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1 **In-host evolution of *Yersinia enterocolitica* during a chronic human infection**

2

3 **Running title:** Human yersiniosis in-host evolution

4

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39

40

41 **Abstract**

42 Following a pacemaker implantation, a 75-years-old patient suffered from five successive
43 bacteremia episodes between in 1999 and 2013 despite long-term antibiotic treatment, with
44 intermittent vegetation apparition on the device atrial lead. Four blood isolates, identified as *Yersinia*
45 *enterocolitica* bioserotype 4/O:3, were further genetically and phenotypically characterized.
46 Phylogenetic reconstruction showed that the patient was chronically infected by the same strain,
47 which evolved within the host for 14 years. Single-nucleotide polymorphism (SNP) analysis indicates
48 that the last two isolates evolved in parallel and formed two independent lineages within the host.
49 Pan-genome analysis and genome comparison showed that their common evolution was
50 characterized by 41 small insertion/deletion events, loss of three large DNA fragments and mutations
51 in 140 genes. A phylogenetic analysis by maximum likelihood identified two genes presenting a
52 positive selection signal, suggesting that these mutations provided a survival advantage to bacteria
53 during chronic infection. Quinolone resistance in the last two isolates was acquired through a so far
54 undescribed deletion in the *gyrA* gene.

55 Mass-spectrometry analysis revealed a strong proteome remodeling in the last two isolates
56 which was correlated with a truncation in the stringent response regulator DksA. A reduced carbon,
57 energy and purine metabolism supports their severe growth defects *in vitro*. 3rd-generation
58 cephalosporin resistance of the last isolate was correlated with a truncation of OmpF, the main porin
59 translocating antibiotics through the outer-membrane, as well as an increased production of BlaA
60 and AmpC β -lactamases.

61 This is the first report of genetic and phenotypic changes associated to within-host
62 adaptation of a pathogenic *Yersinia* species under antibiotic pressure.

63

64 **Keywords** : bacteremia/genomics/proteomics/within-host evolution/yersiniosis

65 Introduction

66 Bacterial exposure to a novel environment in a host creates conditions where evolution
67 might take place. As observed for cancer cells, a bacterial clone can experience selection for an
68 accumulation of genetic variants that promote long-term survival and clonal expansion¹. This
69 scenario has been observed in cystic fibrosis patients who can be chronically infected with
70 pathogenic bacteria. In a study from a single patient infected for 20 years with *Burkholderia*
71 *multivorans*, bacterial adaptation to respiratory airways was associated with mutations affecting
72 metabolism, cell envelope, and biofilm formation². In a different study concerning 474 *P. aeruginosa*
73 isolates from 34 cystic fibrosis patients, convergent evolution of 52 genes involved in antibiotic
74 resistance, motility, and biofilm formation was observed³. Host-adaptation through rapid evolution
75 of a *Salmonella enterica* serotype Enteritidis causing a chronic systemic infection was documented in
76 an immunocompromised patient⁴. Genetic diversification of *Staphylococcus epidermidis* has also
77 been described during a 16-week pacemaker-associated endocarditis, leading to increased biofilm
78 formation, reduced growth rate, and antibiotic tolerance⁵.

79 In-host evolution has not been reported for infections associated with *Yersinia* spp.
80 Yersinioses include fulminant infections such as plague caused by *Yersinia pestis*, as well as mild or
81 severe enteritis caused by *Yersinia pseudotuberculosis* or *Yersinia enterocolitica* (Ye)^{6,7}. The latter
82 represents the third most common cause of diarrhea from bacterial origin in temperate and cold
83 countries⁸. Systemic Ye infections occur mostly in elderly patients with underlying disorders such as
84 diabetes, iron overload, or cirrhosis⁹.

85 Persistent Ye infections in patients causing relapses for several years have been reported¹⁰,
86 but the potential genetic relationships between the isolated strains was not determined. This study is
87 the first report of long-term, in-host evolution of a pathogenic *Yersinia* species in a patient
88 presenting iterative episodes of bacteremia over 14 years.

89

90 Results

91 Case description

92 A 75-year-old woman presented with an atrioventricular block that led to a pacemaker
93 implantation in September 1998. In December 1999, the patient had a first septicemic episode
94 resulting in the isolation of a Ye strain (Ye.1) (Fig 1). She received ceftriaxone and netilmicin
95 treatment for 4 weeks and recovered. In January 2000, the patient experienced a second episode of
96 bacteremia with isolation of another Ye strain (Ye.2). She was treated with the same antibiotics for
97 four weeks, followed by 18 months of ciprofloxacin therapy (until July 2001), during which monthly

98 blood cultures were negative. A third bacteremia occurred in August 2001 with isolation of a third *Ye*
99 (*Ye.X*, not kept in collection) that was resistant to nalidixic acid but susceptible to ciprofloxacin, which
100 had been used for treatment. The patient received a long-term ceftriaxone therapy until July 2005,
101 and monthly blood cultures were negative. In June 2006, the patient was hospitalized for a sepsis but
102 had a negative blood culture. For the first time, echocardiography evidenced vegetations on the
103 pacemaker atrial lead, suggesting endocarditis due to bacterial growth on the cardiac device (Fig S1).
104 A 6-year ceftriaxone treatment was administered, leading to vegetation disappearance. In December
105 2012, the patient suffered from pneumonia, and ceftriaxone treatment was replaced by spiramycin.
106 In January 2013, she presented with another bacteremia with isolation of a fourth *Ye* strain (*Ye.3*)
107 and vegetations reappeared on the pacemaker atrial lead. Strain *Ye.3*, like *Ye.X*, was resistant to
108 nalidixic acid. The patient was treated with piperacillin/tazobactam/amikacin for one week, followed
109 by a 9-month ceftriaxone therapy. The patient had a last bacteremia in October 2013, during which a
110 fifth *Ye* strain (*Ye.4*) was isolated. Minimum inhibitory concentration (MIC) of ceftriaxone increased
111 from 0.19 mg/L (*Ye.3*) to 2 mg/L (*Ye.4*) leading to antimicrobial treatment modification with
112 cotrimoxazole. Three months later, the patient died of heart failure.

113

114 **Phenotypic characterization of *Ye* isolates shows in-host evolution of antibiotic susceptibility** 115 **during long term treatment**

116 The five strains isolated during the iterative bacteremia were initially identified as *Ye* using a
117 VITEK2 GN card. Except for *Ye.X*, which was not kept in collection, strains were subjected to
118 phenotypical characterization: *Ye.1* and *Ye.2* (also referred as “early isolates”) were identified as *Ye*
119 bioserotype 4/O:3; due to a severe growth defect, *Ye.3* and *Ye.4* (also referred as “late isolates”)
120 could not be characterized by phenotypic methods. A substantial increase in mid-exponential phase
121 doubling time at 37°C was measured, from 49 and 44 minutes for *Ye.1* and *Ye.2* to 5h38 and 4h46 for
122 *Ye.3* and *Ye.4*, respectively. Doubling times at 28°C were slightly longer, with 57 minutes for *Ye.1* and
123 *Ye.2*, 6h49 for *Ye.3* and 5h28 for *Ye.4* (Fig S2).

124 After isolation of the four strains, we parallelly assessed the susceptibility of *Ye.1* to *Ye.4* to
125 33 different antibiotics or antibiotics combinations (Table S1). All strains were resistant to the
126 penicillins amoxicillin and ticarcillin. *Ye.1* and *Ye.2* were resistant to first-generation cephalosporin
127 (C1G) (data not shown) and they were susceptible to all other tested antibiotics, as usually observed
128 for *Ye* bioserotype 4/O:3, although *Ye.2* showed a small resistance increase to several β -lactams and
129 netilmicin, the aminoglycoside (AG) which was administered in combination with the 3rd generation
130 cephalosporin (C3G) ceftriaxone between *Ye.1* and *Ye.2* isolation. *Ye.3* and *Ye.4* acquired a resistance

131 to the quinolone nalidixic acid, as observed with Ye.X, and Ye.4 became resistant to the second-
132 generation cephalosporin (C2G) ceftiofur.

133 To further characterize the antibiotic profile of the isolated strains, the MIC of 10 antibiotics
134 and antibiotics combinations was measured (Table 1). While the MIC of different antibiotics classes
135 such as cephalosporin, AG and fluoroquinolone (FQ) slightly increased between Ye.1 and Ye.2, an
136 unexpected AG and FQ MIC decrease was observed for the late isolates Ye.3 and Ye.4 compared to
137 the early isolates Ye.1 and Ye.2. Furthermore, Ye.4 was fully resistant to the C3G ceftriaxone as well
138 as the C2G ceftiofur, as observed before, and was slightly more resistant than Ye.3 to piperacillin-
139 tazobactam, the combination of a penicillin and a β -lactamase inhibitor administered before
140 ceftriaxone between Ye.3 and Ye.4 isolation.

