new species *'Actinomyces ihuae'* (i.hu.ae, N.L. gen. adj., *ihuae,* which is based on the acronym IHU, for Institut Hospitalo-Universitaire in Marseille, France, where the strain was isolated), isolated from stool. Strain SD1^T is the type strain of the new species *'Actinomyces ihuae'* (= CSUR P2006 = DSM 100538).

Description of *'Actinomyces bouchesdurhonensis'* sp. nov.

Cells are Gram-positive bacilli with a length of 1.5 to 1.9 µm and a width of 0.5 to 0.6 µm. This strain did not exhibit catalase and oxidase activity. *Actinomyces bouchesdurhonensis* is nonmotile and non–spore forming. Colonies are smooth and grey with a diameter of 0.6 to 1.5 mm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth.

Marseille-P2825 was susceptible to rifampicin (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and erythromycin (15 IU). The major fatty acid is 9-octadecenoic acid.

The genome of strain Marseille-P2825 is 2 304 249 bp long with 63.28% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT576385 and FQSA01000000, respectively.

We propose the new species *'Actinomyces bouchesdurhonensis'* sp. nov. (bou.ches.du.rho.nen'sis, N.L. neut. adj. *bouchesdurhonensis,* pertaining to Boûches du Rhône, the name of the French territory where the strain was isolated) was isolated from a stomach wash. Strain Marseille-P2825^T is the type strain of the new species *'Actinomyces bouchesdurhonensis'* (= CSUR P2825 = DSM 103075).

Description of *'Actinomyces urinae'* sp. nov.

Marseille-P2225 is a Gram-positive rod-shape with a length of 400 to 600 nm and width of 200 to 400 nm. This strain no catalase and no oxidase activity. *'Actinomyces urinae'* is nonmotile and non–spore forming. Colonies are translucent, beige microcolonies, with a diameter of 100 µm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 48 hours' growth.

Strain Marseille-P2225 was susceptible to gentamicin (500 µg/mL), amoxicillin (25 µg/mL), doxycycline (30 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid.

TABLE 8. dDDH comparison[a]

	A	B	C	D	E	F	G	H	I	J	K
A	100% ± 00										
B	20.80% ± 2.35	100% ± 00									
C	22.30% ± 2.35	21.70% ± 2.35	100% ± 00								
D	21.40% ± 2.35	21.30% ± 2.35	22.90% ± 2.40	100% ± 00							
E	20.70% ± 2.30	22.10% ± 2.35	22.00% ± 2.35	19.80% ± 2.30	100% ± 00						
F	21.40% ± 2.35	27.50% ± 2.40	26.20% ± 2.40	18.10% ± 2.25	23.10% ± 2.40	100% ± 00					
G	21.30% ± 2.35	20.20% ± 2.30	21.60% ± 2.35	23.70% ± 2.40	19.80% ± 2.30	25.50% ± 2.40	100% ± 00				
H	21.20% ± 2.35	24.90% ± 2.40	23.70% ± 2.40	19.50% ± 2.30	25.10% ± 2.40	30.90% ± 2.45	22.70% ± 2.35	100% ± 00			
I	20.40% ± 2.20	20.70% ± 2.35	21.90% ± 2.35	19.60% ± 2.3	34.80% ± 2.45	21.80% ± 2.35	19.00% ± 2.30	22.30% ± 2.35	100% ± 00		
J	20.60% ± 2.35	22.10% ± 2.35	22.40% ± 2.35	19.90% ± 2.35	35.00% ± 2.50	24.50% ± 2.40	19.90% ± 2.30	25.00% ± 2.40	23.50% ± 2.40	100% ± 00	
K	25.90% ± 2.40	19.70% ± 2.30	22.60% ± 2.40	20.90% ± 2.35	20.10% ± 2.30	20.10% ± 2.30	21.50% ± 2.35	20.40% ± 2.35	20.20% ± 2.30	21.40% ± 2.35	100% ± 00

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225; G, *A. cardiffensis*; H, *A. europaeus*; I, *A. johnsonii*; J, *A. naeslundii*; K, *A. odontolyticus*.
dDDH, digital DNA-DNA hybridization; GGDC, genome-to-genome distance; HSP, high-scoring pair.
[a]Confidence intervals indicate inherent uncertainty in estimating DDH values from intergenomic distances based on models derived from empirical test data sets (which are always limited in size). These results are in accordance with 16S rRNA (Fig. 1) and phylogenetic analyses as well as GGDC results.

