Molecular Evolution of the *Yersinia* Major Outer Membrane Protein C (OmpC)

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ABSTRACT: The genus *Yersinia* includes species with a wide range of eukaryotic hosts (from fish, insects, and plants to mammals and humans). One of the major outer membrane proteins, the porin OmpC, is preferentially expressed in the host gut, where osmotic pressure, temperature, and the concentrations of nutrients and toxic products are relatively high. We consider here the molecular evolution and phylogeny of *Yersinia ompC*. The maximum likelihood gene tree reflects the macroevolution processes occurring within the genus *Yersinia*. Positive selection and horizontal gene transfer are the key factors of *ompC* diversification, and intraspecies recombination was revealed in two *Yersinia* species. The impact of recombination on *ompC* evolution was different from that of another major porin gene, *ompF*, possibly due to the emergence of additional functions and conservation of the basic transport function. The predicted antigenic determinants of OmpC were located in rapidly evolving regions, which may indicate the evolutionary mechanisms of *Yersinia* adaptation to the host immune system.

KEYWORDS: porins, *Yersinia*, pathogen adaptation, recombination, outer membrane

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Introduction

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and includes 17 species. The three species that are pathogenic to humans (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) are very well studied; the other 14 species are considered nonpathogenic to humans but can cause infections in fish (*Yersinia ruckeri*), insects (recently described *Yersinia entomophaga*), and mammals (*Yersinia intermedia*, *Yersinia frederiksenii*, and *Yersinia kristensenii*).[1,2] Pathogenic *Yersinia* species cause zoonoses that circulate in a natural focus, with humans as an accidental host. In that case, man provides a new, unusual ecological niche for the pathogens.[3] Survival of bacteria in the new environment will depend on the presence of adaptation factors that appeared in the previous habitat as random events of evolution. In this context, *Yersinia* is a model genus for the study of evolutionary events that contribute to the emergence and development of pathogenic potential.[1,4]

Nonspecific general porins are β-barrel proteins and predominant among the outer membrane proteins (OMPs) of gram-negative bacteria. Porins provide passive transport of small molecules through the outer membrane and modulate the membrane's permeability to harmful molecules, such as bile salts, antibiotics, and biocides. Furthermore, some porins could induce bacterial invasion and create protective immunity against bacterial infections.[5–8] The reciprocal expression of two general porins, OmpC and OmpF, is one of the common ways that bacterial cells adapt to changing environmental conditions.[9–11] For example, OmpF (which has a large pore size) is mainly expressed at a temperature of 4 °C–25 °C and when there is a lack of nutrients in the medium, while OmpC (which has a smaller pore size) is primarily expressed at the temperature of a warm-blooded organism (37 °C) and during nutrient excess,[12] OmpC plays a more significant role in bile resistance than OmpF.[13] Therefore, these proteins may contribute to the adaptation of bacterial pathogens to different hosts and different environmental conditions.

Previously, we found that the *ompF* gene of *Yersinia* is characterized by pronounced interspecific and intraspecific polymorphisms. The interspecies and intraspecies recombination and positive selection within the gene play important roles in its diversification. Our analysis demonstrated that *ompF* evolved with a nonrandom mutation rate under overall purifying selection; however, the regions encoding the surface loops of OmpF contain sites subjected to positive selection.[14,15] Therefore, the study of evolution of OmpC, the second main porin of *Yersinia*, which is predominantly expressed in warm-blooded organisms, will allow us to advance our understanding of the mechanisms of pathogen adaptation and survival in the host environment.

In this study, we defined the mechanisms of molecular evolution of OmpC based on an investigation of gene sequence divergence among all currently known *Yersinia* species.

Materials and Methods

Bacterial strains and data collection. A total of 90 nucleotide coding sequences that represent 15 different species of *Yersinia* and *Serratia marcescens* as the out-group were used in this study. These sequences were obtained from the DNAs of the 39 previously described strains[14] and from the genomes of 51 strains retrieved from the GenBank and SRA databases (http://www.ncbi.nlm.nih.gov/). All row data were assembled in the CLC Genomics Workbench (Qiagen), followed by annotation on the RAST server.[16] Some properties of these strains and the ID numbers of the genomes and sequences are presented in Supplementary Table 1. We identified OmpC orthologs in the genomes by examining the neighbor genes on both sides of the *ompC* gene. The flanking genes were a penicillin-binding protein, ampH (COG1680), a small regulatory RNA gene, micF, and an Major facilitator superfamily (MFS) transporter, HlyD (COG0845).[17] Strain selection was intended to include the strains of all known *Yersinia* species with a high degree of diversity (Supplementary Table 1).