141

142 **Genetic analysis supports a chronic *Ye* infection, unravels a new quinolone resistance mutation in** 143 ***gyrA* and reveals a OmpF-mediated cephalosporin resistance**

144 Taxonomic assignment of the 4 isolated strains was obtained based on a core-genome
145 multilocus sequence typing (cgMLST) with 500 core genes using whole-genome *de novo* sequence,
146 confirming that strains Ye.1 to Ye.4 belong to the *Ye* genotype 4, corresponding to bioserotype 4/O:3.

147 To investigate whether the multiple infection episodes were independent or due to a chronic
148 colonization by a unique evolving bacterial strain, we studied the genetic relatedness of strains Ye.1
149 to Ye.4 by a core-genome single-nucleotide polymorphism analysis, with inclusion of 259 additional
150 *Ye* biotype 4 strains isolated between 1963 and 2016 (Table S2). The phylogenetic tree
151 reconstruction based on analysis of 4,738 SNPs showed that Ye.1 to Ye.4 were closely related and
152 formed a unique clade separated from other *Ye* strains (Fig 2A). This result strongly suggests that the
153 patient suffered from a chronic infection due to a unique evolving *Ye* 4/O:3 strain.

154 We next analyzed the molecular events that characterized the in-host evolution of Ye.1 to
155 Ye.4. Genomic comparison and pan-genome phylogenetic analysis did not evidence any gene
156 acquisition or small insertion/deletion events that were common and specific to the four *Ye* isolates.
157 Ye.1 (isolated in December 1999) and Ye.2 (isolated in January 2000) were identical except for the
158 loss in Ye.1 of a 1.5 kb region (between nucleotides 4,282,424 and 4,283,952 from the Y11 reference
159 genome), indicating that evolution had already started within the first months of infection. The use
160 of the least-squares dating tool (<https://lsdating.pasteur.fr>) estimated the closest internal node for
161 Ye.1 and Ye.2 in October 1999, suggesting that the patient was contaminated at least two months
162 before the first bacteremic episode (Fig 2B). The closest internal node for Ye.3 (isolated in January
163 2013) and Ye.4 (isolated in October 2013) was January 2011 (Fig 2B). However, a truncation in the
164 mutation frequency decline *mfd* gene and the slow growth phenotype of the late isolates should be

165 considered, thus the divergence between Ye.3 and Ye.4 could have happened earlier. Their common
166 ancestor had therefore evolved in the patient for at least 11 years and accumulated 285 SNPs before
167 diverging into Ye.3 and Ye.4, which since accumulated additional 31 and 52 SNPs, respectively (Table
168 S3). 140 genes containing mutations vertically acquired from their common ancestor
169 (synapomorphies) were identified in the Ye.3 and Ye.4 genomes, including the *gyrA* gene (see below
170 and Table S4). Ye.3 and Ye.4 shared three identical large deletions: a 2-gene deletion of 2.5 kb, a 9-
171 gene deletion of 9.9 kb, and a 28-gene deletion of 32.7 kb (Table 2). These strains also shared 41
172 small insertions/deletions (Table 2). Whereas 6 genes did not exhibit any frameshift, 35 genes
173 displayed a mutation leading to a truncated form of the product, and thus to loss-of-function.
174 Truncated genes and deleted regions were found to be involved in essential physiological functions
175 such as transcription, translation, replication, respiration, division, and carbohydrate and iron
176 metabolism (Table 2).

177 A positive selection signal was detected in all isolated strains, in the acyl-CoA thioesterase II,
178 which regulates levels of acyl-CoAs, free fatty acids, and coenzyme A. In position 280 of this enzyme,
179 a glycine is normally conserved in diverse eukaryotic and bacterial species¹¹ while in Ye.1 to Ye.4 a
180 serine was present. Position 280 is located next to important residues involved in two catalytic triads
181 (Q278 together with N204 and T228, as well as E279 together with H58 and S107) and a dimer
182 interface (V277 and V281) and could possibly affect the catalytic and functional activities of the acyl-
183 Coa thioesterase. Another positive selection signal was present in a phosphate transporter, for which
184 the C-terminal region displayed a deletion observed only in Ye.3 and Ye.4 that affects two
185 cytoplasmic and two transmembrane domains. While we did not investigate the specific contribution
186 of this deletion to the functionality of the phosphate transporter, the positive selection signal
187 suggests that the promoted changes provided a survival advantage (Table S5).

188 The genetic basis of antibiotic resistance was investigated upon bacterial genome
189 sequencing. Comparison of *gyrA* sequences of Ye.1 to Ye.4 with those of the reference strain Y11 and
190 strain IP38477, which are susceptible to nalidixic acid, showed that Ye.3 and Ye.4 displayed the same
191 3-nucleotide deletion at positions 245 to 247 in the quinolone resistance-determining region (QRDR)
192 of *gyrA* (Fig S3A). This so far undescribed deletion leads to replacement of aspartic acid 82 and serine
193 83 with a unique glycine without any frameshift, explaining the genetic basis of the resistance to
194 quinolones in Ye.3 and Ye.4 (Fig S3B). Additionally, a nucleotide substitution introduced a stop codon
195 in position 78 of the Ye.4 *OmpF* porin, truncating the protein at one fifth of its length. Porin
196 mutations are usually associated with a decreased antibiotics uptake through the outer-membrane
197 and could explain cefoxitin and ceftriaxone resistance.

198 **Comparative proteomics of *Ye* isolates reveals an extensive metabolic rewiring, reduced virulence**
199 **determinant expression and possibly OmpF-mediated antibiotics resistance**

200 To further characterize *Ye* evolution during the course of infection, we analyzed proteomes
201 of the four isolates by mass spectrometry. We measured between 2,086 and 2,287 proteins in the 20
202 samples (5 replicates of the 4 strains), for a total of 2,408 different proteins identified among all
203 samples against a total database containing 4,424 unique proteins of the 4 isolates. All pairwise
204 comparisons for protein differential abundance between strains were performed. 277 proteins were
205 differentially abundant between the two early isolates *Ye*.1 and *Ye*.2 and 323 proteins between the
206 two late isolates *Ye*.3 and *Ye*.4. In contrast, 845 to 1,070 proteins were differentially abundant in the
207 4 pairwise comparisons of early versus late isolates, which account for almost half of the identified
208 proteome (Dataset S1). These proteome differences correlate with the genetic distances of early and
209 late isolates.

210 The annotated proteins from *Ye*.1 to *Ye*.4 were mapped to the *Ye* genotype 4 reference
211 strain Y11 (Table S6). Gene set enrichment analysis (GSEA) on biological process gene ontology (GO)
212 terms revealed isolate-specific variations as well as common trends in early versus late isolate
213 comparisons (Fig 3). The loss of the pYV virulence plasmid (probably a laboratory artifact) in all
214 strains but *Ye*.1 could be observed through the enrichment of the type III secretion system term in
215 *Ye*.1. *Ye*.2 was specifically characterized by an enriched galactose metabolism. Translation and tRNA
216 processing terms were strongly upregulated in late isolates and even more in *Ye*.4. Amino acid
217 biosynthetic process was the most specific enriched term in late isolates versus early isolates.
218 Mapping of the protein abundance comparisons on the global metabolic map of Y11 (Fig S4, S5) and
219 on specific KEGG pathways (Fig S6) shows a strong metabolic rewiring from early to late isolates, such
220 as a decreased purine, pyrimidine or riboflavin metabolism, one-carbon pool by folate, carbohydrate
221 metabolism and oxidative phosphorylation, and a perturbed amino acid metabolism such as
222 increased histidine, serine, tryptophan or leucine biosynthesis, or reduced arginine or methionine
223 biosynthesis and glycine cleavage (Fig S6).

224 Virulence-associated proteins such as the attachment locus Ail, the fimbriae MyfA, the serine
225 protease ecotin, superoxide dismutases or the positive regulator RovA were less abundant in late
226 isolates, correlating with the increased abundance of the negative virulence regulators RovM or
227 YmoA (Table 3). Additionally, a mutation in the initiation codon of the invasin *Inv* completely
228 abrogated its production in *Ye*.4. On the other hand, the chemotactic operon *cheAWYZ* was more
229 expressed in late isolates, along an increased expression of quorum sensing autoinducer-1 *yenI* and a
230 decreased expression of autoinducer-2 operon *IsrGFBDcARK*. The invasin *Inv* was only slightly
231 downregulated in *Ye*.3 but strongly in *Ye*.4 compared to early isolates.

232 Iron import systems such as the heme import system encoded by the *hmuRSTUV* operon or
233 the TonB/ExbB/ExbD transport system were more abundant in the late isolates, while iron storage
234 abundance such as the non-heme ferritin FtnA or the bacterioferritin Bfr was decreased. All these
235 iron metabolism protein changes correlated with a decreased abundance of the iron metabolism
236 repressor Fur, showing a probable adaptation to the iron-scarce environment during *Ye* evolution in
237 the bloodstream.