The genome of strain Marseille-P2225 is 1 946 897 bp long with 56.13% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LN870295 and FPKP01000000, respectively.

We propose the creation of the new species *'Actinomyces urinae'* sp. nov. (u.ri.na'e, N.L. gen. fem. *urinae,* 'of urine'), which was isolated from urine. Strain Marseille-P2225^T is the type strain of the new species *'Actinomyces urinae'* (= CSUR P2225 = DSM 100700).

Description of *'Actinomyces marseillensis'* sp. nov.

Cells are Gram positive and rod shaped, with a length of 2.0 to 2.2 µm and a width of 0.4 to 0.5 µm. This strain exhibited no catalase or oxidase activity. *'Actinomyces marseillensis'* is nonmotile and non–spore forming. Colonies are smooth and shiny with a diameter of 0.5 to 1.5 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth.

Strain Marseille-P2818 was susceptible to gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), amoxicillin (25 µg/mL), tobramycin (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL), rifampicin (30 µg/mL), doxycycline (30 IU) erythromycin (15 IU) and vancomycin (30 µg/mL). The major fatty acid is 9-octadecenoic acid.

The genome of strain Marseille-P2818 is 2 007 553 bp long with 57.09% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT576400 and FTLP00000000, respectively.

'Actinomyces marseillensis' sp. nov. is proposed as new species (mar.sei.ll.en'sis, L. gen. neut. adj., from *marseillensis,* pertaining to Marseille, where the strain was isolated). It was isolated from the sputum. Strain Marseille-P2818^T is the type strain of the new species *'Actinomyces marseillensis'* (= CSUR P2818 = CCUG 71898).

Description of *'Actinomyces mediterranea'* sp. nov.

Marseille-P3257 cells are Gram-positive bacilli with a length of 2.3 to 2.6 µm and a width of 0.53 to 0.61 µm. This strain did not exhibit catalase and oxidase activity. *'Actinomyces mediterranea'* is nonmotile and non–spore forming. Colonies are smooth and white in colour, with a diameter of 0.4 to 1.1 mm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain Marseille-P3257 was susceptible to rifampicin (30 µg/mL), fosfomycin (50 µg/mL), doxycycline (30 IU), erythromycin (15 IU), gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), ceftriaxone (30 µg/mL), amoxicillin (25 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and

vancomycin (30 µg/mL). The major fatty acid is 9-octadecenoic acid. The genome of strain Marseille-P3257 is 2 395 621 bp long with 63.79% GC content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT598588 and FTPB00000000, respectively.

We propose the new species *Actinomyces mediterranea* sp. nov. (me.di.ter.ra.ne.a, L. fem. adj. *mediterranea,* the Latin name of the Mediterranean Sea bordering Marseille, where the strain was isolated), which was isolated from the duodenum wash. Strain Marseille-P3257^T is the type strain of the new species *'Actinomyces mediterranea'* (= CSUR 3257 = CCUG 70143).

Description of *'Actinomyces oralis'* sp. nov.

Cells are Gram positive and rod shaped, with a length of 1.6 to 1.8 µm and a width of 0.6 to 0.7 µm. This strain no exhibited catalase or oxidase activity. *'Actinomyces oralis'* is nonmotile and non–spore forming. Colonies are smooth and grey, with a diameter of 0.3 to 1.2 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain Marseille-P3109 was susceptible to rifampicin (30 µg/mL), gentamicin (15 µg/mL), gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), ceftriaxone (30 µg/mL), amoxicillin (25 µg/mL), penicillin G (10 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid. The genome of strain Marseille-P3109 is 3 149 233 bp long with 68.40% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT627670 and OOHN00000000, respectively.