PCR and sequencing. PCR amplification of the *ompC* gene was performed with the universal primers YeC-seq1 5′-CCATTGGGATTATATGCTCG-3′ and ompC-Rent 5′-CYTRTWATCAGRATTAGAACTGG-3′, designed in this study using Vector NTI Advance v. 11.0 (Invitrogen Corp.). The expected amplicon sizes were 1100–1152 bp. The PCR conditions were as follows: initial denaturation at 95 °C for 5 minutes, then 25 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 45 seconds, followed by a final extension step at 72 °C for 5 minutes. The PCR fragments were evaluated on a 1.5% agarose gel stained with ethidium bromide. Unincorporated primers and Deoxynucleotide triphosphates (dNTPs) were removed from PCR products with a GeneJET PCR Purification Kit (Thermo Scientific). The purified PCR fragments were sequenced on a 3130xl Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses. The primary screening of the obtained nucleotide sequences was performed using Sequence Scanner v. 1.0 (Applied Biosystems). A multiple alignment was performed using ClustalW implemented in MEGA v. 6.06.[18] Phylogenetic trees were constructed using MEGA v. 6.06. All three coding positions were examined and the neighbor-joining and maximum likelihood methods with the Kimura 2-parameter model were applied. The reliability of the phylogenetic trees was checked with a bootstrap test (2000 replications).

Evolution analysis. We used the server Datamonkey (www.datamonkey.org)[19] and the RDP4 program,[20] for recombination detection. In Datamonkey, the calculations were performed using the GARD method.[21] In the RDP4, recombinant sequences were detected via four automated recombination detection methods: RDP,[22] MaxChi,[23] Chimera,[24] and GENECONV.[25] For the RDP method, internal reference sequences were used, the window size was set to 20, and the sequence identity of 0%–100% was used. For both the MaxChi and the Chimera methods, the number of variable sites was set to 40. For the GENCONV method, we used standard settings. A maximum *P*-value of 0.01 and the Bonferroni correction were used.

Positive or negative selection was calculated as the proportion of synonymous (silent; dS) to nonsynonymous (amino acid changing; dN) substitution rates. The analysis was performed on the Datamonkey web server using SLAC, FEL, REL,[26] and IFEL[27]; $P < 0.05$ was considered statistically significant for these tests.

Nucleotide divergence (Pi) – the number of nucleotide differences in the site[28] – was determined using DnaSP v. 5.10,[29] using the sliding window with a length of 5 and a step size of 1.

The prediction of B-cell epitopes in the protein-coding sequences was carried out on the IEDB Analysis Resource server (http://tools.immuneepitope.org) using the BepiPred method.[30]

Results and Discussion

Phylogenetic and recombination analyses of ompC. We used the previously obtained DNA of 39 *Yersinia* strains[14] for the sequencing of the full-length coding sequences of *ompC*; these sequences were combined with the sequences from the GenBank and SRA databases. Overall, we analyzed 90 sequences that represented 15 species of *Yersinia* and *S. marcescens* as the out-group (Supplementary Table 1). All sequences were aligned and analyzed in MEGA. Figure 1 shows a maximum likelihood tree of *ompC*. For the neighbor-joining method, we obtained lower bootstrap values (shown in parentheses in Fig. 1). The tree was formed by 11 ancient clades (indicated as short perpendicular lines in Fig. 1), but the degree of evolutionary relatedness between them was impossible to identify because of the low bootstrap values. In general, the *ompC* tree topology reflects the macroevolution processes occurring within the genus *Yersinia*; all the species form distinct monophyletic groups within the ancient clades, excepting *Y. pestis* and *Y. pseudotuberculosis*, which merge in one *Y. pseudotuberculosis* complex. The tree topology correlates well with previously constructed phylogenetic trees for the genus *Yersinia*.[4,31]

From our data and data in the literature, porin genes often contain signatures of past intra- and intergenic recombination.[14,32,33] To understand the role of horizontal gene transfer in *ompC* evolution, we analyzed the sequences for the presence of recombination using the GARD method on the Datamonkey server. When we analyzed all the unique sequences ($n = 77$), the method did not reveal any recombination events. Then, we decided to divide these sequences into seven clusters, based on the phylogeny and genetic distance between the most divergent species. Five clusters presented distinct species