238 We looked at the proteins level of resistance and susceptibility factors such as β -lactamase,
239 porin or efflux pump. An upregulation of the category A β -lactamase BlaA was observed for the late
240 strains, negatively correlating with the regulator AmpE. More importantly, the category C BlaB (or
241 AmpC) β -lactamase was more abundant in the ceftriaxone-resistant isolate Ye.4 compared to all
242 other isolates. In addition to its truncation in Ye.4, the porin OmpF was less abundant in Ye.2
243 compared to Ye.1, probably causing a decreased permeability of the outer-membrane to antibiotics
244 (Table 3). The OmpC porin showed variable abundance levels, correlating with OmpR/EnZ two
245 components system abundance, while other porins such as OmpW and two other porins (Y11_17651
246 and Y11_12201) were strongly upregulated in the late isolates.

247

248 **Truncation of the stringent response regulator DksA is one of the main drivers of proteome** 249 **changes**

250 To better understand which genetic difference could be the cause of the overall changing
251 proteome, the correlation of the 6 pairwise comparisons was assessed against 57 published *Ye*
252 transcriptome comparisons (Table S7) that we previously gathered on the Yersiniomics database¹².
253 Interestingly, early vs late isolate comparisons had a correlation coefficient ranging from -0.29 to -
254 0.43 with the comparisons of a *Ye* WT vs $\Delta dksA$ mutant and was one of the most correlated
255 experiments in our dataset (Table S8, Fig S7). These transcriptomes originated from a recent RNA-
256 Sequencing study exploring the *Ye* stringent response on *Ye* bioserotype 1B/O:8 in WT, $\Delta dksA$,
257 $\Delta relA\Delta spoT$ and $\Delta dksA\Delta relA\Delta spoT$ strains¹³. We considered the correlations with our proteomes as
258 high considering the variation in the *Ye* genotype and culture conditions used, as well as the analytic
259 differences between RNA-sequencing based transcriptomics and mass spectrometry-based
260 proteomics. Strikingly, a truncation in *dksA* gene is also observed in our late isolates (Table 2), where
261 only the 72 first amino acids (AA) out of 153 AA remains. This truncation could explain the
262 differential abundance of ribosome-associated proteins such as the ribosome maturation factor
263 RimM, ribosome hibernation promoting factor Hpf or ribosome-associated translation inhibitor RaiA,
264 ultimately leading to ribosomal protein upregulation. tRNA-associated proteins were also more
265 abundant in late isolates in a *dksA*-dependent way (Table S9). Other protein abundance changes

266 correlating with *dkSA* inactivation include the more abundant previously mentioned hemin
267 transporter subunits HmuR, HmuS, HmuT, magnesium/cobalt transporter CorA, sulfurtransferase
268 TusA, the shikimate and proline pathway enzyme AroB, AroK, ProA and ProB, DNA-binding
269 transcriptional regulator Fis, two of the porins (Y11_12201, Y11_17651) or the acetate uptake
270 transporter SatP among other. On the other hand, *dkSA* inactivation was associated to the
271 downregulation of the previously mentioned *lsr* operon as well as the urease *ureABCDEFGD* and
272 glycogen utilization *glgBXCAP* operons, of the ferritins FtnA and Bfr, stress response protein ElaB,
273 superoxide dismutase SodC, entericidin A/B, glycine cleavage system enzyme GcvT or fatty acid
274 degradation enzymes FadA and FadB (Table S9). Interestingly, *dkSA* deletion also correlated with a
275 strong decrease in *acs* and *actP* genes, which were described as truncated in late isolates.
276 Most importantly, the stationary phase sigma factor RpoS (σ^5) expression was *dkSA*-dependent, and
277 probably explain most of the regulated proteins such as ribosomal and metabolic enzymes.

278

279 Discussion

280 This is the first report of a chronic *Ye* infection case due to a strain evolving in the same
281 patient. Between December 1999 and October 2013, antibiotics were apparently successful in the
282 treatment of five independent *Ye* bacteremia episodes; however, discontinuation of antibiotics was
283 always followed by a new bacteremia. The appearance of vegetations observed by echocardiography
284 in 2006, and their disappearance during therapy, argue for *Ye* growth on the atrial lead of the
285 pacemaker and release into the bloodstream during the antibiotic discontinuation periods.
286 Genomic and proteomic analyses of four *Ye* strains (Ye.1 to Ye.4) isolated from the patient allowed us
287 to identify different mechanisms to resist to various antibiotic classes. Resistance to nalidixic acid
288 was already observed from August 2001 after long-term ciprofloxacin treatment. Resistance to
289 quinolones usually involves mutations in the QRDR of the *gyrA* gene, leading either to (i) a change of
290 glycine 81 into cysteine, (ii) a change of serine 83 into arginine, isoleucine, or cysteine, or (ii) a
291 change of aspartic acid 87 into a tyrosine, asparagine, or glycine¹⁴⁻¹⁷. In Ye.3 and Ye.4, we observed a
292 previously undescribed deletion of 3 nucleotides in the *gyrA* QRDR, leading to substitution of aspartic
293 acid 82 and serine 83 by a unique glycine. Our work thus identifies a novel mutation in *gyrA* likely
294 leading to quinolone resistance.
295 After the long-term ciprofloxacin treatment was changed for the C3G ceftriaxone, Ye.4 became
296 resistant to cefoxitin and ceftriaxone by abrogating OmpF porin production, a widely shared strategy
297 to reduce membrane permeability to antibiotics¹⁸ and shown to be induced in *Escherichia coli* upon
298 exposure to ceftriaxone¹⁹. A previous work showing OmpF mutations (the highest molecular weight

299 called YOMP-C in this previous work) selected by cefoxitin treatment in *Ye* and leading to increased
300 β -lactam resistance supports the antibiotic import via this porin²⁰. β -lactamase induction has long
301 been studied in *Ye*²¹ and a recent *in silico* study suggested an increased BlaB inducibility in *Ye* biotype
302 4²². The class A β -lactamase BlaA and class C β -lactamase BlaB (or AmpC) were more abundant in the
303 *Ye*.4 isolate, probably playing a synergistic role together with porin inactivation, as both β -lactamases
304 have been shown to be involved in ceftriaxone resistance in *Ye* biotype 1B²³. Moreover, the non-
305 specific MIC increased for various antibiotics classes in *Ye*.2 could also be explained by a slightly
306 reduced OmpF expression compared to *Ye*.1. Interestingly, the macrolide efflux pump MacA was
307 more expressed in *Ye*.3, which was isolated during the pneumonia treatment with the macrolide
308 spiramycin. However, no specific genetic event could be linked to all these proteome variations.

309 While no ciprofloxacin resistance was observed, *Ye*.X (which was isolated after ciprofloxacin
310 treatment), *Ye*.3 and *Ye*.4 displayed a severe growth defect *in vitro*, which could account for
311 increased antibiotics tolerance. Two major biological processes contribute indeed to antibiotic
312 tolerance: biofilm formation^{24,25} and slow growth and/or metabolic dormancy^{5,26,27}. In the late
313 isolates, we identified mutations in genes involved in metabolism or growth in several gene clusters.
314 The cumulative mutations in genes encoding the RNA polymerase subunits RpoC²⁸ and RpoD, the
315 pDNA topoisomerase ParE²⁹, the 50S ribosomal protein RplF^{30,31}, the rRNA methyltransferase
316 RImE/RrmJ/FtsJ^{32,33} or the stringent response regulator DksA^{34,35} could account for slow growth.
317 Proteomics analysis of the isolates revealed a strong metabolic rewiring with reduced carbohydrate
318 utilization and reduced purine, pyrimidine and riboflavin folate metabolism, together with decreased
319 respiration, which overall corroborate the slow growth phenotype.

320 Vegetations detected in the pacemaker argue for the formation of a biofilm by the *Ye* strains.
321 *Ye* biofilm production has been reported on medical devices such as catheters or feeding tubes^{25,36}.
322 We identified several mutations in *Ye*.3 and *Ye*.4 which can be associated with loss of motility and
323 planktonic growth, favoring biofilm formation: for example, mutation of the flagellar genes *flgD* in
324 both *Ye*.3 and *Ye*.4, and *flgG* and *flgA* in *Ye*.4. Of note, loss-of-function mutations associated with
325 decreased motility have been identified in *Burkholderia spp.* and *P. aeruginosa* isolates from cystic
326 fibrosis patients^{2,3}. In addition, flagellar assembly is repressed in adverse and nutrient-poor
327 environments owing to its energetic cost³⁷. In this direction, no flagellar proteins were detected for
328 any isolate in our culture conditions, in agreement with the reported low flagellar expression in
329 *Yersinia* species at 37°C³⁸. Additionally, a decreased expression or inactivation of adherence and
330 invasion factors to host cells such as Ail, MyfA or Inv could favor adhesion to an abiotic surface by
331 other surface factors. However, proteomics observations regarding higher chemotaxis and quorum
332 sensing AI-1 as well as lower quorum sensing AI-2 protein levels in late isolates prevent us to draw

333 definitive conclusions on biofilm formation capacities of these isolates. A recent study characterizing
334 the *Ye* stringent response also showed reduced motility and biofilm formation in a $\Delta dksA$ mutant,
335 while a (p)ppGpp mutant $\Delta relA\Delta spoT$ had an increased biofilm capability³⁴, suggesting that our late
336 isolates, truncated in DksA due to the third large deletion described in these isolates, had reduced
337 abilities to form biofilms. However, these experiment from the literature were performed at 26°C
338 and are not representative of bacterial *in vivo* behavior at 37°C.