We propose the creation of the new species *'Actinomyces oralis'* sp. nov. (o.ra'lis, N.L. neut. adj. *oralis,* 'from the mouth,' from which the strain was isolated), which was isolated from human sputum. Strain Marseille-P3109^T is the type strain of the new species *'Actinomyces oralis'* (= CSUR P3109 = DSM 103942).

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.nmni.2018.06.004.

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5.Discussion :

5.1.Répertoire des bactéries urinaires humaines :

562 espèces bactériennes différentes ont été identifiées grâce à l'étude de la littérature comme ayant été isolées en culture ou découvertes par métagénomique à partir d'urines humaines [35]. Parmi ces 562 espèces, 423 étaient isolées en culture, 100 avaient un métabolisme anaérobie strict (17,8%) ce qui représente moins de bactéries anaérobies en proportion que dans le microbiote du tube digestif (34,0%) et 350 étaient communes avec le microbiote digestif déjà décrit (62,3%). Les espèces décrites par culture avaient un métabolisme anaérobie strict (9,4%) dans une moindre proportion que les espèces décrites par méthodes de génétique moléculaire (30,8%), ainsi il est probable qu'il n'y ait pas une telle différence de composition en termes de bactéries aérointolérantes strictes entre le microbiote urinaire et le microbiote digestif mais qu'il y ait un biais du fait de l'occultation prolongée du microbiote urinaire et de méthodes de culture préalables ayant favorisées la croissance d'espèces bactériennes non fastidieuses de croissance rapide en condition aérobie.

Dans le cadre de la recherche bibliographique automatisée qui visait à identifier l'ensemble des bactéries isolées dans des urines humaines, quelques biais sont à relever. Nous avons réalisé la recherche bibliographique à partir d'une liste de bactéries isolées chez l'homme dressée dans un article précédemment publié dans notre laboratoire en 2014 auquel nous avons ajouté les bactéries décrites dans notre laboratoire entre 2014 et la date de réalisation de la recherche automatisée du 15 au 17 février 2018. Nous n'avons donc pas inclus les bactéries décrites dans l'intervalle entre la recherche bibliographique réalisée et la date à laquelle le manuscrit a été accepté le 7 novembre 2018. Certaines bactéries isolées dans des urines et décrites dans cet intervalle ont donc pu nous échapper. De même, notre répertoire est une image à un temps donné de l'état de la littérature et n'est pas dynamiquement mis à jour en fonction des nouvelles parutions. De plus les résultats ont été triés manuellement et certains articles dont le titre était peu informatif ont pu ne pas être pris en compte.

Dans la littérature, les échantillons décrivant des espèces bactériennes retrouvées dans les urines humaines étaient généralement prélevés chez des patients présentant des symptômes urinaires ou infectieux évoquant une infection. Il est donc difficile d'inférer les résultats de cette recherche au microbiote de patients sains.

5.2.Description du microbiote urinaire humain :

Nous avons testé 441 échantillons d'urine au sein desquels nous avons isolé 459 espèces bactériennes différentes. Parmi ces 459 espèces, 264 n'avaient jamais été décrites dans les urines auparavant et 18 sont des nouvelles espèces ce qui a permis d'implémenter le répertoire de bactérie connues dans les urines de 39%. Il est à noter que 161 espèces bactériennes étaient aéro-intolérantes (35%). En contraste, aucune des bactéries identifiées dans le laboratoire de microbiologie clinique n'étaient aéro-intolérantes et seulement 9,4% des bactéries identifiées en culture décrites dans la littérature étaient de métabolisme anaérobique strict. Ceci reflète l'intérêt d'utiliser des milieux de culture spécifiques permettant la croissance d'espèces bactériennes fastidieuse de métabolisme anaérobique strict [5, 10, 17].