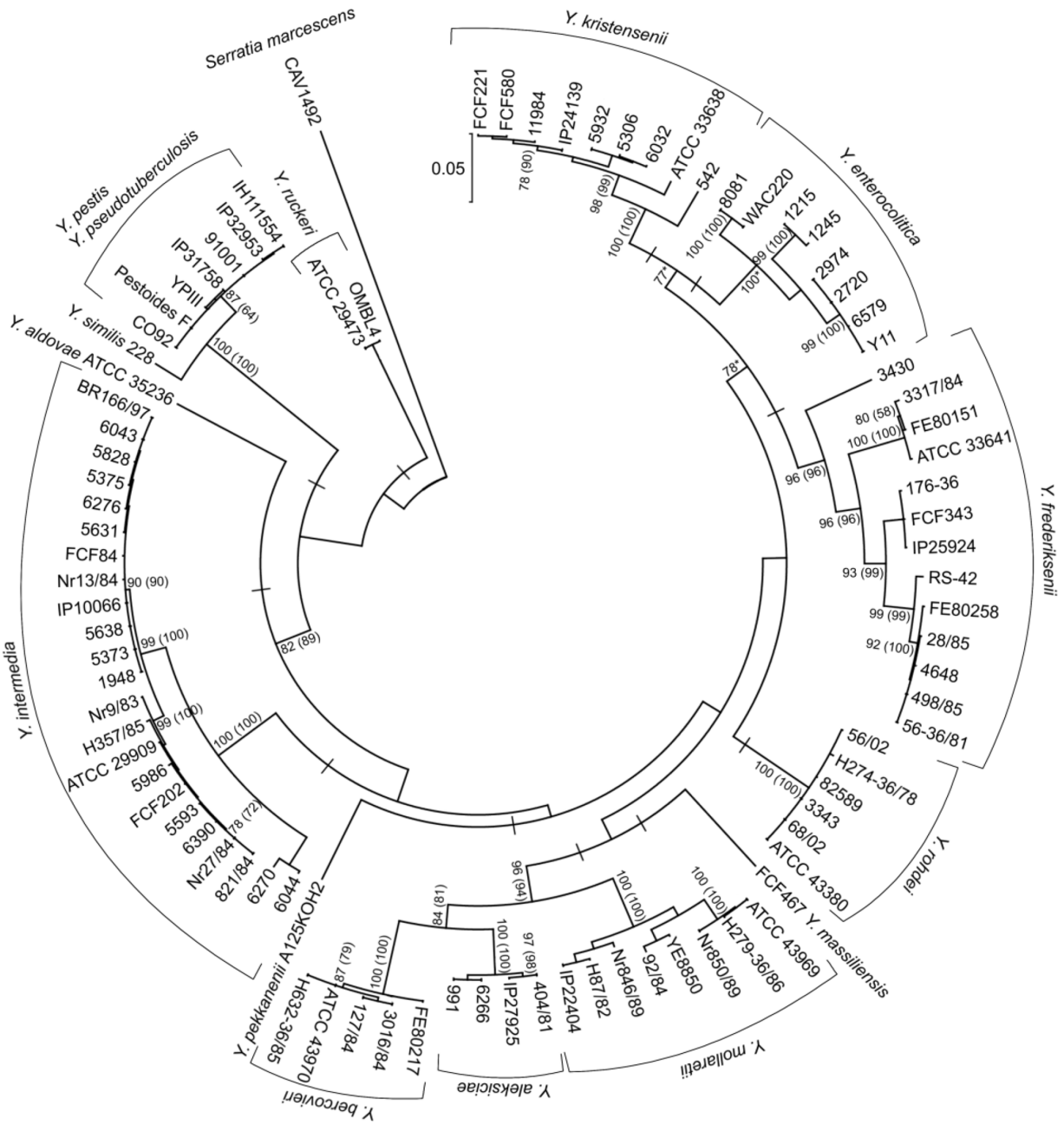

Figure 1. Maximum likelihood tree of *Yersinia ompC*. The scale displays the number of nucleotide substitutions per nucleotide site. Numbers at nodes of the tree – the bootstrap value in percentage (75% cutoff). Bootstrap values obtained for the neighbor-joining method are in parentheses.
Note: *Difference in the tree topology when compared with the neighbor-joining one. *S. marcescens* CAV1492 – out-group.