339 Strikingly, late isolates seem more sensitive to various aminoglycosides and
340 fluoroquinolones. DksA truncation could explain this phenotype, as a *dksA* mutation increased
341 susceptibility to ciprofloxacin in *Y. pseudotuberculosis*³⁵ and to chloramphenicol and ampicillin in
342 *Ye*³⁴. However, this effect is species and condition specific as, in *Acinetobacter baumannii*, conflicting
343 results have been reported concerning ciprofloxacin susceptibility^{39,40}. Interestingly, a *dksA* mutation
344 in *Pseudomonas aeruginosa* increased on the other hand survival when exposed to ciprofloxacin,
345 strengthening the interest in studying bacterial tolerance in addition to resistance⁴¹. Finally, a *rlmE*
346 (or *rrmJ/ftsJ*) mutation, found in the late isolates, also led to an increased susceptibility to
347 chloramphenicol, gentamicin, spectinomycin and other antibiotics in *E. coli*³².

348 In addition to the evolutive pressure of antibiotics, *Ye* faced the host immune system,
349 nutritional immunity as well as a specific environment requiring a metabolic switch to adapt to a new
350 lifestyle in the blood⁴². Amino acid requirements such as tryptophan for survival in the blood could
351 explain the perturbed amino acid biosynthesis pathways in the late isolates. Recent studies also
352 highlighted the role of glycine and other amino acid in complement susceptibility, and metabolism
353 adaptation could be a way to better survive the complement cascade⁴³⁻⁴⁵. Iron scarcity in the
354 bloodstream favored the upregulation of iron transport and reduced abundance of iron storage
355 proteins. Interestingly, these regulations were also observed in the transcriptome of the *dksA*
356 mutant¹³.

357 DksA regulation of ribosome production, modulation of regulators such as RpoS or Fis or
358 amino acid promoters is well documented in model bacteria⁴⁶⁻⁵¹ and was recently validated in *Ye* by
359 transcriptomics¹³. We could observe these effects in the proteome of the late isolates lacking a
360 functional DksA. DksA regulates the stringent response that the bacteria can experience during
361 nutrient starvation, but is also linked to antibiotic exposure or immune stress^{52,53}. The pppGpp
362 pyrophosphatase GppA (or Ppx) was also mutated in the late isolates, probably reinforcing
363 unbalanced (p)ppGpp levels and a dysregulated stringent response. As a substantial number of
364 protein changes are associated with DksA truncation and that a strong phenotypic change is induced
365 by *dksA* inactivation^{13,34,54}, this mutation could have been specifically selected during in-host
366 evolution, for example for the previously described iron transport. However, as the deletion has a

367 pleiotropic impact on the global transcriptome, it does not allow to distinguish a specific selection
368 process from side-effects of the deletion. Moreover, it remains to be deciphered if the main selection
369 pressure was the antibiotic treatment or the adaptation to the host environment, to temper a
370 detrimental stringent response on the long run for example. Additionally, the Ye.4 isolate showed an
371 even closer proteome to a $\Delta dksA$ or $\Delta relA\Delta spoT$ mutants than Ye.3, perceptible in the higher
372 ribosomal content or decreased fatty acid degradation pathway for example, suggesting that
373 ceftriaxone-resistant Ye.4 possess a more dysregulated stringent response which could be due
374 to *OmpF* inactivation, reducing nutrient import. Alternatively, stringent response being an adaptation
375 mechanism to a varying environment, its inactivation could have been simply selected by the
376 evolution of the bacteria in a controlled and non-changing environment, the blood. This mechanism
377 could synergistically happen along genome size reduction, a hallmark of environment specialization.

378 Interestingly, loss of the pore-forming toxin *YaxAB* (*Y11_10331/Y11_10321*) as well as
379 reduced virulence factor abundance such as *Ail*, *MyfA*, superoxide dismutases, *ecotin*, *invasin* or the
380 regulator *RovA* suggest that bacteria defective for pathogenic functions that carry a fitness cost can
381 be selected to favor host adaptation, as argued for *Staphylococcus aureus*^{31,55,56}. Finally, *Ye* late
382 isolates displayed a mutation in *mfd*, previously showed to be required for diversification and
383 antibiotics resistance^{57,58}. This suggest that the late slow-growing isolates accumulated less
384 mutations and evolved even slower than expected, while most of the in-host evolving strains
385 previously described showed increased mutation rate⁵⁹.

386

387 In conclusion, we report the unusual clinical case of a patient with iterative bacteremia and
388 endocarditis due to a *Ye* bioserotype 4/O:3 strain evolving over a period of 14 years. The last two
389 isolates, which were simultaneously evolving in the patient, accumulated mutations that allowed
390 antibiotic resistance (a previously unknown substitution conferring resistance to quinolones) and
391 possibly antibiotic tolerance. The latter phenotype was associated with a reduction in genome size
392 and loss of essential functions for growth *in vitro*, while retaining the ability to survive in the host.
393 The last isolated strains caused bacteremia despite the continuity of the antibiotic treatment,
394 highlighting the transition occurring between early onset tolerance and resistance, a critical problem
395 in antibiotic treatment that should urgently be addressed⁶⁰⁻⁶². Our findings have implications in case
396 management, as they reinforce the need for early pacemaker removal in addition to
397 antibiotherapy⁶³. They may also guide future design of more effective treatments for chronic and
398 biofilm-associated bacterial infections. If the infected material cannot be removed as it was the case
399 in our study, the choice of the right antibiotic is crucial. It must be well tolerated by the patient, be
400 active on the biofilm and should not promote the emergence of resistance nor tolerance. It appears

401 here that long-term ceftriaxone has been well tolerated, however it induced tolerance and resistance
402 to this antibiotic. Investigation of the mechanisms modulating antibiotic tolerance is therefore a
403 critical issue for the treatment of long-term infections.

404

405 **Materials and Methods**

406 **Phenotypic characterization**

407 Bacterial strains were identified using the VITEK2 GN card (bioMérieux) and were sent to the *Yersinia*
408 National Reference Laboratory (Institut Pasteur) for species confirmation/characterization as
409 described⁶⁴. For the growth curves, bacteria were precultured in 5 mL lysogeny broth (LB) at 37°C
410 under agitation at 200 rotation per minute (rpm) for 16 to 96 hours. Precultures were diluted to
411 OD_{600nm}=0.1, and 2.5 mL were inoculated in 250 mL LB. Incubations were performed either at 28°C or
412 37°C under agitation at 200 rpm. Optical density at 600 nm was recorded at different time points to
413 monitor growth of the isolates. Mid-exponential phase growth rate was calculated between 2 and 4
414 hours for Ye.1 and Ye.2, and between 7 and 31 hours for Ye.3 and Ye.4.

415

416 **Antibiotic resistance profiling**

417 The antibiotic resistance profiles of the bacterial strains were first determined using VITEK2
418 (bioMérieux) and by disk diffusion method on Mueller-Hinton agar supplemented with 5% horse
419 blood (bioMérieux) for growth deficient isolates during the course of the clinical case. A more
420 comprehensive antibiotic resistance profiling and minimal inhibitory concentration (MIC)
421 measurement was respectively performed by disk diffusion method and Etest on Mueller-Hinton
422 agar supplemented with 5% horse blood (bioMérieux) for the four strains kept in collection at the
423 end of the clinical case. Interpretation was performed according to the recommendations of the
424 Antibiogram Committee of the French Society for Microbiology.

425

426 **Whole genome sequencing and analysis**

427 Bacterial genomes were sequenced and whole genome-based taxonomic assignment was obtained
428 using a 500-gene cgMLST-*Yersinia* as previously described⁶⁵.
429 229 epidemiologically unrelated *Ye 4/O:3* strains isolated between 1982 and 2016 were also
430 sequenced and public genomes of 34 additional strains were used for subsequent analysis (Table S2).
431 Paired-end FASTQ files were used for variant calling using the Y11 reference strain (accession
432 number: NC_017564) with Snippy version 4.1.0⁶⁶. Putative recombinogenic regions were detected
433 and masked with Gubbins version 2.3.4⁶⁷. A maximum-likelihood phylogenetic tree was built from an

434 alignment of 4,738 chromosomal SNPs with RAxML version 8.2.8⁶⁸. Tree visualization was created
435 with iTOL⁶⁹.

