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Genome sequencing of a virulent avian *Pasteurella multocida* strain GX-Pm reveals the candidate genes involved in the pathogenesis

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ABSTRACT

Pasteurella multocida (*P. multocida*) was first shown to be the causative agent of fowl cholera by Louis Pasteur in 1881. First genomic study was performed on an avirulent avian strain Pm70, and until 2013, two genomes of virulent avian strains X73 and P1059 were sequenced. Comparative genome study supplied important information for further study on the pathogenesis of fowl cholera. In the previous study, a capsular serotype A strain GX-Pm was isolated from the liver of a chicken, which died during an outbreak of fowl cholera in 2011. The strain showed multiple drug resistance and was highly virulent to chickens. Therefore, the present study performed the genome sequencing and a comparative genomic analysis to reveal the candidate genes involved in virulence of *P. multocida*. Sequenced draft genome sequence of GX-Pm was 2,292,886 bp, contained 2941 protein-coding genes, 5 genomic islands, 4 IS elements and 2 prophage regions. Notability, all the predicted drug-resistance genes were included in predicted genomic islands. A comparative genome study on virulent avian strains P1059, X73 and GX-Pm with the avirulent avian strain Pm 70 indicated that 475 unique genes were only identified in either of virulent strains but absent in the avirulent strain. Among these genes, 20 genes were contained within genomes of all three virulent strains, including a few of putative virulence genes. Further characterization of the pathogenic functions of these genes would benefit the understanding of pathogenesis of fowl cholera.

1. Introduction

Fowl cholera is an acute, fatal septicemic disease of various domestic and wild bird species, which is responsible for significant loss in poultry husbandry (Christensen and Bisgaard, 2000). In chickens, common forms of the disease are acute and chronic. In acute fatal cases, chickens often show no clinical prodrome, sudden onset and rapid death. Pathological examination can find that there is a lot of yellow pericardial fluid in dead chickens and a large number of gray, needle-size necrosis points distributed in the liver (Christensen and Bisgaard, 1997, 2000). In chronic disease, signs are principally due to localized infections of leg or wing joints, comb, wattles and subcutaneous tissue of the head (Christensen and Bisgaard, 2000).

The pathogen, *Pasteurella multocida* (*P. multocida*) can be divided into five serogroups (A, B, D, E, and F) and 16 somatic types based on differences in capsular antigens and lipopolysaccharide antigens respectively (Carter, 1955; Heddleston et al., 1972). Serotype of *P. multocida* is designated by its capsular type followed by the somatic type, and the most common serotypes causing fowl cholera are A:5, A:8, A:9 and A:1 in China and A:1, A:3, A:3.4 in the United States (Zheng, 1984; Rhoades and Rimler, 1990; Wang et al., 2009).

There is wide variation in virulence of different isolates of *P. multocida* (Wilkie et al., 2012). Therefore, genomic study could supply essential information for understanding the virulence factors. First genomic study was performed on avirulent avian strain Pm70, and identified 104 putative virulence-associated genes, including filamentous hemagglutinin pfhB1 and pfhB2, which were involved in adherence of bacterial cells to host cell surfaces, and 53 genes, which were involved in iron acquisition and metabolism (May et al., 2001). Since then, there was no new whole genome sequence of *P. multocida* available during the past decade. Until 2013, a comparative genomic study was performed on virulent strains X73, P1059 and avirulent strain Pm70 (Johnson et al., 2013). As a result, 336 unique genes were found only

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in virulent strains but absent in the avirulent strain Pm70, and these genes included L-fucose transport and utilization system, several novel sugar transport systems, and several novel hemagglutinins including PfhB4 (Johnson et al., 2013). Undoubtedly, the comparative genomic analysis on more virulent and avirulent strains would also benefit for identification of the candidate genes involved in the pathogenesis of fowl cholera.

Therefore, in the present study, a capsular serotype A of *P. multocida* strain GX-Pm, which was isolated from the liver of a dead chicken during the outbreak of fowl cholera in China in 2011 and showed high virulence to chickens (Yu et al., 2013) was used for whole genome sequencing. Then, a comparative genomic study on virulent strains of GX-Pm, P1059, X73 and the avirulent strain Pm70 was performed to identify the genes involved in the pathogenesis of fowl cholera.

2. Materials and methods

2.1. Bacterial strains

Strain GX-Pm was a pathogenic strain isolated from the liver of a chicken that died of acute fowl cholera in 2011 in Guangxi, China (Yu et al., 2013). The GX-Pm strain was maintained on tryptic soy agar (Becton, Dickinson and Company, USA) plus 10% bovine serum or cultured in tryptic soy broth medium (Becton, Dickinson and Company, USA) plus 10% bovine serum to mid-log phase at 37 °C under aerobic conditions. Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany). The concentration was determined by Nanodrop2000 and the quality of the extracted total genomic DNA was evaluated by agarose gel electrophoresis.