Les 684 espèces bactériennes au total cultivées dans urines partage 62% de leur composition avec le microbiote digestif déjà connu et 32% avec le microbiote vaginal, ce qui est plutôt en faveur d'une origine digestive du microbiote urinaire [10, 36]. D'autre part il n'a pas été mis en evidence une difference majeure de composition du microbiote urinaire entre les sexes féminin et masculin en dehors d'une fréquence plus importante d'*Escherichia coli* ainsi qu'un nombre d'uropathogènes par échantillons plus important chez les femmes. Nos résultats conduisent à un changement de paradigme dans la compréhension du microbiote urinaire, soulignant que son origine est l'intestin plutôt que le vagin. Il est à noter que le microbiote des voies urinaires peut être modifié après une transplantation de matière fécale par voie digestive haute, l'ingestion orale de probiotique ou des viandes contaminées ce qui laisse penser qu'une origine digestive au microbiote urinaire est probable [37-41]. Cela appuie la nécessité de mener des études afin de comprendre le mécanisme de passage du microbiote du tube digestif vers les autres microbiotes (urine [37-41], sein [42], etc).

Dans la sous-population pédiatrique de notre étude, il n'a pas été mis en évidence de différence significative en termes de nombre d'espèces bactériennes différentes et de richesse bactérienne (index de Shannon) entre les urines considérées « stériles » par les techniques de culture standard dans le laboratoire de microbiologie clinique et celles avec un développement microbien considéré significatif par culturomics, hormis concernant la population de patients étant sous antibiotiques lors du prélèvement d'urine. Il n'a pas été non plus mis en évidence de différence significative entre les sexes concernant la richesse bactérienne. En revanche les échantillons d'urines étaient significativement plus riches chez les sujets plus âgés et chez les sujets avec un poids plus important, d'autres études devraient être menées afin de comprendre les déterminants de l'évolution du microbiote urinaire chez l'enfant au cours de son développement. De multiples études portant sur le microbiote digestif indiquent que celui-ci est

en construction les deux premières années de vie et est de ce fait très sensible aux agressions qui peuvent le modifier durablement contrairement au microbiote de l'enfant plus grand et de l'adulte qui serait beaucoup plus résilient [43-46]. Il est probable qu'il en soit de même concernant le microbiote urinaire.

Nous avons par cette étude montré qu'il existe dans les urines humaines de sujets malades et sains, de sujets féminins et masculins, de nouveau-né, d'enfants et d'adulte, un microbiote identifiable par des techniques de culture spéciale.

Des études récentes montrent l'intérêt des urines comme engrais en agriculture. Ainsi on peut facilement imaginer que c'est un lieu propice au développement d'un microbiote spécifique [47].

5.3.Culturomics :

Notre méthodologie de culture spéciale appliquée aux urines est dérivée de celle décrite par Jean-Christophe Lagier et al. pour les selles appelée culturomics [5, 10, 17]. Dans la méthodologie culturomics, 18 conditions de culture permettant la croissance de la plus grande diversité possible d'espèces bactériennes identifiables à partir des prélèvements de selles ont été sélectionnées.

Au total pour l'étude des échantillons d'urine 17 conditions de culture ont été utilisées.

Concernant la sous-population pédiatrique, nous avons utilisé au total 8 conditions de culture différentes chez les enfants de moins de 3 mois et 10 conditions chez les enfants de 3 à 18 ans. Parmi celles-ci, la condition de préincubation en flacon d'hémoculture anaérobie dans lequel avait été ajouté du jus de rumen de mouton microfiltré et qui était inoculée sur Columbia agar + sang à 30 jours d'incubation était celle qui permettait d'isoler la plus grande diversité bactérienne. La condition d'inoculation directe sur Columbia agar + sang incubée en milieu anaérobie était la 2e plus productive.