436

437 **Pan-genome phylogenetic analysis**

438 A total of 96 additional *Ye* strains were randomly selected together with the *Ye*.1-4 for Phylogenetic
439 Analysis by Maximum Likelihood (PAML)⁷⁰. A pan-genome was constructed using PPanGGOLiN⁷¹.
440 Each gene of the pan-genome (3,824 genes) was translated to proteins and aligned using MAFFT
441 v7.407⁷², allowing to search for genes specific to *Ye* isolates. Back translation to nucleotide was
442 performed to obtain codon alignment, and a phylogenetic tree was built for each gene using IQ-TREE
443 v1.6.7.2⁷³. IQ-TREE's ModelFinder⁷⁴ was used to estimate the best variant of the General Time
444 Reversible (GTR) model. The 3,612 genes for which the strains *Ye*.3 and *Ye*.4 are not sisters in the
445 tree were removed. Synapomorphies were analyzed using a dedicated script
446 (<https://gitlab.pasteur.fr/GIPhy/findSynapomorphies/>). PAML tests detected genes under positive
447 selection pressure.

448

449 **Proteomics sample preparation**

450 *Ye*.1 and *Ye*.2 strains were grown overnight in LB at 37°C under agitation at 200 rpm. 200 µL of the
451 culture were plated on 5 tryptic soy agar (TSA) plates and incubated for 24 hours at 37°C. *Ye*.3 and
452 *Ye*.4 were grown for 3 days in LB at 37°C under agitation at 200 rpm, then 200 µL of the culture were
453 plated on 5 TSA plates and incubated for 48 hours at 37°C. For all *Ye* strains, the bacteria were
454 resuspended with an inoculation loop from the TSA plates in 1 mL phosphate buffered saline (PBS)
455 and washed two times by centrifugation and resuspension in PBS. Bacterial proteins were extracted
456 as explained previously⁷⁵. Briefly, bacteria were lysed with 20 µL trifluoroacetic acid (TFA) for 10
457 minutes and transferred to a sterile 1.5 mL protein low-binding tube. 200 µL of 2M Tris were added,
458 then 24 µL of 100mM tris(2-carboxyethyl)phosphine (TCEP), 400 mM chloroacetamide (CAA) for a
459 final concentration of 10 mM TCEP and 40 mM CAA. Samples were incubated at 95°C for 5 minutes
460 and proteins were quantified using tryptophan fluorescence in a Synergy H1M microplate reader
461 (BioTek) with an excitation wavelength of 280 nm and an emission wavelength of 360 nm. 100 µg of
462 proteins were aliquoted for each sample, and 5 volumes of water were added.
463 Proteins were digested for 11 hours at 37°C under agitation at 600 rpm on a ThermoMix (Thermo)
464 using Sequencing Grade Modified Trypsin (Promega - V5111) with a 1:50 ratio (enzyme:protein)
465 before stopping the digestion by addition of TFA to reach a final pH lower than 2.
466 20 µg of digested peptides were desalted using the Stage-Tips method⁷⁶ using C18 Empore disc,
467 eluted with acetonitrile (ACN) 80%, formic acid (FA) 0.1%. Finally, the peptide solutions were speed-

468 vac dried and resuspended in ACN 2%, FA 0.1% buffer. For each sample, absorbance at 280 nm was
469 measured with a Nanodrop™ 2000 spectrophotometer (Thermo Scientific) to inject an equivalent of
470 DO = 1.

471 For the spectral library preparation for data-independent analysis (DIA), 2 µg of each digested sample
472 were pooled before proceeding to a peptide fractionation. The fractionation was performed with a
473 poly(styrene-divinylbenzene) reverse phase sulfonate (SDB-RPS) Stage-Tips method as previously
474 described^{76,77}. 20µg of the pooled sample was loaded into 3 SDB-RPS (Empore™, 66886-U) discs
475 stacked on a P200 tip and 8 serial elutions were applied as following: elution buffer 1 (Ammonium
476 formate (AmF) 60 mM, ACN 20%, FA 0.5%), elution buffer 2 (AmF 80 mM, ACN 30%, FA 0.5%), elution
477 buffer 3 (AmF 95 mM, ACN 40%, FA 0.5%) , elution buffer 4 (AmF 110 mM, ACN 50%, FA 0.5%),
478 elution buffer 5 (AmF 130 mM, ACN 60%, FA 0.5%), elution buffer 6 (AmF 150 mM, ACN 70%, FA
479 0.5%) and elution buffer 7 (ACN 80%, ammonium hydroxide 5%). All fractions were speed-vac dried
480 and resuspended with ACN 2%, FA 0.1% before injection. For all fractions and individual samples, iRT
481 peptides were spiked as recommended by Biognosys (Biognosys - Ki-3002-1).

482

483 **LC-MS/MS proteomics**

484 For the creation of the spectral library, a nanochromatographic system (Proxeon EASY-nLC 1200 -
485 Thermo Scientific) was coupled online with a Q Exactive™ HF mass spectrometer (Thermo Scientific).
486 Peptides from each fraction and from the pooled sample were injected into a capillary column
487 picotip silica emitter tip (home-made column, 39cm x 75 µm ID, 1.9 µm particles, 100 Å pore size,
488 ReproSil-Pur Basic C18 - Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) after an equilibration
489 step in 100 % mobile phase A (H₂O, FA 0.1%). Peptides were eluted with a multi-step gradient from 2
490 to 7% mobile phase B (ACN 80% / FA 0.1%) in 5 min, 7 to 23% B in 70 min, 23 to 45% B in 30 min and
491 45 to 95% B in 5 min at a flow rate of 250 nL/min for up to 132 min. Column temperature was set to
492 60°C. Mass spectra were acquired using Xcalibur software using a data-dependent Top 10 method
493 with a survey scans (300-1700 m/z) at a resolution of 60,000 and MS/MS scans (fixed first mass 100
494 m/z) at a resolution of 15,000. The AGC target and maximum injection time for the survey scans and
495 the MS/MS scans were set to 3.0x10⁶, 100 ms and 1.0x10⁵, 45 ms respectively. The isolation window
496 was set to 1.6 m/z and normalized collision energy fixed to 28 for HCD fragmentation. We used a
497 minimum AGC target of 2.0x10³ for an intensity threshold of 4.4x10⁴. Unassigned precursor ion
498 charge states as well as 1, 7, 8 and >8 charged states were rejected and peptide match was disable.
499 Exclude isotopes was enabled and selected ions were dynamically excluded for 30 seconds.

500 For the DIA analysis, mass spectra were acquired in DIA mode with the same nanochromatographic
501 system coupled on-line to a Q Exactive™ HF mass spectrometer. For each sample, 1 µg of peptides

502 was injected into the same capillary column picotip silica emitter tip after an equilibration step in 100
503 % mobile phase A (H₂O, FA 0.1%). Peptides were eluted using the same multi-step gradient and
504 temperature for up to 125min. MS data was acquired using the Xcalibur software with a scan range
505 from 295 to 1170 m/z. The DIA method consisted in a succession of one MS scan at a resolution of
506 60,000 and 36 consecutive MS/MS scans with 1 Da overlapping windows (isolation window = 25 m/z)
507 at 30,000 resolution. The AGC (Automatic Gain Control) target and maximum injection time for MS
508 and MS/MS scans were set to 3.0×10^6 , 60 ms and 2.0×10^5 , auto respectively. The normalized collision
509 energy was set to 28 for HCD fragmentation.

510

511 **Proteome bioinformatic analysis**

512 MaxQuant analyses: DDA raw files were processed using MaxQuant software version 1.6.6.0⁷⁸ with
513 Andromeda search engine⁷⁹. The MS/MS spectra were searched against a personal *Yersinia*
514 *enterocolitica* database (4,424 entries). The database was created by combining the four different
515 *Yersinia enterocolitica* strains (Ye.1, Ye.2, Ye.3 and Ye.4). Sequences with at least one different amino
516 acid were considered as unique and specific of the corresponding strain. Sequences with 100%
517 similarity were reported once. FASTA headers contain the traceability of each strain.

518 Variable modifications (methionine oxidation and N-terminal acetylation) and fixed modification
519 (cysteine carbamidomethylation) were set for the search and trypsin with a maximum of two missed
520 cleavages was chosen. The minimum peptide length was set to 7 amino acids and the false discovery
521 rate (FDR) for peptide and protein identification was set to 0.01. The main search peptide tolerance
522 was set to 4.5 ppm and to 20 ppm for the MS/MS match tolerance. Second peptides were enabled to
523 identify co-fragmentation events.

