Genomic analysis of a *Raoultella ornithinolytica* strain causing prosthetic joint infection in an immunocompetent patient

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We sequenced the genome of *Raoultella ornithinolytica* strain Marseille-P1025 that caused a rare case of prosthetic joint infection in a 67-year-old immunocompetent male. The 6.7-Mb genome exhibited a genomic island (RoGI) that was unique among *R. ornithinolytica* strains. RoGI was likely acquired by lateral gene transfer from a member of the *Pectobacterium* genus and coded for a type IVa secretion system found in other pathogenic bacteria and that may have conferred strain Marseille-P1025 an increased virulence. Strain Marseille-P1025 was also able to infect, multiply within, and kill *Acanthamoeba castellanii* amoebae.

In 2001, the analysis of the 16S rRNA and *rpoB* gene sequences enabled reclassification of some *Klebsiella* species within the genus *Raoultella*¹. Formerly known as *Klebsiella ornithinolytica*, *Raoultella ornithinolytica* is a Gram-negative, non-motile and encapsulated bacillus¹ that inhabits aquatic environments and can also be found in hospital water circuits⁴. Reports of human *R. ornithinolytica* infections, initially rare, are increasing and mostly include biliary or urinary tract infections, and bacteremias³⁵. Bone and joint infections caused by *R. ornithinolytica* are seldom reported¹⁰. We recently reported a case of chronic prosthetic joint infection caused by *R. ornithinolytica* in a 67-year-old immunocompetent male¹¹. In this study, the causative strain, Marseille-P1025, was isolated from the peri-prosthetic pus¹¹.

Herein, in order to determine whether this strain had specific virulence factors, we sequenced its genome and compared it to those of other *R. ornithinolytica* strains available in public databases.

Results

**General genomic features.** The draft genome sequence of *R. ornithinolytica* strain Marseille-P1025 consisted of 38 scaffolds after assembly and finishing. No putative plasmid sequence was detected. The chromosome size, G+C content, and CDS content were 5,644,584 bp, 55.6% and 5,260, respectively. A total of 86 RNA genes were identified, including one complete rRNA operon, a second 23S rRNA, eight other 5S rRNAs and 74 tRNAs. Of the 5,260 predicted CDSs, 4,391 genes were assigned a putative function (83.48%) and 869 (16.52%) were annotated as hypothetical proteins. A total of 4,438 (84.37%) genes were assigned a COG functional category.

**Genome comparison.** The genomic comparison is summarized in Table 1. Strain Marseille-P1025, with 5,260 CDs, had a smaller genome than those of strains 10–5246, 2–156_04_S1_C1, 2-156-04_S1_C2, TNT, 811_RORN and BAL286 (5,288, 5,281, 5,284, 5,281, 5,314 and 5,646 CDs, respectively) but larger than those of strains NBRC 105727, B6, A14, CMUL058, CB1 and Yanglingl2 (5,108, 4,907, 4,933, 5,202, 4,953 and 5,033 CDs, respectively) (Table 1). Strain Marseille-P1025 exhibited 95 specific genes (Fig. 1, Table 2) when compared to all other studied *R. ornithinolytica* strains. In contrast, 37 genes present in at least 7 strains were absent in strain Marseille-P1025 (Fig. 1, Table S1).

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The thirteen studied strains exhibited a pangenome and a core genome of 9,815 and 3,822 genes, respectively (Fig. 1). Figure 1 shows the dispersion of the pangenome of *R. ornithinolytica*. The phylogenetic analysis based on accessory genes clustered strains Marseille-P1025 and NBRC 105727.

**Functional annotation.** The COG functional classification of the 95 genes specific of strain Marseille-P1025 demonstrated that 23 of the Marseille-P1025-specific genes were grouped in a 11,473-kb genomic island located in scaffold 21 (Fig. 2). This genomic island, which we named RoGI, exhibited a G+C content of 49.5% (vs 55 to 56% for the genomes of *R. ornithinolytica* strains, Table 1), and was absent from other *R. ornithinolytica* (Fig. 2).