(*Y. frederiksenii*, *Y. intermedia*, *Y. enterocolitica*, *Yersinia rohdei*, and *Y. kristensenii*). Two clusters combined several closely related species: the YPS cluster, which included *Y. pseudotuberculosis*, *Y. pestis*, and *Yersinia similis*, and the YM cluster, which included *Yersinia mollaretii*, *Yersinia bercovieri*, and *Yersinia aleksiciae*. As a consequence of this division, we could detect recombination in the four clusters (Table 1). We also used the RDP4 program to identify parental sequences and determine their contribution to a mosaic structure of *ompC*. We revealed two recombination events in the *Y. frederiksenii* cluster and one in the *Y. enterocolitica* cluster. Thus, we found evidence for intraspecies recombination in at least two species.

Apparently, recombination in *ompC* occurs only between closely related strains. Despite the same levels of polymorphism in the two main porin genes, *ompC* was not involved in horizontal gene transfer between species of *Yersinia*, whereas both intra- and interspecific recombination were found in *ompF*.[14,15]

Thus, we can suppose that the *ompC* gene of *Yersinia* has evolutionary constraints on recombination between different species. Homologous recombination is common for many bacterial porin genes and is one of the mechanisms of their diversification[34,35]; however, the comparison of the two main nonspecific porins (OmpC and OmpF) of *Yersinia* revealed very different contributions of recombination to their evolu-

Table 1. Results of recombination detecting.

CLUSTER	GARD			RDP4	
	RB* NUMBER	RB LOCATION IN ALIGNMENT, BP	*P*-VALUE	RE** NUMBER	RE LOCATION IN ALIGNMENT, BP
Y. frederiksenii	1	480	0.05	2	308–393, 661–865
Y. enterocolitica	1	1053	0.01	1	626–914
Y. kristensenii	2	162,924	0.01		
YM	1	859	0.01		

Notes: *Recombination break point. **Recombination event.

tion that could be due to the emergence of additional functions, while the basic transport function remained intact for both proteins.

Evolutional analysis. The *ompC* genes of *Yersinia* are orthologous, and their evolution are determined by microevolution processes, like mutations and selection through speciation. The evolutionary divergence of the analyzed *ompC* sequences was 0.145 ± 0.006. We detected 475 variable nucleotide sites, which comprised 41% (475/1152 bp) of the length of a multiple alignment that included unique sequences (n = 77). The domain organization of *Yersinia* OmpC was determined based on the structural data of *Escherichia coli* OmpC (Osmoporin, 2 J1 N, RCSB PDB). It was unexpected that the distribution of variable sites was uniform throughout the *ompC* length, regardless of the protein structure (Fig. 2). We previously showed that the *ompF* gene of *Yersinia* is characterized by an uneven distribution of variable sites, which are preferentially localized in the regions encoding the external loops.[14] The nonrandom distribution with variable and conserved domains is common for the porin genes of different bacteria.[35,36]

We consider that the difference in the evolutionary rates of different parts of orthologs is associated with evolutionary pressure acting as a purifying or positive selection. As Zheng et al.[37] showed, rapidly evolving gene regions may result in the functional divergence of proteins, significantly contributing to the phenotypic differences between closely related organisms.

We tested the effects of adaptive evolution on the *ompC* gene of *Yersinia*. The analysis was performed on the Datamonkey server, using SLAC, FEL, IFEL, and REL. We detected the sites under positive and negative selection in seven previously identified clusters. The positive codons were found in the six clusters excepting the *Y. rohdei* cluster (Fig. 3); positive sites were preferentially located within regions encoding the external loops of the protein. For example, in the YM cluster, seven out of eight positive codons were focused in the coding regions corresponding to the L2, L5, L6, and L8 loops. In the *Y. enterocolitica* group, we found four sites, and two of them were in the L4 loop region. In the *Y. frederiksenii* cluster, we identified six codons located in the L4, L5, L6, and L7 regions (Fig. 3). These sites were mapped onto three-dimensional structural model of OmpC, which is presented in Figure 4. The positive sites were in different loops of different *Yersinia* species and may be the evidence of the functional divergence of these loops in speciation. The negative codons found in all seven clusters encoded amino acid residues that maintain the β-barrel structure (Fig. 3).