Ce travail est un travail exploratoire destiné à montrer l'utilité du concept de description du microbiote urinaire par des techniques de culture spéciales. Il est innovant par l'étude d'échantillons d'urines, notamment pédiatriques, l'utilisation d'un milieu de culture liquide destiné à l'enrichissement en bactéries fastidieuses et l'utilisation de milieux de culture solides artisanaux contenant des antioxydants destinés à améliorer la culture de bactéries considérées comme anaérobies, du jus de rumen a pour objectif de favoriser le développement de bactéries fastidieuses en recréant les conditions du tube digestif [7-9]. Le processus de préparation du jus de rumen implique l'utilisation de filtres dont le diamètre des pores est de 0.2µm. Le but de ce traitement est de supprimer toutes les bactéries dont le diamètre est supérieur au diamètre des

pores. Néanmoins, certaines espèces bactériennes semblent avoir une taille inférieure au diamètre des pores. Ces bactéries appelées ultramicrobactéries ont une taille moyenne de 0.009±0.002 μm3 [48]. Afin d'éliminer le risque de mettre en évidence des bactéries issues du jus de rumen, nous avons utilisé un témoin négatif qui n'a pas montré de développement microbien.

Les méthodes de culture ont pour inconvénient d'être opérateur dépendant et il est probable que la technique développée entre le début de l'étude et la fin se soit améliorée, rendant la comparabilité des résultats entre échantillons délicates.

Une des limites de l'utilisation des méthodes de culture spéciale sur les prélèvements d'urine d'enfant de moins de 3 mois est la quantité de matériel biologique à disposition. En effet, dans certains cas les mictions recueillies n'excédaient pas 3 ml, l'ECBU diagnostique étant prioritaire, il restait parfois trop peu d'échantillon pour pouvoir ensemencer l'urine sur un grand nombre de milieux différents et notamment aucun milieu halophile n'a été testé dans ce sous-groupe.

Nous avons modifié à plusieurs reprises le protocole utilisé pour la culture des bactéries. Initialement, les conditions de préincubation dans des flacons d'hémoculture n'étaient pas réalisées dans le sous-groupe d'enfants de moins de 3 mois de peur de ne pas avoir suffisamment de quantité d'urine pour faire l'ensemble des conditions. Cependant devant les très bons résultats que ce type de culture donnait chez les enfants de 3 à 18 ans, ces conditions ont été secondairement réalisées pour les urines de 1 à 9. Elles ont ainsi été mises en culture en préincubation dans un flacon d'hémoculture après décongélation de 1 ml qui avait été initialement conservé à -80°C. Et à partir de l'échantillon 10 tous les échantillons étaient inoculés frais dans les flacons d'hémoculture. De plus à partir de l'urine 25 en ensemencement direct et pour tous les ensemencements sur gélose après préincubation en flacon d'hémoculture, les milieux solides faits maisons (R-Medium et Columbia agar + Sang + Rumen) ont été réalisés avec doublement de la quantité d'agar pour rendre la gélose plus rigide et ainsi ralentir la diffusion du *Proteus mirabilis*. En effet, le *Proteus mirabilis* étant une bactérie mobile, il a entrainé des difficultés lors du repiquage des urines 1, 4, 7, 8, 20 et 24 lors de l'ensemencement direct sur gélose solide car il formait un tapis de bactéries en moins de 24h ce qui gênait le repiquage et l'isolement des différentes espèces bactériennes présentes dans l'échantillon.

Un des biais possibles expliquant la moindre performance de notre méthode de culturomics par rapport à la métagénomique pourrait aussi concerner l'identification par spectrométrie de masse MALDI-TOF qui n'est parfois pas suffisamment précise pour le diagnostic d'espèce. Il aurait été intéressant de vérifier en séquençage 16S les souches obtenues

par culture et identifiées par spectrométrie de masse cependant le temps de travail et le coût que cela engendre ne sont pas négligeables et rendent cela peu réalisable.