Of these 23 genes, nine coded type IVa secretion system proteins (Table 2), including seven VirB proteins (VirB4 to 11, Table 2) and two proteins related to bacterial conjugation, including a type IVa secretion system conjugal transfer protein and a conjugal transfer protein (Table 2). Moreover, the RoGI island contained a gene coding a second CP4-57 prophage integrase (intA) (Table 2). The genes coding the VirB1, VirB2, VirB3 and VirD4 proteins were identified at other locations of the genome from strain Marseille-P1025, thus supporting the assumption that this strain had a complete and putatively functional type IVa secretion system.

In addition, seven (7.4%) proteins were involved in intracellular trafficking and secretion, seven (7.4%) in replication and repair, four (4.2%) in cell wall/membrane/envelope biogenesis and four (4.2%) had a general functional prediction only (Table 2). Finally, three genes coded integrases including a CP4-57 prophage integrase (intA), two genes coded integrating conjugative element proteins, and two genes coded a CP4-57 prophage regulatory protein AlpA and a transposase, respectively (Table 2).

**Table 1.** Genomic comparison of *Raoultella ornithinolytica* strains.

| Strains          | GenBank accession numbers | Number of genes | Number of protein-coding genes | Number of RNAs | G+C content (%) |
|------------------|---------------------------|-----------------|--------------------------------|----------------|-----------------|
| Strain Marseille-P1025 | FTLL01000000             | 5,346           | 5,260                          | 86             | 55.6            |
| Strain 10-5246 | AGDM0000000000             | 5,353           | 5,288                          | 65             | 55.5            |
| Strain NBRC 105727 | BCRX0000000000             | 5,186           | 5,108                          | 78             | 55.7            |
| Strain B6 | CP004142                  | 5,018           | 4,937                          | 81             | 55.9            |
| Strain A14 | CP008886                  | 5,012           | 4,933                          | 79             | 55.9            |
| Strain CMUL058 | CVRH0000000000             | 5,288           | 5,202                          | 86             | 55.7            |
| Strain TNT | JHQC0000000000             | 5,363           | 5,281                          | 82             | 55.5            |
| Strain 2-156-04_SI_C1 | JNPC0000000000             | 5,380           | 5,281                          | 99             | 55.6            |
| Strain 2-156-04_SI_C2 | JNPD0000000000             | 5,386           | 5,284                          | 102            | 55.6            |
| Strain 811_RORN | JURX0000000000             | 5,332           | 5,314                          | 18             | 55.6            |
| Strain BAL286 | JXXF0000000000             | 5,706           | 5,646                          | 60             | 55              |
| Strain CB1 | LFBW0000000000             | 5,017           | 4,953                          | 64             | 55.9            |
| Strain Yangling I2 | CP013338                | 5,125           | 5,033                          | 92             | 55.7            |

**Figure 1.** Pan-genome analysis of *R. ornithinolytica* whole-genome sequences. A maximum likelihood tree was constructed from the accessory genome elements (left). The presence (blue) and absence (white) of accessory genome elements is presented on the right.