2.2. Genome sequencing and assembly

Sequencing of strain GX-Pm was performed using Illumina Miseq platform at Sangon Biotech Co., Ltd. A paired-end sequencing library was constructed with insert sizes of approximately 500 base pairs (bp), and a mate-pair sequencing library was constructed with average insert size of approximately 5000 bp. The low-quality reads were filtered and only high-quality reads were used for *de novo* assembly with Velvet 1.1 (Zerbino and Birney, 2008). In order to fill the intra-scaffold gaps, we used paired-end and mate-pair information to retrieve read pairs that had one read well aligned on the contigs and the other read located in the gap region. The obtained scaffolds were ordered relative to the genome of Pm70 (GenBank accession number AE004439.1) with MUMmer3 (Kurtz et al., 2004). The draft genome sequence of strain GX-Pm was deposited under the accession numbers: GenBank: JZXO00000000.

2.3. Genome annotation

Open reading frame (ORF) prediction was performed with RAST database (http://www.nmpdr.org/FIG/wiki/view.cgi/FIG/RapidAnnotationServer), which was a fully-automated service for annotating bacterial and archaeal genomes. Besides, all predicted ORFs were compared against the databases of non-redundant protein (nr), Swiss-Prot, TrEMBL, CDD, Pfam and Cluster of Orthologous groups (COG). tRNAs and rRNAs were identified using tRNAscan-SE (Lowe and Eddy, 1997) and RNAmmer1.2 (Lagesen et al., 2007), respectively. The genome plot of GX-Pm was generated with the DNAPlotter tool (Carver et al., 2009).

2.4. Genome analysis and identification of horizontal gene transfer

Insertion sequence (IS) elements were searched with IS Finder (Siguier et al., 2006). Genome islands (GIs) were identified using IslandViewer (Langille and Brinkman, 2009), which was a computational tool that integrates three different genomic island prediction methods: IslandPick, IslandPath-DIMOB, and SIGI-HMM, followed by manual inspection. In order to identify the drug-resistance and virulence-associated genes, we compared the genome sequences against the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009). The prophage sequences were analyzed using PHAge Search Tool (PHAST) (Zhou et al., 2011).

2.5. Comparison of genome structure of GX-Pm with other avian *P. multocida* strains

To gain a better understanding of genome sequences among different avian *P. multocida* isolates, three additional sequenced *P. multocida* genome sequences were obtained from NCBI. The selected strains were Pm70 [GenBank: AE004439.1], P1059 [Accession: NZ_CM001581.1] and X73 [Accession: NZ_CM001580.1]. The four genomes were compared to each other using progressive Mauve with default parameters (Darling et al., 2010). In order to analyze the shared and unique genes in the *P. multocida* genomes sequenced, BlastP strategy was used with a similarity cutoff of 90% identity as a designation for similar or dissimilar coding proteins.

3. Results and discussion

3.1. General features of the sequenced GX-Pm genome

Prior to perform genome sequencing, the paired-end (PE) and mate-pair (MP) libraries were constructed. The result of genome sequencing indicated that the ratio of obtained high-quality (>Q20) data was 97.39% (PE sequencing) and 81.06% (MP sequencing), and sequencing depth was 717 and 560, respectively. The low-quality reads were filtered and only high-quality reads were used to draft assemble, resulting in 8 scaffolds (>500 bp). The size of the largest scaffold was 2,275,759 bp, according to 99.3% of the whole genome.

Draft genome sequence of strain GX-Pm was 2,292,886 bp, with average GC % content of 40.75%. According to the annotation with RAST database, the draft genome of strain GX-Pm contained 2941 ORFs, 52 tRNA-carrying genes, with a coding percentage of 87.11% (Table S1). All genome sequences were deposited in NCBI database (accession number: JZXO00000000). In addition, general genome features of strain GX-Pm were consistent with previous studies (Table S2) (May et al., 2001; Liu et al., 2012; Johnson et al., 2013). The schematic circular diagram of the GX-Pm strain was generated with the DNAPlotter tool (Fig. 1).

3.2. Genomic Islands and prophage regions in GX-Pm genome

Because GIs often contained genes involved in virulence, antibiotic resistance or other important adaptations, and were considered to play a critical role in pathogenicity of bacteria (Dobrindt et al., 2004), it prompted us to analyze the GIs in GX-Pm genome. Five GIs were found in the genome of GX-Pm by using IslandViewer (Table S3) (Langille and Brinkman, 2009). It was interesting that all the predicted drug-resistance genes by the ARDB database (2 streptomycin resistance genes, 1 chloramphenicol resistance and 1 sulfonamide resistance gene) were included in predicted genomic islands. Because GX-Pm strain showed resistance to gentamycin, cephradine, danamycin, neomycin, streptomycin, doxycycline, clindamycin, ampicillin, amikacin, vancomycin, lincomycin, azithromycin and spectinomycin (Yu et al., 2013), the present study may suggest that getting horizontal transfer elements may be one of the important reasons for the Chinese current strain GX-Pm to become multiple drug resistance.

By blasting IS database, 4 IS elements were found (Table S4). It was very interesting that all IS elements were included in the GIs, and it suggested that the GIs of GX-Pm may be involved in several functions of this bacterium.

Prophages, including defective ones, contributed to important biological properties to their bacterial hosts and are common in bacterial genomes that had been sequenced (Casjens, 2003), and various studies