We have previously shown that intensive diversification of the *ompF* gene of *Yersinia* occurred in different external loops of different species[14]; the same feature was described in several studies of other bacterial porins.[38,39] Apparently, the presence of both negative and positive selection is the com-

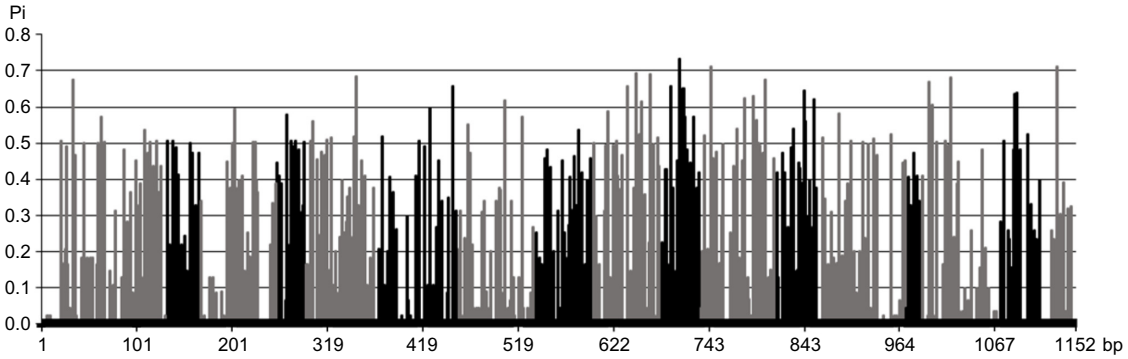

Figure 2. Nucleotide divergence (Pi) of *ompC*. Regions encoding the periplasmic loops and transmembrane strands are indicated by gray shading, the regions encoding the external loops (L1–L8) are colored by black shading.

Figure 3. Multiple alignment of OmpC sequences from *Yersinia* species.
Notes: β1–β16 – transmembrane β-strands; β16 – part of β16-strand; L1–L8 – external loops; P1–P8 – periplasmic loops. Amino acid residues that demonstrated negative selection are marked by light gray shading; amino acid residues that demonstrated positive selection are marked by dark gray shading.

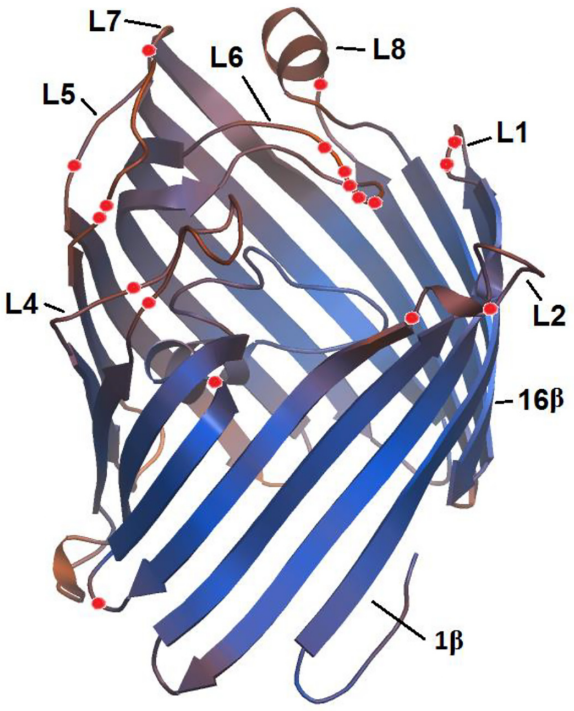

Figure 4. Location of positively selected sites in simulated structure OmpC porin of *Y. pseudotuberculosis*.
Note: Sites that show positive selection are marked by red spheres.

mon characteristic of bacterial porins. Thus, the *ompC* gene of *Yersinia* evolves with a conserved protein structure, which is essential for the basic transport function, and diversification of the external loops, which allow the protein to develop additional functions.

Antigenic determinant analysis. Porins are well known as antigenic and immunogenic proteins.[7,8] Their rapidly evolving regions that generally correspond to the external loops can serve as a molecular basis for the emergence of antigenic variation and escaping from the host immune system.[40] We analyzed OmpC sequences from each species on the IEDB Analyses Resource server to predict linear B-cell epitopes in a protein. Figure 5 schematically represents the predicted antigenic determinants along the peptide chain. The number and localization of epitopes were almost the same among the different species, whereas their sequence heterogeneity was remarkably different. We suggest that this high sequence variability may result in the immunological heterogeneity of OmpC in different *Yersinia* species. Differential levels of cross-reactivity between the different porin variants were previously reported.[35,41] In the experiments, the authors demonstrated that antibodies prepared against one porin variant either weakly bind or do not interact with other variants, confirming that the variable external loops contain