The thirteen studied strains exhibited a pangenome and a core genome of 9,815 and 3,822 genes, respectively (Fig. 1). Figure 1 shows the dispersion of the pangenome of *R. ornithinolytica*. The phylogenetic analysis based on accessory genes clustered strains Marseille-P1025 and NBRC 105727.
| Genes   | Locus         | Putative function (COGs category)                  |
|---------|--------------|--------------------------------------------------|
| bicA_2  | PROKKA_04516 | sulfate permease (P)                             |
| can_2   | PROKKA_04515 | calcium ABC transporter ATPase (P)               |
| cot3A   | PROKKA_01597 | Glycosyltransferase (M)                         |
| group_2403 | PROKKA_02443 | hypothetical protein (S)                        |
| group_5276 | PROKKA_00062 | hypothetical protein (S)                        |
| group_5277 | PROKKA_00063 | hypothetical protein (S)                        |
| group_5288 | PROKKA_01595 | Glycoside hydrolase (Not in Cogs)               |
| mshA    | PROKKA_01596 | Glycosyltransferase (M)                         |
| group_5291 | PROKKA_01598 | hypothetical protein (S)                        |
| group_5295 | PROKKA_01607 | MATE efflux family protein, flippase (Not in Cogs) |
| group_5296 | PROKKA_01608 | Glycosyltransferase (G)                         |
| epsJ_2  | PROKKA_01609 | putative glycosyltransferase EpsJ (M)           |
| group_5299 | PROKKA_01612 | pyruvyl transferase (M)                         |
| group_5302 | PROKKA_01684 | site-specific DNA-methyltransferase (L)        |
| group_5305 | PROKKA_01688 | hypothetical protein (Not in Cogs)              |
| group_5311 | PROKKA_01727 | competence CoiA-like family protein (R)        |
| group_5312 | PROKKA_01728 | hypothetical protein (S)                        |
| group_5319 | PROKKA_01736 | molecular chaperone Tir (Not in Cogs)           |
| group_5322 | PROKKA_01739 | hypothetical protein (Not in Cogs)              |
| group_5332 | PROKKA_02397 | hypothetical protein (E)                        |
| group_5341 | PROKKA_02408 | integrating conjugative element protein (Not in Cogs) |
| group_5342 | PROKKA_02409 | carbamoyl transferase (Not in Cogs)             |
| group_5343 | PROKKA_02410 | Glutamate synthase (Not in Cogs)                |
| group_5344 | PROKKA_02411 | HNH endonuclease (Not in Cogs)                  |
| group_5345 | PROKKA_02412 | hypothetical protein (S)                        |
| group_5348 | PROKKA_02415 | Zinc-binding dehydrogenase. (Not in Cogs)      |
| group_5349 | PROKKA_02417 | DNA adenine methylase (Not in Cogs)             |
| intA_3  | PROKKA_02432 | Prophage CP4-57 integrase, Bacteriophage P4 integrase (L) |
| group_5360 | PROKKA_02433 | Prophage CP4-57 regulatory protein (AlpA) (K)  |
| group_5361 | PROKKA_02434 | hypothetical protein (S)                        |
| group_5363 | PROKKA_02436 | integrase (Not in Cogs)                         |
| group_5365 | PROKKA_02438 | hypothetical protein (Not in Cogs)              |
| group_5368 | PROKKA_02441 | DNA primase (R)                                 |
| group_5369 | PROKKA_02442 | hypothetical protein (S)                        |
| group_5370 | PROKKA_02564 | hypothetical protein (S)                        |
| group_5371 | PROKKA_02565 | hypothetical protein (S)                        |
| group_5372 | PROKKA_02566 | hypothetical protein (S)                        |
| group_5382 | PROKKA_02576 | hypothetical protein (Not in Cogs)              |
| group_5393 | PROKKA_02589 | Repressor (Not in Cogs)                         |
| group_5395 | PROKKA_02591 | hypothetical protein (Not in Cogs)              |
| group_5396 | PROKKA_02592 | hypothetical protein (S)                        |
| ltrA    | PROKKA_03114 | Group II intron-encoded protein LtrA (X)        |
| group_5405 | PROKKA_03344 | hypothetical protein (S)                        |
| group_5406 | PROKKA_03345 | hypothetical protein (S)                        |
| group_5407 | PROKKA_03346 | hypothetical protein (Not in Cogs)              |
| group_5408 | PROKKA_03347 | Helix-turn-helix (E)                            |
| group_5409 | PROKKA_03348 | hypothetical protein (S)                        |
| group_5410 | PROKKA_03349 | tRNA, anti-like protein (Not in Cogs)           |
| group_5412 | PROKKA_04074 | Reverse transcriptase (RNA-dependent DNA polymerase) (L) |
| group_5417 | PROKKA_04503 | hypothetical protein (Not in Cogs)              |
| smc     | PROKKA_04512 | Chromosome partition protein Smc (R)             |
| group_5422 | PROKKA_04752 | hypothetical protein (Not in Cogs)              |
| group_5423 | PROKKA_04753 | hypothetical protein (Not in Cogs)              |
| group_5424 | PROKKA_04754 | Integrase (L)                                   |
| group_5426 | PROKKA_05105 | hypothetical protein (S)                        |

Continued
ClonalFrameML and Phylogenetic Analysis. To verify whether the RoGI island was acquired by lateral gene transfer, we used a recombination and phylogenetic analysis. Figure 3 shows the recombination events of external origin marked by a dark blue horizontal line. ClonalFrameML identified 170 recombination events on all branches of the clonal genealogy, including 23 recombination events in the genome of strain Marseille-P1025 (Fig. 3). These 23 regions appeared to be possible recombination hotspots (Fig. 3). Three of these recombination hotspots (red circle) were located in scaffold 21 of strain Marseille-P1025 (located from nucleotides 5,425,000 to 5,612,500) (Fig. 3), close to the RoGI island that coded the type IVa secretion system (located from nucleotides 5,504,317 to 5,515,790, Fig. 3).