524 Spectronaut analyses: Spectronaut v. 16.0.220606 (Biognosys AG)⁸⁰ was used for DIA-MS data
525 analyses. Data extraction was performed using the default BGS Factory Settings. Briefly, for
526 identification, both precursor and protein FDR were controlled at 1%. For quantification, Qvalue was
527 used for precursor filtering and global imputation strategy was used; peptides were grouped based
528 on stripped sequences. Cross Run Normalization was enabled.

529

530 **Proteome statistical analysis**

531 To find the proteins more abundant in one condition than in another, the intensities quantified using
532 Spectronaut were compared. Only proteins identified with at least one peptide and with at least four
533 intensity values in one of the two compared conditions were kept for further statistics. First, proteins
534 absent in a condition and present in another are put aside. These proteins can directly be assumed
535 differentially abundant between the conditions. Then, intensities of the remaining proteins were first

536 log-transformed (\log_2). Intensity values were normalized by median centering within conditions
537 (section 3.5 in this reference⁸¹). Missing values were imputed using the `impute.slsa` function of the R
538 package `imp4p`⁸². Statistical testing was conducted using a `limma` t-test thanks to the R package
539 `limma`⁸³. An adaptive Benjamini-Hochberg procedure was applied on the resulting p-values thanks to
540 the function `adjust.p` of the `cp4p` R package⁸⁴ using the robust method described in this reference⁸⁵
541 to estimate the proportion of true null hypotheses among the set of statistical tests. The proteins
542 associated to an adjusted p-value inferior to a FDR level of 1% and an absolute \log_2 (fold-change)
543 superior to 1 have been considered as significant and differentially abundant proteins. Finally, the
544 proteins of interest are therefore those which emerge from this statistical analysis supplemented by
545 those which are present from one condition and absent in another (“ID table” in the S1 Dataset).
546 Annotated proteins of Ye.1 to Ye.4 strains were blasted on the Y11 reference proteome (RefSeq
547 annotation from December 2022, accession GCF_000253175.1), and the best hit for each Y11 protein
548 was kept. For each pairwise comparison based on the Ye.1 to Ye.4 loci database describe above, the
549 duplicated Y11 loci were removed by keeping the less differentially expressed value, assuming the
550 duplicated loci came from an annotation of a protein carrying a SNP. The R package `clusterProfiler`
551 v4.6.2 was used for enrichment and gene set enrichment analysis⁸⁶. The GSEA function was used
552 based on the Y11 loci by mapping to an in-house annotation file extracted from the Y11 Gene
553 Ontology terms downloaded from the QuickGo website accessed on May 25th 2023⁸⁷. The `gseKEGG`
554 function was used with the online KEGG dataset for the “yey” organism accessed on May 25th 2023.
555 Analysis was performed with the detected proteins as statistical background. Foldchange data were
556 transformed to be uploaded on the iPath3 website⁸⁸. Foldchange data were also mapped to specific
557 KEGG pathways using the R package `pathview`⁸⁹. Published transcriptomes were downloaded from
558 the Yersiniomics website¹². Ye 8081, Y1 and Y11 reference genomes were blasted to find
559 homologous loci, and proteomes and transcriptomes were fused in a unique dataframe. The
560 `rquery.cormat` function was used to compute the Pearson correlations of the foldchanges and display
561 the correlation matrix.

562

563 **Data Availability Section**

564 The datasets and computer code produced in this study are available in the following databases:

- 565 • Genome sequences: ENA PRJEB19854 (sample ID starting with ERS and accession numbers
566 starting with FWC) for *Y. enterocolitica* 1 (Ye.1) (ERS1580350, FWCF02000001-
567 FWCF02000181), Ye.2 (ERS1580351, FWCE02000001-FWCE02000227), Ye.3 (ERS1580352,
568 FWCB02000001-FWCB02000195), and Ye.4 (ERS1580353, FWCD02000001-FWCD02000173)

- 569 • The mass spectrometry proteomics data have been deposited to the ProteomeXchange
570 Consortium via the PRIDE partner repository⁹⁰ with the dataset identifier PXD043567

571

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582 Some figures were created with BioRender.

583 **Conflict of interest**

584 The authors declare no competing interests.

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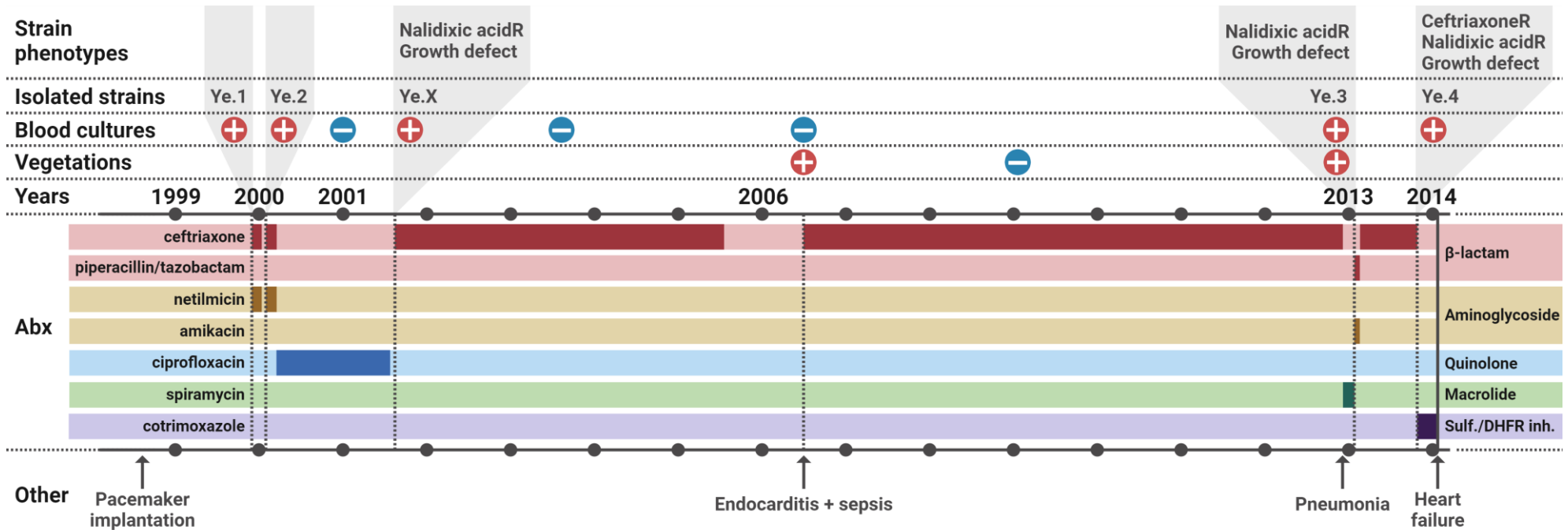
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800 **AUTHOR CONTRIBUTIONS**