Concernant l'étude en sous-groupe chez les enfants de moins de 3 mois, incapables de continence urinaire, les échantillons urinaires collectés étaient prélevés soit par Urinocol (majorité des cas), soit par sondage vésical par voie transurethral (parmis les patients inclus, 11 (35,5%) ont eu un sondage urinaire pour réalisation d'un examen diagnostic ou pour prise en charge thérapeutique, à l'occasion duquel un échantillon d'urine a été prélevé) après un nettoyage soigneux de la région pelvi-perinéale. Ce sont les techniques les plus couramment utilisées pour recueillir des échantillons urinaires chez les jeunes enfants n'ayant pas acquis la « propreté » (âge moyen d'acquisition de la propreté diurne : 30-36 mois). Il est à noter que le mode de recueil par Urinocol (poche stérile collée sur le périnée après désinfection périnéale et laissée en place jusqu'à l'émission des urines) est considéré comme à risque de contaminations périnéales [49], la poche devait donc être changée à intervalles réguliers en l'absence d'émission d'urine pour éviter au maximum le risque de contamination. Réaliser des sondages vésicaux ou des ponctions sus-pubiennes systématiques n'aurait pas été acceptable en raison de considérations éthiques. Les patients sélectionnés n'auraient eu aucun bénéfice individuel direct à subir ces procédures qui sont douloureuses et les exposent à certaines complications (sténoses urétrales, hémorragies). Il paraît d'autant plus difficile d'imposer ces procédures à des enfants dans le cadre d'une étude exploratoire. Néanmoins, les procédures invasives de prélèvement urinaire, si elles permettent de cibler le microbiote vésical et d'exclure en partie les bactéries colonisant l'urètre ou les contaminations périnéales, sont éloignées de la réalité clinique pour la plupart des patients. Elles permettraient de décrire un microbiote vésical et non urinaire de la même façon que des prélèvements de selles permettent de décrire un microbiote fécal et non intestinal.

De plus nous voulions pouvoir comparer le nombre d'espèces bactériennes isolées dans notre étude avec celui de la totalité des espèces bactériennes déjà décrite dans les urines dans la littérature. La recherche bibliographique utilisée ne permettait pas de trier les résultats en fonction du type de prélèvements effectués. Mais en l'absence de précisions, il est probable que la plupart des échantillons urinaires utilisés pour décrire les espèces bactériennes isolées dans des urines soient des échantillons d'urine mictionnelle de milieu de jet, réalisés après désinfection périnéale, ou même de prélèvements issus de recueils d'urine par Urinocol ce qui rend possible la comparaison avec les espèces bactériennes que nous avons isolées.

Nos résultats sont cohérents par rapport aux données de la littérature. Hilt *et al*. [29] ont montré une croissance bactérienne sur des échantillons d'urines obtenus par sondage vésical

dans 80% des cas grâce à des techniques de culture spéciale contre 92% des urines sans développement microbien significatif dans les conditions de culture standard.

Nous avons également isolé une grande variété d'espèces considérées anaérobie strictes. D'autres études portant sur la culture des urines ont également isolé des bactéries anaérobies [29-32] en forte proportion.

Nos résultats ouvrent la possibilité d'une étude systématique du microbiote urinaire par des techniques de culture spéciales. En incluant un plus grand nombre d'échantillons, en standardisant et en simplifiant notre protocole, on pourrait décrire et comparer le microbiote dans différentes situations physiologiques (âge, sexe, activité sexuelle, alimentation) et pathologiques (antibiothérapie, traitements immunosuppresseurs, pathologies urologiques, pathologies néphrologiques, hémodialyse, pathologies auto-immunes, pathologiques tumorales) voire éluder une partie des processus à l'origine de ces pathologies, comme pour le cancer de la vessie par exemple [50-53].

L'existence d'un microbiote urinaire appuie les recommandations récentes qui visent à ne pas prescrire d'antibioprophylaxies au long court car elles sélectionnent des bactéries résistantes au sein des différents microbiotes ce qui peut conduire à des infections par des germes résistant chez l'hôte pour un bénéfice non démontré sur la réduction de cicatrices rénales à long terme [54].