The phylogenetic analysis of nucleotide sequences from the RoGI island supported the assumption that it was acquired by lateral gene transfer by identifying close phylogenetic neighbours in Pectobacterium atrosepticum strain JG10-08, Pectobacterium sp. strain SCC3193, two Pectobacterium wasabiae (strain CFBP-3304 and strain RNS08.42.1 A), Cedecea neteri strain ND14b and Citrobacter amalonaticus strain Y19 (Fig. S1).

Table 2. Functional annotation of the 95 specific genes of strain Marseille-P1025 among R. ornithinolytica strains. Genes from the RoGI genomic island are in bold.

| Genes    | Locus           | Putative function (COGs category)                      |
|----------|-----------------|--------------------------------------------------------|
| group_5427 PROKKA_05106 | hypothetical protein (Not in Cogs)                     |
| group_5429 PROKKA_05109 | FemK-like protein (s)                                 |
| group_5434 PROKKA_05117 | DNA polymerase V subunit UmuC (Not in Cogs)            |
| group_5440 PROKKA_05131 | hypothetical protein (S)                              |
| group_5441 PROKKA_05181 | hypothetical protein (S)                              |
| group_5443 PROKKA_05184 | hypothetical protein (S)                              |
| group_5444 PROKKA_05187 | hypothetical protein (S)                              |
| group_5445 PROKKA_05188 | hypothetical protein (S)                              |
| group_5446 PROKKA_05189 | hypothetical protein (Not in Cogs)                     |
| group_5447 PROKKA_05190 | hypothetical protein (Not in Cogs)                     |
| group_5448 PROKKA_05191 | IS110 family transposase (X)                           |
| group_5450 PROKKA_05193 | hypothetical protein (R)                              |
| traC_3 PROKKA_05194 | DNA primase TraC (L)                                   |
| group_5452 PROKKA_05195 | cysteine desulfurase activator complex subunit SuFD (Not in Cogs) |
| group_5453 PROKKA_05196 | Type IV secretory system Conjugative DNA transfer (Not in Cogs) |
| group_5454 PROKKA_05197 | conjugal transfer protein (Not in Cogs)                 |
| group_5455 PROKKA_05198 | Type IV secretion system protein VirB11 (U)            |
| group_5456 PROKKA_05199 | Type IV secretion system protein VirB10 (U)            |
| virB9 PROKKA_05200 | Type IV secretion system protein virB9 precursor (U)   |
| pflE PROKKA_05201 | Type IV secretion system protein PflE (VirB8) (U)      |
| group_5459 PROKKA_05202 | Type IV secretion system proteins VirB7 (Not in Cogs)   |
| group_5460 PROKKA_05203 | TrbL/VirB6 plasmid conjugal transfer protein (U)       |
| group_5461 PROKKA_05204 | Integrating conjugative element protein (Not in Cogs)   |
| group_5462 PROKKA_05205 | Type IV secretion system proteins (VirB5) (U)          |
| virB4 PROKKA_05206 | Type IV secretion system protein virB4(ATPase) (U)     |
| group_5465 PROKKA_05209 | hypothetical protein (Not in Cogs)                      |
| intA_5 PROKKA_05210 | Prophage CP4-57 integrase, Bacteriophage P4 integrase (L) |
| group_5475 PROKKA_05235 | hypothetical protein (Not in Cogs)                      |
| group_5483 PROKKA_05243 | hypothetical protein (Not in Cogs)                      |
| group_5484 PROKKA_05244 | hypothetical protein (Not in Cogs)                      |
| group_5485 PROKKA_05245 | hypothetical protein (L)                               |
| group_5488 PROKKA_05248 | hypothetical protein (S)                               |
| group_5493 PROKKA_05254 | hypothetical protein (Not in Cogs)                      |
| group_5518 PROKKA_05291 | hypothetical protein (S)                               |
| group_5536 PROKKA_05325 | inner membrane protein (s)                             |
| group_5541 PROKKA_05330 | hypothetical protein (Not in Cogs)                      |
| group_5542 PROKKA_05333 | ATPase (D)                                             |
| group_5543 PROKKA_05334 | hypothetical protein (Not in Cogs)                      |
| group_5545 PROKKA_05338 | hypothetical protein (Not in Cogs)                      |
| group_5566 PROKKA_01605 | hypothetical protein (Not in Cogs)                      |
Conjugative pilus. It is known that type IVa secretion systems elaborate pili to establish a host contact for substrate secretion or bacterial conjugation. In order to confirm that strain Marseille-P1025 elaborates a conjugative pilus, electron microscopy was performed on cells after 24 h of incubation. Figure 4 shows that strain Marseille-P1025 possesses a conjugative pilus.