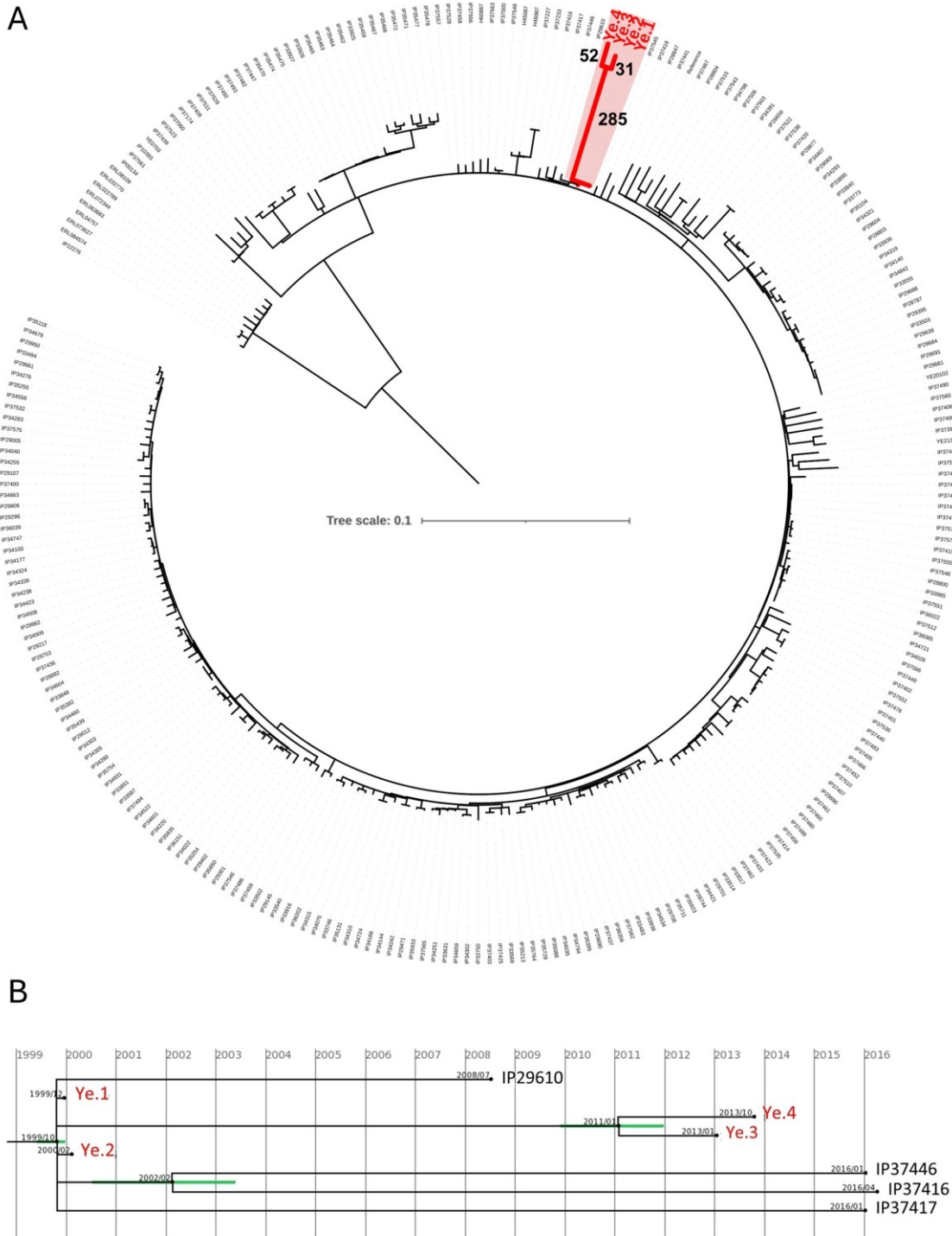
801 C.S. and P.L.-B. planned, performed and analyzed experiments with the help of L.M., N.C. and L.G.
802 J.G. set up and performed pan-genome together with PAML analysis. R.B., C.L.-B., F.B., M.F. and B.M.
803 are the physicians who took care of the patient and shared all the clinico-epidemiological data. L.D.
804 performed antibiograms and MIC measurement. T.D. performed mass spectrometry-based
805 proteomics. P.L.-B. and O.D. were involved in proteome analysis and identification of antibiotic
806 tolerance mechanism. C.S., P.L.-B. and J.P.-C. interpreted data and wrote the manuscript. E.C., O.D.,
807 M. M., P.L. and J.P.-C. supervised the work. C.S. and P.L.-B. are shared first authors and P.L. and J.P.-
808 C. are shared last authors.
809

810 **Figures**



811 **Figure 1.** Clinical case description of Ye chronic infection. Positive and negative symbols reflect the blood culture results, or the observation of vegetations
 812 on the pacemaker atrial lead. Periods of antibiotics treatment are shown in a darker color on the chronograph, with the associated treatment names on the
 813 left and antibiotic classes on the right. Abx: antibiotics. Ceftriaxone^R: resistant to ceftriaxone. Nalidixic acid^R: resistant to nalidixic acid. Sulf: sulfonamide.
 814 DHFR inh: dihydrofolate reductase inhibitor.

815



816 **Figure 2.** Phylogenetic reconstruction. (A) Maximum likelihood phylogeny reconstructed with RAXML
817 under GTR model with 100 bootstraps based on 4,738 SNPs identified in 262 strains. The genome of
818 the Y11 strain (NC_017564) was used as reference for variant calling. Numbers close to the branches
819 indicate SNPs numbers. (B) Estimation of the date of internal nodes using least-squares dating
820 (<https://lsdating.pasteur.fr>) among the 4 strains isolated from the patient. Green bars represent the
821 standard deviation of the estimated date.



822 **Figure 3.** Gene set enrichment analysis (GSEA) of biological process gene ontology terms across
 823 pairwise comparisons of *Ye* proteomes. Positive normalized enrichment scores (NES) are shown in
 824 red and correspond to enriched terms in the first strain in the comparison, while negative NES are
 825 shown in blue and correspond to enriched term in the second strain in the comparison. Multiple
 826 testing is adjusted by the Benjamini-Hochberg procedure.

827

828

Table 1. Minimum inhibitory concentration (mg/L) of the 4 Ye strains on 10 antibiotics and antibiotics combinations assessed by Etest.

Antibiotic class	Antibiotic	Ye.1	Ye.2	Ye.3	Ye.4
β-lactam	ceftriaxone ¹²³	0,047	0,094	0,094	>256
	cefoxitin	4	12	12	>256
	piperacillin-tazobactam ³	0,094	0,25	<0,016	0,094
aminoglycosides and aminoglycosides-like	netilmicin ¹²	0,75	3	0,06	0,06
	amikacin ³	3	3	2	1
	tobramycin	1	1	0,09	0,09
	gentamicin	0,5	1	0,09	0,09
	spectinomycin	12	24	0,09	0,09
Quinolone	ciprofloxacin ²	0,008	0,012	0,002	0,032
	levofloxacin	0,032	0,032	0,008	0,032

¹Administred treatment between Ye.1 and Ye.2 isolation

²Administred treatment between Ye.2 and Ye.3 isolation

³Administred treatment between Ye.3 and Ye.4 isolation

Table 3. Log₂(foldchange) of the proteome pairwise comparisons for selected proteins. Proteins were mapped on the Y11 reference genome. P-value and adjusted p-value can be found in Table S6. 0 corresponds to proteins not detected in any conditions of the comparison. +15 and -15 corresponds to proteins detected only in the first or second condition of the comparison, respectively.