Au sein du laboratoire de l'IHU méditerranée-infection plusieurs travaux sont encore en cours sur microbiote urinaire des transplantés rénaux adultes, de patients atteints de cancer de la vessie, sur l'évolution du microbiote urinaire avant et après consommation de probiotiques, sur l'évolution du microbiote urinaire avant et après greffe rénale ainsi que sur la recherche de bactéries extrémophiles et d'archées au sein d'échantillons d'urine [55]. Il est intéressant d'expérimenter les performances de nouveaux milieux ou de nouvelles conditions de culture, ciblées sur certains *Phyla* ou certains groupes bactériens considérés fastidieux, notamment des bactéries halophiles ou des archées. Ces expériences ont déjà été menées avec succès dans notre laboratoire sur des échantillons de selles et de lait maternel et progresse sur les autres liquides biologiques humains.

D'autre part il pourrait être intéressant de comparer les résultats de plusieurs techniques de recueil d'urines chez un même patient afin de comprendre quelles espèces sont spécifiques de quelle portion du tractus urinaire.

Et enfin, étudier pour un même enfant plusieurs échantillons provenant de différents sites du corps humain (méconium, selles, liquide gastrique, urine, peau, bouche…) mais aussi de son environnement (lait maternel, peau maternelle et paternelle, liquide amniotique,

prélèvement vaginal, bouches des parents, selles maternelles, urines maternelles…) permettrait d'établir une cartographie du microbiote humain et d'établir les liens d'un site à un autre notamment au cours de la croissance (colonisation d'un site par translocation bactérienne) et d'un individu à un autre (transmission par contact).

5.4.Métagénomique :

Dans les 38 échantillons d'urine pédiatriques analysés à la fois en culture et en métagénomique, la métagénomique est apparue plus précise et complète que la culture. 413 OTUs ont été identifiées au rang d'espèce (428 sous-espèces), parmis lesquelles 259 n'étaient pas décrites dans la literature auparavant et 328 n'avaient pas été cultivées dans ces échantillons. Notamment, cette méthode a permis l'identification de plusieurs bactéries halophiles et d'archées. Cependant parmi les 149 espèces bactériennes trouvées en culture dans ces 38 échantillons analysés par les 2 techniques, 85 l'étaient aussi par métagénomique mais 64 ne l'étaient pas. Ainsi ces deux méthodes semblent complémentaires.

Dans notre étude, l'analyse métagénomique des échantillons s'est faite dans un second temps. Il y aurait cependant un intérêt à réaliser la métagénomique avant la culture (bien que ceci soit difficilement réalisable du fait de la nécessité d'ensemencer les urines dans les 6 heures). En effet cela permettrait d'orienter le type de milieux à concevoir pour mieux cultiver les espèces supposées présentes dans l'échantillon et notamment certaines espèces très fastidieuses (exemple : *Akkermansia muciniphila*), certaines espèces halophiles (exemple : *Herminiimonas glaciei)* ou des archées (exemple : *Methanobrevibacter smithii*).

Toutefois, il est fort probable qu'il y ait de nombreux faux positifs parmi les résultats de métagénomique. En effet, on ne connait pas la clairance de l'ADN bactérien et d'autres organismes dans les urines (constituées en grande partie de matières dégradés plus ou moins toxiques à éliminer par l'organisme). Un exemple flagrant au cours de notre étude est la détection en métagénomique de *Pseudomallada prasinus* qui est un arthropode et dont on peut être presque certain de l'absence au sein de l'urine d'un nourrisson.

Par ailleurs, il y a aussi des faux négatifs lors de l'analyse des prélèvements par métagénomique dus au biais de profondeur. Seuls les organismes présents en grandes quantités sont identifiés.