Interaction of R. ornithinolytica with A. castellanii trophozoites. Acanthamoeba castellanii is a free-living amoeba that has previously been used as a eukaryote model to study the virulence of pathogenic microorganisms, including Acinetobacter baumannii, mycobacteria and streptococci. To determine whether strain Marseille-P1025 can multiply in eukaryotic cells, triplicate co-culture assays were performed with Acanthamoeba castellanii amoebae. Raoultella ornithinolytica strain P2310, isolated from the feces of a healthy individual, was used as a control for this experiment (Figs 5 and 6). We observed that the numbers of both uninfected and infected A. castellanii trophozoites incubated into PAS at 32 °C decreased over time. However, the mean percentages of remaining live amoebae at day 3 were 45.0±1.33%, 19.69±1.44% and 27.83±4.82% for uninfected amoebae, amoebae infected with strain P2310 and amoebae infected with strain Marseille-P1025, respectively. Therefore, the number of infected amoebae decreased significantly more than those of uninfected amoebae (p < 0.05) in presence of both R. ornithinolytica strains (Figs 5A, S2), and strain Marseille-P1025 caused a higher amoebal mortality than strain P2310, although this difference was not statistically different (p = 0.17).

We also evaluated the numbers of CFUs obtained from intra-amoebal bacteria at H0 and H72 of co-culture (Figs 5B, S2). At H0 and H72, a mean 3.57×10⁵ CFUs/mL and 9.67×10⁷ CFUs/mL, respectively, were cultivated for strain Marseille-P1025 versus 5.73×10⁵ CFUs/mL and 9.33×10⁷ CFUs/mL, respectively, for strain P2310. The growth rate of both strains (270±97.5 and 174±29.3, respectively) was significantly higher for strain Marseille-P1025 (p < 0.05). Hence, these experiment demonstrated that strain Marseille-P1025 exhibited a higher pathogenicity for amoebae than the control strain (Fig. 5B). To confirm these observations, we examined bacteria within amoebae by Gimenez staining. Optical microscopy observations were consistent with the CFU evaluations. We observed that after 5h of co-culture, most A. castellanii cells were infected by R. ornithinolytica strain Marseille-P1025 (Fig. 6A). Not only was R. ornithinolytica strain Marseille-P1025 able to survive within A. castellanii, but it began to multiply after 24 hours of co-culture (Fig. 6B). At day 2 of co-culture, strain Marseille-P1025 continued to multiply within amoebae. Furthermore, at day 3 of co-culture, infected amoebae started to lyse (Fig. 6D) whereas strain Marseille-P1025 kept multiplying. The lysis of A. castellanii amoebae was complete after 5 days of co-culture with bacteria (Fig. S3). We also observed that R. ornithinolytica survived in PAS medium without amoebae but did not multiply from day 0 to day 3.

Discussion
Infections due to R. ornithinolytica are under-reported, possibly because this bacterium is difficult to identify using conventional phenotypic methods. Raoultella ornithinolytica is currently regarded as an emerging hospital-acquired infection agent, particularly after invasive procedure. Few pathogenic factors are recognized in R. ornithinolytica compared to other members of the family Enterobacteriaceae. These include the ability to adhere to human tissues, to form biofilms in urinary catheters and to convert histidine to histamine in scombroid fishes, thus causing redness and flushing of the skin.