Locus	Old Locus	Gene	Product	Log ₂ (foldchange)					
				Ye.1	Ye.1	Ye.1	Ye.2	Ye.2	Ye.3
				vs	vs	vs	vs	vs	vs
Virulence-associated proteins									
Y11_RS00110	Y11_00221	<i>ail</i>	attachment invasion locus protein Ail	0	2,62	3,25	2,63	3,25	0,62
Y11_RS01220	Y11_02591	<i>eco</i>	serine protease inhibitor ecotin	-0,05	1,35	1,66	1,39	1,71	0,32
Y11_RS06755	Y11_14481	<i>inv</i>	autotransporter invasin Inv	0,53	1,28	4,99	0,75	4,47	3,71
Y11_RS01540	Y11_03301	<i>myfA</i>	fimbrial polyadhesin major subunit MyfA	-0,27	0,62	1,18	0,89	1,47	0,57
Y11_RS01020	Y11_02141	<i>rovM</i>	virulence transcriptional regulator RovM	1,02	-1,57	-2,01	-2,59	-3,03	-0,44
Y11_RS14390	Y11_30461	<i>sodA</i>	superoxide dismutase [Mn]	0,33	1,09	1,13	0,75	0,79	0,04
Y11_RS03855	Y11_08321	<i>sodB</i>	superoxide dismutase [Fe]	0,06	1,73	1,96	1,67	1,91	0,23
Y11_RS10840	Y11_22981	<i>sodC</i>	superoxide dismutase family protein [Cu-Zn]	0,7	3,08	3,5	2,38	2,8	0,42
Y11_RS09565	Y11_20331	<i>ymoA</i>	expression modulating protein YmoA	-0,19	-2,41	-3	-2,22	-2,81	-0,59
Chemotaxis proteins									
Y11_RS06745	Y11_14461	<i>flgM</i>	anti-sigma-28 factor FlgM	-0,56	-1,01	-1,56	-0,44	-1	-0,55
Y11_RS06785	Y11_14551	<i>cheZ</i>	protein phosphatase CheZ	-0,6	-2,08	-2,86	-1,48	-2,26	-0,78
Y11_RS06790	Y11_14561	<i>cheY</i>	chemotaxis response regulator CheY	-0,06	-1,74	-1,96	-1,68	-1,9	-0,22
Y11_RS06795	Y11_14571		chemotaxis response regulator protein-glutamate methyltransferase	0	-0,34	-0,14	-0,85	-0,76	0,15
Y11_RS06805	Y11_14591		methyl-accepting chemotaxis protein	0	-15	-15	-2,19	-2,48	-0,31
Y11_RS06815	Y11_14621	<i>cheW</i>	chemotaxis protein CheW	0	-2,6	-3,26	-2,59	-3,26	-0,67
Y11_RS06820	Y11_14641	<i>cheA</i>	chemotaxis protein CheA	0	-0,75	-2,55	-0,94	-2,71	-1,8
Y11_RS11490	Y11_24361	<i>flgM</i>	flagellar biosynthesis anti-sigma factor FlgM	0,08	1,54	1,43	1,45	1,35	-0,11
Quorum sensing proteins									
Y11_RS02270	Y11_04881	<i>yenR</i>	LuxR family transcriptional regulator YenR	-0,31	0,58	1,3	0	0	0
Y11_RS02275	Y11_04891	<i>yenI</i>	acyl-homoserine-lactone synthase	-0,36	-2,42	-2,17	-2,06	-1,79	0,26
Y11_RS10410	Y11_22111	<i>luxS</i>	S-ribosylhomocysteine lyase	-0,04	0,16	0,38	0,2	0,42	0,22
Y11_RS17855	Y11_37571	<i>lsrG</i>	(4S)-4-hydroxy-5-phosphonooxypentane-2,3-dione isomerase	-0,3	1,2	1,94	1,49	2,23	0,74
Y11_RS17860	Y11_37581	<i>lsrF</i>	3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase	-0,51	2,05	2,68	2,56	3,18	0,62
Y11_RS17865	Y11_37591	<i>lsrB</i>	autoinducer 2 ABC transporter substrate-binding protein LsrB	0,03	2,81	3,84	2,78	3,81	1,03
Y11_RS17870	Y11_37601	<i>lsrD</i>	autoinducer 2 ABC transporter permease LsrD	0	0	0	0	0	0
Y11_RS17880	Y11_37621	<i>lsrA</i>	autoinducer 2 ABC transporter ATP-binding protein LsrA	-0,45	15	15	15	15	0
Y11_RS17885	Y11_37631	<i>lsrR</i>	transcriptional regulator LsrR	0,15	1,25	1,43	1,1	1,28	0,18
Y11_RS17890	Y11_37641	<i>lsrK</i>	autoinducer-2 kinase	-0,15	0,81	1,3	0,96	1,45	0,49
Iron import and storage proteins									
Y11_RS02915	Y11_06251	<i>fcuA</i>	Ferrichrome receptor protein FcuA	1,51	-0,54	-1,43	-2,04	-2,94	-0,9
Y11_RS03015	Y11_06491	<i>ftnA</i>	non-heme ferritin	-0,27	1,46	2,04	1,73	2,31	0,58
Y11_RS05035	Y11_10881	<i>tonB</i>	TonB system transport protein TonB	0,98	-1,07	-1,96	-2,05	-2,94	-0,89
Y11_RS05710	Y11_12361		siderophore-interacting protein	0,76	-0,55	-1,22	-1,31	-1,98	-0,67
Y11_RS08900	Y11_18931	<i>fur</i>	ferric iron uptake transcriptional regulator	-0,18	0,43	1,37	0,61	1,55	0,94
Y11_RS11645	Y11_24711	<i>exbD</i>	TonB system transport protein ExbD	0,37	-1,41	-2,68	-1,78	-3,06	-1,28
Y11_RS11650	Y11_24721	<i>exbB</i>	tol-pal system-associated acyl-CoA thioesterase	1,79	0,31	-0,86	-1,49	-2,65	-1,16
Y11_RS15495	Y11_32861	<i>bfr</i>	bacterioferritin	0,68	3,87	4,66	3,19	3,98	0,79
Y11_RS16005	Y11_33831	<i>hemY</i>	protoheme IX biogenesis protein HemY	0,13	1,29	1,56	1,17	1,44	0,27
Y11_RS16010	Y11_33841	<i>hemX</i>	uroporphyrinogen-III C-methyltransferase	0,51	1,09	1,57	0,57	1,06	0,48
Y11_RS16920	Y11_35641		TonB-dependent siderophore receptor	0,49	-2,35	-3,5	-2,84	-3,99	-1,15
Y11_RS16930	Y11_35671	<i>hmuV</i>	Hemin import ATP-binding protein HmuV	0	0	0	0	0	0
Y11_RS16935	Y11_35681	<i>hmuU</i>	Hemin transport system permease protein HmuU	0	0	0	0	0	0
Y11_RS16940	Y11_35691	<i>hmuT</i>	hemin ABC transporter substrate-binding protein	0,88	-1,2	-2,82	-2,08	-3,7	-1,62
Y11_RS16945	Y11_35701	<i>hmuS</i>	hemin-degrading factor	0,29	-2,71	-4,85	-2,99	-5,13	-2,14
Y11_RS16950	Y11_35711	<i>hmuR</i>	TonB-dependent hemoglobin/transferrin/lactoferrin family receptor	1,85	-1,42	-2,95	-3,27	-4,79	-1,52
Y11_RS18015	Y11_37891	<i>fecA</i>	Iron(III) dicitrate transport protein FecA	2	0,26	-0,74	-1,73	-2,75	-1,01
Beta-lactamase associated proteins									
Y11_RS04605	Y11_09911	<i>blaA</i>	class A beta-lactamase BlaA	0,45	-1,37	-3,01	-1,83	-3,46	-1,63
Y11_RS05870	Y11_12651	<i>blaB</i>	class C beta-lactamase BlaB (AmpC)	1,03	-0,03	-0,97	-1,05	-1,99	-0,94
Y11_RS05875	Y11_12661	<i>ampR</i>	LysR family transcriptional regulator AmpR	0,46	-2,15	-0,65	-2,6	-1,11	1,49
Y11_RS18675	Y11_39241	<i>ampD</i>	1,6-anhydro-N-acetylmuramyl-L-alanine amidase AmpD	0	-0,06	0,19	-0,15	0,05	0,24
Y11_RS18680	Y11_39251	<i>ampE</i>	beta-lactamase regulator AmpE	-0,49	0,82	2,58	1,31	3,06	1,75
Porins									
Y11_RS01275	Y11_02741	<i>ompC</i>	porin OmpC	-1,48	0,9	-0,15	2,38	1,34	-1,04
Y11_RS02065	Y11_04441	<i>ompF</i>	porin OmpF	1,4	-1,37	3,58	-2,77	2,18	4,95
Y11_RS02150	Y11_04631	<i>ompA</i>	porin OmpA	-0,24	0,53	0,26	0,77	0,5	-0,27
Y11_RS05005	Y11_10821	<i>ompW</i>	outer membrane protein OmpW	0,92	-2,55	-1,52	-3,47	-2,44	1,03
Y11_RS05640	Y11_12201		porin	1,22	-2,84	-2,99	-4,07	-4,19	-0,15
Y11_RS08265	Y11_17621	<i>aqpZ</i>	aquaporin Z	-0,79	2,64	4,48	3,43	5,27	1,84
Y11_RS08275	Y11_17651	<i>ompF2</i>	porin OmpF2	0,19	-0,96	-1,92	-1,15	-2,12	-0,96
Y11_RS15180	Y11_32151	<i>ompR</i>	two-component system response regulator OmpR	-1,92	0,83	0,58	2,75	2,5	-0,25
Y11_RS15185	Y11_32161	<i>envZ</i>	two-component system sensor histidine kinase EnvZ	-1,26	0,69	0	1,95	1,26	-0,69
Efflux pumps									
Y11_RS00155	Y11_00321	<i>acrD</i>	multidrug efflux RND transporter permease AcrD	-0,54	-2,03	-0,85	-1,49	-0,31	1,18
Y11_RS00315	Y11_00681	<i>macA</i>	macrolide-specific efflux protein MacA	0	-15	0	-15	0	1,4
Y11_RS09485	Y11_20131	<i>acrR</i>	multidrug efflux transporter transcriptional repressor AcrR	0	0	0	0	0	0
Y11_RS09490	Y11_20141	<i>acrA</i>	efflux RND transporter adaptor subunit AcrA	0,22	0,82	0,87	0,6	0,65	0,05
Y11_RS09495	Y11_20151	<i>acrB</i>	efflux RND transporter permease AcrB	0,14	1,2	1,08	1,06	0,94	-0,11
Y11_RS10240	Y11_21811	<i>emrA</i>	multidrug efflux MFS transporter periplasmic adaptor subunit EmrA	0	-0,33	-2,77	-0,39	-2,86	-2,45
Y11_RS10245	Y11_21821	<i>emrB</i>	multidrug efflux MFS transporter permease subunit EmrB	0	0	0	0	0	0
Y11_RS11740	Y11_24901	<i>tolC</i>	outer membrane channel protein TolC	0,05	-0,18	-0,18	-0,23	-0,23	0
Y11_RS18200	Y11_38261	<i>robA</i>	MDR efflux pump AcrAB transcriptional activator RobA	-0,86	0,76	1,2	0,13	2,06	0,44
Y11_RS20600	Y11_43311	<i>mdtC</i>	multidrug efflux RND transporter permease subunit MdtC	-0,16	-0,51	-0,37	-0,34	-0,21	0,14
Y11_RS20605	Y11_43321	<i>mdtB</i>	MdtB/MuxB family multidrug efflux RND transporter permease subunit	-0,06	0,11	0,28	0,17	0,34	0,17
Y11_RS20610	Y11_43331	<i>mdtA</i>	MdtA/MuxA family multidrug efflux RND transporter periplasmic adaptor subunit	-0,53	-0,64	-0,04	-0,12	0,49	0,61

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