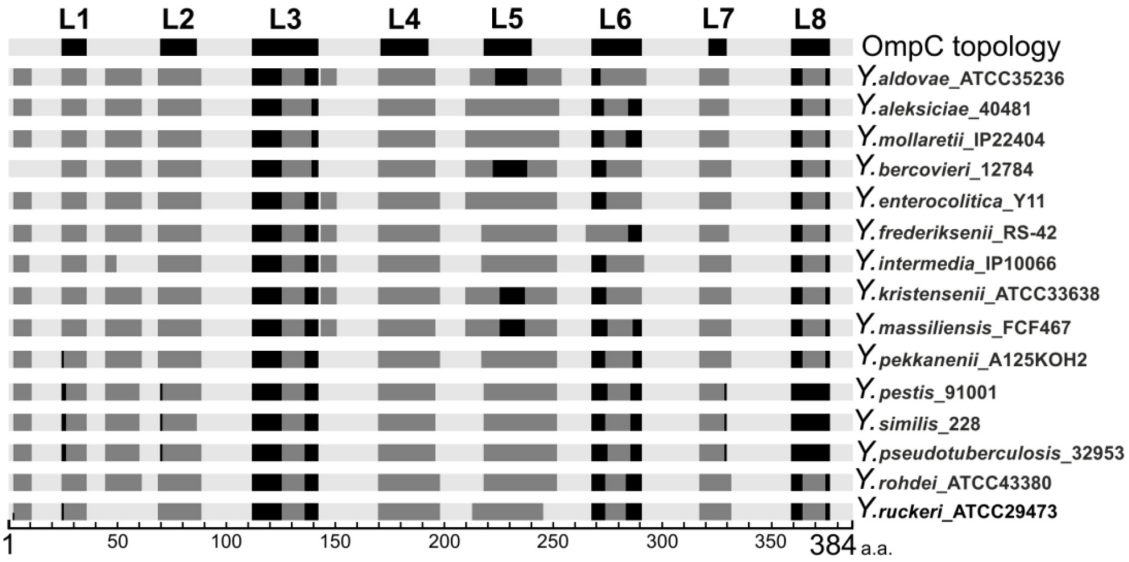

Figure 5. Location of antigenic determinant regions along OmpC of *Yersinia* species.
Notes: External loops (L1–L8) are indicated by black shading; periplasmic loops and transmembrane β-strands are indicated by light gray shading; and antigenic determinants are indicated by dark gray shading.

regions suitable for the development of OmpC-based vaccines and diagnostic assays.

Conclusion

The genus *Yersinia* includes a diverse group of species from environmental isolates (*Yersinia aldovae*, *Yersinia massiliensis*, etc.) to pathogens of insects (*Y. entomophaga*), fish (*Y. ruckeri*), mammals (*Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii*), and humans (*Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*). In this work, we investigated macro- and microevolutionary processes contributing to the divergence of the *ompC* gene, which encodes one of the major *Yersinia* porins. Our findings indicate that the patterns of *ompC* that change across the genus *Yersinia* are consistent with those of the shaping of the genus. Positive selection observed in external loops and homologous recombination provided the exchange of novel *ompC* variants within *Yersinia* populations. It can be speculated that homological recombination between porin genes is a strategy for the survival of *Yersinia* species under various stress conditions, such as a new niche, starvation, or bactericides. Taking into account the fact that OmpC is one of the predominant proteins in the outer membrane at a warmblood condition, the identification of antigenic determinants in rapidly evolving regions may indicate the evolutionary mechanisms used by *Yersinia* species to adapt to the pressures of the host immune system. The results of this study reveal the role of recombination and positive selection in the expansion of adaptability of bacterial populations. Further experiments are needed to establish the role of the individual variants in the pathophysiology of *Yersinia*.

Author Contributions

Conceived and designed the experiments: AMS, MPI. Analyzed the data: AMS. Wrote the first draft of the manuscript: AMS, EPB. Contributed to the writing of the manuscript: AMS, EPB, MPI, AVR. Agreed with manuscript results and conclusions: AMS, EPB, KVG, AVR, MPI. Jointly developed the structure and arguments for the paper: AMS, EPB, MPI, KVG. Made critical revisions and approved the final version: AVR, MPI. All the authors reviewed and approved the final manuscript.

Supplementary Material

Supplementary Table 1. Characteristics of strains and sequences.

Notes: [1]Collection of Research Institute of Epidemiology and Microbiology, Siberian Branch of Russian Academy of Medical Sciences (Vladivostok, Russia). [2]Collection of Max von Pettenkofer Institute for Hygiene and Clinical Microbiology, Ludwig Maximilian University (Munich, Germany), represented by bold text.

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