By comparing the genome of strain Marseille-P1025 that had caused a chronic prosthetic joint infection in an immunocompetent patient, to those of other R. ornithinolytica strains, we identified a unique 11-kb genomic island (RoGI) among R. ornithinolytica strains. This genomic island coded nine proteins from the type IVa...
secretion system (SS) (Fig. 2, Table 2), four proteins related to bacterial conjugation and two integrases. In addition, the genome from strain Marseille-P1025 contained another four components of the type IVa SS, which suggests that this system was complete. The presence of a conjugative pilus and the ability of strain Marseille-P1025 to invade, survive and multiply in an amoeba (Acanthamoeba castellanii) confirms the presence of type IVa secretion system.

The type IVa SS is involved in various mechanisms of bacterial pathogenesis such as the transfer of Agrobacterium tumefaciens oncogenic DNA into plants leading to tumours. The Type IVa SS is also involved in conjugation and thus plays a crucial role in genomic plasticity, notably by enabling the transfer of plasmids carrying antibiotic resistance or virulence genes among pathogenic bacteria. In addition, conjugation systems may also contribute significantly to the development of infections by promoting surface pilus adhesion-mediated attachment, colonization and biofilm formation. It is also reported that the type IVa secretion system, particularly the virB operon, is essential for survival and intracellular multiplication. Siddiqui et al. have shown that pathogenic bacteria, but not those who are weakly or not pathogenic, can survive within A. castellanii cysts. The ability of R. ornithinolytica to grow and cause cytopathic effect in A. castellanii seems to be correlated with its virulence. We demonstrated that strain Marseille-P1025 was not only able to survive within amoebae but could also multiply more efficiently than the control strain and kill amoebae, supporting its virulent behavior.

We also demonstrated that the RoGI genomic island of strain Marseille-P1025 was closely related to sequences from the Pectobacterium atrosepticum strain JG10-08, Pectobacterium sp. strain SCC3193, two Pectobacterium
wasabiae (strains CFBP-3304 and RNS08.42.1A), Cedecea neteri strain ND14b and Citrobacter amalonaticus strain Y19 (Fig. S1). As the genomes of P. wasabiae strains CFBP-3304 and RNS08.42.1A, P. atrosepticum strain JG10-08 and Pectobacterium sp. strain SCC3193 exhibit genomic G + C contents of 50.6%, 50.4%, 51.1% and 50.4%, respectively, which is closer to that of the RoGI island (49.5%) than that of strain Marseille-P1025 (55.6%), we assume that it may have been transferred from a Pectobacterium or a phylogenetically-close species. Pectobacterium species (P. wasabiae and P. atrosepticum) are phytopathogens 22,23 that belong to the Enterobacteriaceae family like R. ornithinolytica.

We also detected the presence of three specific genes carried by the CP4-57 prophage, including two integrases (intA) and alpA, a transcriptional regulator of intA.24 The IntA integrase has been shown to intervene in biofilm formation. In E. coli, the deletion of the intA gene reduces early biofilm formation24 whereas the increased synthesis of IntA leads to excision of the CP4–57 prophage24 which, in turn, increases biofilm formation24. Therefore, IntA may play a role in biofilm formation in strain Marseille-P1025, thus facilitating its adhesion to foreign material such as the patient’s joint prosthesis.

In conclusion, R. ornithinolytica strain Marseille-P1025, that caused a rare case of chronic prosthetic joint infection in a 67-year-old immunocompetent male, exhibited a complete type IVa secretion system that was unique among R. ornithinolytica strains and was able to infect, multiply within, and kill amoebae. These properties may explain its particular virulence. In addition, this type IVa SS was mostly coded by a genomic island (RoGI) that was probably acquired by lateral gene transfer from Pectobacterium species.

Material and Methods
DNA extraction and Genome sequencing. Strain Marseille-P1025 was cultivated on Columbia agar (bioMérieux, Marcy-l’Etoile, France) at 37 °C in aerobic atmosphere for 24 hours. Then, after a pre-treatment with lysozyme at 37 °C for 2 hours, the DNA was extracted using an EZ1 biorobot and the EZ1 DNA tissue kit (Qiagen, hilden, Germany). The elution volume was 50 µL. Genomic DNA (gDNA) was quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA) to 8 ng/µl, prior to being sequenced on a MiSeq sequencer (Illumina, San Diego CA, USA) with the Paired-End and barcode strategy in order to be mixed with 20 other projects constructed according to the Nextera XT library kit (Illumina).