5.5.Description des nouvelles espèces bactériennes cultivées au sein d'échantillons d'urine humaine :

L'approche couplée de la méthode culturomics et de la taxonogénomique nous a permis de décrire les 6 nouvelles espèces bactériennes découvertes dans les échantillons d'urine d'enfant sur les 18 nouvelles espèces découverte au sein d'échantillons d'urine humaine dans notre laboratoire IHU Méditerranée-Infection. Pour 3 d'entre elles seule la description rapide de la séquence 16S, du spectre de masse et des caractéristiques de culture et de morphologie a été réalisée, la description du génome, des caractéristiques physicochimiques et de la sensibilité aux antibiotiques sera réalisée prochainement. Pour deux d'entre elles la description rapide et la description complète ont été publiées. Pour l'une d'entre elles la description courte a été publiée tandis que la description complète est en cours de finition.

Ces nouvelles espèces viennent augmenter le nombre d'espèces bactériennes connues chez l'homme. Il est notable qu'une fois découvertes ces bactéries sont ensuite mieux identifiées en pratique clinique comme on peut le voir par exemple pour l'espèce bactérienne *Corynebacterium urinapleomorphum* qui depuis sa découverte a été retrouvée dans un échantillon clinique [56].

6.Conclusion et perspectives :

Le microbiote urinaire peut être décrit par des techniques de culture spéciales. On peut isoler des bactéries dans des échantillons urinaires de patients ne présentant pas les critères habituels définissant l'infection urinaire, y compris lorsque l'ECBU est considérée stérile. Les techniques de culture spéciales permettent d'identifier des espèces qui ne sont pas mises en évidence par les techniques de culture standard mais aussi de nouvelles espèces bactériennes non décrites dans la littérature. Cependant d'autres espèces ne sont pas cultivées avec les milieux de culture qui ont été utilisés (notamment certaines bactéries halophiles, des archées, des bactéries très aéro-intolérantes), ainsi des études ultérieures utilisant d'autres conditions de culture seraient nécessaires afin de poursuivre la description du microbiote urinaire.

Nous avons dressé un panorama des espèces bactériennes isolées dans des échantillons urinaires à travers la littérature scientifique. Cette liste a vocation à fournir un point de départ à la description de la composition du microbiote urinaire en condition physiologique ou pathologique. Elle devra être complétée par de futurs travaux d'analyse du microbiote urinaire mais aussi par les publications rapportant des infections urinaires à germes nouvellement identifiés dans des urines.

L'origine intestinale du microbiote bactérien urinaire humain semble très probable. De futurs travaux devraient explorer les mécanismes qui y conduisent.

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Résumé :

L'urine a longtemps été considérée stérile.

Ce travail visait à établir le répertoire des bactéries connues dans les voies urinaires humaines par une revue de la littérature et à implémenter ce répertoire en analysant des échantillons d'urine par culturomics et métagénomique.

562 espèces bactériennes ont été décrites dans des échantillons d'urine humains dans la littérature. 62,6% ont été associées à un cas d'infection humaine.

Parmi les 441 échantillons d'urine analysés par culturomics, 459 espèces bactériennes différentes ont été isolées, dont 264 jamais décrites dans l'urine, 18 nouvelles espèces.

Parmi les 684 espèces bactériennes isolées au moins une fois en culture à partir d'échantillons d'urine, 424 (62%) avaient déjà été isolées du microbiote intestinal.

Parmi les espèces retrouvées uniquement en métagénomique figurent des bactéries extrêmophiles.

Il existe un microbiote des voies urinaires humaines qui peut être décrit par culturomics et métagénomique.

Abstract :

Urine was usually considered sterile.

This work aimed to establish the repertoire of known bacteria in the human urinary tract through a review of the literature and to implement this repertoire by analyzing urine samples by culturomics and metagenomics.

In literature, 562 bacterial species have been described in human urine samples, in which 62.6% were associated with one case of human infection.

Of the 441 urine samples analyzed by culturomics, 459 different bacterial species were isolated, of which 264 never described in the urine, 18 new species.

Of the 684 bacterial species isolated at least once in culture from urine samples, 424 (62%) had already been isolated from the gut microbiota.

Extremophilic bacteria and archaea are identified among the species found only with metagenomics method.

There is a microbiota of the human urinary tract that can be described by culturomics and metagenomics.