One ng of gDNA was used as input and augmented for the fragmentation step. Then, limited cycle PCR amplification completed the tag adapters and introduced dual-index barcodes. The libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina), pooled into a single library and then loaded onto the reagent cartridge. Automated cluster generation and Paired-End sequencing with dual index reads was performed in a single 39-hour run in a 2 × 251-bp.

Total information of 9.8 Gb was obtained from a 1,165 K/mm 2 cluster density with 88% (18,993,000 clusters) of the clusters passing quality control filters. Within this pooled run, the index representation of R. ornithinolytica strain Marseille-P1025 was determined to be 5.51%. The 1,046,713 Paired-End reads were filtered according to the read qualities.
Genome annotation and comparison. The sequencing reads were assembled using the A5 assembler\textsuperscript{25}. Then, a step of finishing was done using the Mauve software\textsuperscript{26} and CLC bioserver. After assembly and finishing, the genome size was 5.6 Mb. Open reading frames (ORFs) were predicted using the Prodigal tool (http://prodigal.ornl.gov) with defaults parameters. The prediction of protein function was performed by searching against the GenBank database using BLASTP algorithm\textsuperscript{27}. Functional classification of gene families (COG ID and Letters) was obtained using COGnitor against the COG database\textsuperscript{28}. tRNAs and rRNAs were detected using tRNAscan-SE v.1.21\textsuperscript{29} and RNAmmer v.1.2\textsuperscript{30}, respectively. The presence or absence of plasmids was verified both by searching the gene annotation for any plasmid-related gene and by mapping all contigs against previously published \textit{Raoultella} sp. plasmid sequences.

We compared the genome sequence of \textit{R. ornithinolytica} strain Marseille-P1025 to those of other strains of this species found in public databases. As of August 30\textsuperscript{th}, 2016 13 \textit{R. ornithinolytica} genomes were available in public databases. Of these, we used 12 genomes for comparative analysis and excluded that of strain S12 due to its insufficient quality. The twelve comparator genomes were those from strains 10-5246 (AGDM00000000), NBRC 105727 (BCYR00000000), B6 (CP004142), A14 (CP008886), CMUL058 (CVRH00000000), TNT (JHQH00000000), 2-156-04_S1_C1 (JNPC00000000), 2-156-04_S1_C2 (JNPD00000000), 811_RORN (JURX00000000), BAL286 (JXXF00000000), CB1 (LFBW00000000) and Yangling I2 (CP013338). All genomes were re-annotated using the Prokka software, version 1.11\textsuperscript{31}. Comparisons between all selected genomes were done using Roary, a tool that rapidly builds large-scale pangenomes\textsuperscript{32}, with a blast identity cut-off of 97% for the comparison between \textit{R. ornithinolytica} species. In addition, Roary identified the specific and missing genes from strain Marseille-P1025. Specific genes were checked by BLASTP and TBLASTN against the other studied genomes. Missing genes were checked by TBLASTN against the genome of strain Marseille-P1025, using a coverage and identity of 60% and 80% as thresholds, respectively, as described by Kuenne \textit{et al.}\textsuperscript{33,34}. Easyfig\textsuperscript{35} was used to visualise the coding regions and colour the specific genes of strain Marseille-P1025.

Recombination and Phylogenetic analysis. The genome of strain Marseille-P1025 was used as a reference for whole-genome alignment\textsuperscript{36} using Mugsy\textsuperscript{37}. Then, a phylogenetic tree based on whole genome sequence alignment was done using the FastTree software\textsuperscript{38} and the maximum likelihood method (Fig. 1). ClonalFrameML was used to search recombination hotspots in \textit{R. ornithinolytica} genomes by analyzing both the whole genome alignment and the phylogenetic tree\textsuperscript{39}.

Unique sequences were detected by a BLASTN search for homologous sequences and multiple sequence alignment using the Mafft software algorithm\textsuperscript{40}. Phylogenetic analysis of these unique sequences was performed using MEGA version 7\textsuperscript{41} and the maximum likelihood (ML) algorithm, with 1,000 bootstrap replicates.
Electron microscopy.  Electron microscopy was performed with detection Formvar coated grids. Forty 40 μL of bacterial suspension were deposited on a grid and incubated at 37 °C for 30 min, followed by a 10 sec incubation on ammonium molybdate 1%. Grids were then observed using a Morgagni 268D transmission electron microscope (Philips) at an operating voltage of 60 kV.

Culture of Raoultella ornithinolytica and A. castellanii.  Raoultella ornithinolytica strain P2310, isolated from the feces of a healthy individual, was used as a control in co-culture experiments. Raoultella ornithinolytica strains Marseille-P1025 and P2310 were grown on 5% sheep blood-enriched Columbia agar (BioMérieux) at 35 °C for 24 hours in anaerobic atmosphere. Bacteria were then harvested, centrifuged at 4,000 × g during 5 minutes, washed twice and suspended in PAGE modified Neff’s amoeba saline (PAS). The PAS medium was prepared as follows: solution A (for 100 mL of sterile distilled water), 1.2 g NaCl + 0.04 g MgSO4.7H2O + 1.42 g Na2HPO4 + 1.36 g KH2PO4 solution B (for 100 mL of sterile distilled water), 0.04 g CaCl2, 2H2O; PAS solution − 10 mL of solution A + 10 mL of solution B + 980 mL of sterile distilled water. The inoculum density was determined by the McFarland method.

*Acanthamoeba castellanii* strain Neff (ATCC 30010) was grown in 175 cm2 culture flasks containing 30 mL peptone-yeast extract-glucose (PYG) at 28 °C. When a monolayer was formed, it was harvested by shaking the flasks and centrifuged at 500 × g for 10 min. The pellet was suspended in 30 mL PAS medium. The quantification of the *A. castellanii* population was performed using a COVA® slide cell counting chamber.

Co-culture experiments.  The amoebal trophozoite suspension (5 × 105 amoeba/mL) was inoculated in 24-well plates and allowed to adhere for 30 minutes at 32 °C. Then, *R. ornithinolytica* suspensions were inoculated on amoebae to achieve ratios of infection of 10 *R. ornithinolytica* cells/amoeba. As controls, *A. castellanii* and *R. ornithinolytica* strains were cultivated separately in PAS. After incubation for 2.5 h at 32 °C under a 5% CO2 atmosphere, the co-culture wells were washed three times with PAS to remove any remaining extracellular or adherent bacteria. Incubation at 32 °C under 5% CO2 was then performed for 3 days. The presence of viable *Raoultella* inside amoebal trophozoites was documented by sub-culturing at 0, 24, 48 and 72 h of incubation. For each time point, the *A. castellanii* monolayer from a well was lysed by three passages through a 25-gauge needle. Serial dilutions of the lysate were carried out, plated onto COS medium and incubated for 2 days at 32 °C under anaerobic atmosphere to determine the numbers of intracellular *R. ornithinolytica* colony forming units (CFU). Multiplication rate of the bacterial invasion was calculated as follows: recovered *R. ornithinolytica* (CFU)/*R. ornithinolytica* (CFU) at time 0. The *A. castellanii* population was also monitored during the 3-day experiment: counting and viability check of amoebae, cultivated alone and in co-culture, was performed using COVA® slides after Trypan Blue 0.4% coloration (Sigma-Aldrich, Taufkirchen, Germany). All experiments were reproduced three times, each time in duplicate. The standard error of the mean (SEM) was used to evaluate the experiment value distribution. To compare the intra-amoebal growth of the two tested bacterial strains, we also estimated the daily multiplication rate of bacteria.

The presence of *R. ornithinolytica* within amoeba was also monitored for 5 days by Gimenez staining42. The observation was performed with a LEICA DM 2500 LED microscope.

Statistical analyses.  Statistical analyses mentioned in this study were performed using the Student's t-test and Chi-square test, with a significance level of *P* inferior or equal to 0.05.

Nucleotide sequence accession numbers.  The genome sequence from *R. ornithinolytica* strain Marseille-P1025 was deposited in GenBank under accession number FTLF01000000.
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Author Contributions
Mamadou Beye wrote the manuscript and performed the phenotypic and genomic analyses; Issam Hasni performed the amoebal infection experiments and imaging analyses and edited the manuscript; Piseth Seng took care of the patient and edited the manuscript; Caroline Michelle performed the genomic sequencing and, assembly and edited the manuscript; Bernard La Scola performed the amoebal infection experiments and edited the manuscript; Didier Raoult designed the study, analysed the data and wrote the manuscript; Pierre-Edouard Fourrier designed the study, analysed the data and wrote the manuscript.

Additional Information
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