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Comparative Genomics of *Streptococcus pyogenes* M1 isolates differing in virulence and propensity to cause systemic infection in mice

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ABSTRACT

Streptococcus pyogenes serotype M1 is a frequent cause of severe infections in humans. Some M1 isolates are pathogenic in mice and used in studies on infection pathogenesis. We observed marked differences in murine infections caused by M1 strain SF370, 5448, 5448AP or AP1 which prompted us to sequence the whole genome of isolates 5448 and AP1 for comparative analysis. Strain 5448 is known to acquire inactivating mutations in the CovRS two-component system during mouse infection, producing hypervirulent progeny such as 5448AP. Isolates AP1 and 5448AP, more than 5448, caused disseminating infections that became systemic and lethal. SF370 was not pathogenic. Phages caused gross genetic differences and increased the gene content of AP1 by 8% as compared to 5448 and SF370. Each of six examined M1 genomes contained two CRISPR-Cas systems. Phage insertion destroyed a type II CRISPR-Cas system in AP1 and other strains of serotypes M1, M3, M6 and M24, but not in M1 strains 5448, SF370, MGAS5005, A20 or M1 476. A resulting impaired defence against invading genetic elements could have led to the wealth of phages in AP1. AP1 lacks genetic features of the MGAS5005-like clonal complex including the streptodornase that drives selection for hypervirulent clones with inactivated CovRS system. Still, inactivating mutations in *covS* were a common genetic feature of AP1 and the MGAS5005-like isolate 5448AP. Abolished expression of the cysteine proteinase SpeB, due to CovRS inactivation could be a common cause for hypervirulence of the two isolates. Moreover, an additional protein H-coding gene and a mutation in the regulator gene *rofA* distinguished AP1 form other M1 isolates. In conclusion, hypervirulence of *S. pyogenes* M1 in mice is not limited to the MGAS5005-like genotype.

1. Introduction

Streptococcus pyogenes is a Gram-positive bacterium that causes a broad spectrum of infections in humans, including relatively mild purulent infections of the throat and the skin, erysipelas, as well as severe necrotizing soft tissue, invasive infections and streptococcal toxic shock syndrome (STSS) (Chhatwal and Graham, 2008). Naturally, *S. pyogenes* infects humans and has no known animal reservoir. However, to investigate the pathogenesis of *S. pyogenes* infections several murine models have been developed, which use specific mouse virulent isolates. *S. pyogenes* is divided into different M serotypes, correlating with *emm* genotypes of which more than 220 are known today (Sanderson-Smith et al., 2014).

Genomic population structure analysis of more than 3600 *S. pyogenes* isolates distinguished two groups of serotype M1 (*emm*-type 1), SF370-like and MGAS5005-like isolates. In MGAS5005-like isolates horizontal gene transfer led to increased virulence and spread of these M1 strains since the 1980s (Nasser et al., 2014). Serotype M1 is one of the most frequent M-types isolated from invasive infections worldwide (Steer et al., 2009) and most of the murine models of infection use M1 isolates such as strains AP1 and 5448.

Strain AP1 derives from an invasive clinical isolate that binds human IgG via protein H (Åkesson et al., 1990). Furthermore, the virulence factor SIC (Streptococcal Inhibitor of Complement) was first discovered in AP1 (Åkesson et al., 1996). The M1 protein of this strain induces vascular leakage in the lungs after intravenous injection into mice (Herwald et al., 2004) and subcutaneous inoculation of AP1 evokes a systemic infection in Balb/c (Oehmcke et al.,

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2009; Oehmcke et al., 2013) as well as in C57BL/6 (Shannon et al., 2010) mice. Strain 5448, a MGAS5005-like isolate (Maamary et al., 2012), also derives from a clinical isolate and was employed in a murine model of necrotizing fasciitis (Buchanan et al., 2006) and in other mouse models of skin and tissue infections in several studies (Cole et al., 2006; Zinkernagel et al., 2012, 2008). This strain acquires functionally inactivating mutations in genes that code for the (control of virulence) CovRS two component regulatory system, which increases virulence as in the progeny 5448AP (Aziz et al., 2004a, Cole et al., 2006; Maamary et al., 2012; Li et al., 2013). Strain SF370 was not virulent after subcutaneous injection in mice (Maamary et al., 2012).

Animal experiments complement studies that use isolates and clinical data and from human infections. Other than in natural infections in humans, infection aetiology is controlled in animal models and opportunism of infection as well as differential host predisposition can be excluded in these studies. Despite some limitations and the availability of primate models (Nasser et al., 2014) murine models remain important for vaccine and pathogenesis research. They are comparatively inexpensive, offer a homogeneous genetic background and genetically modified mice are available for studies on molecular infection pathogenesis and host interactions. Previous infection experiments with different M1 isolates in mice were not comparable as they differed in infection doses, culture conditions of the initial inoculum and the infected mouse strain, prompting us to investigate the outcome of infection in C57BL/6 mice under comparable conditions. By comparative genome sequence analysis we investigated the genetic basis for the marked differences between AP1, 5448 (5448AP) and SF370 in virulence and propensity to cause systemic infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *S. pyogenes* strain AP1 (40/58) of serotype M1 belongs to the collection of the World Health Organisation (WHO) Collaborating Center for Reference and Research on Streptococci (Prague, Czech Republic). Strain 5448 of serotype M1 was originally isolated from a patient with necrotising fasciitis and toxic shock syndrome (Chatellier et al., 2000). Its progeny 5448AP was obtained after animal passage (Aziz et al., 2004a). Strain SF370 of M1 serotype was described and sequenced earlier (Ferretti et al., 2001). Bacteria were grown overnight in Todd–Hewitt-Broth (THB) supplemented with 0.5% yeast extract (THY) at 37 °C and 5% CO_2 without shaking. For infection experiments bacteria were harvested in mid-logarithmic phase (O.D. 0.5–0.6) and washed with sterile PBS. The bacterial concentration of 5×10^8 CFU/ml in PBS was adjusted if required.

2.2. Mouse infection model and determination of bacterial loads

Eight to twelve week old female C57BL/6 mice (Harlan, Venray, The Netherlands) were housed in a pathogen-free animal facility at the Helmholtz Centre for Infection Research under standard conditions according to institutional guidelines. All animal experiments were approved by the appropriate ethical committee for animal experimentation (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany; permit 33.9-42502-04-12/1009). Mice were infected with 5×10^7 CFU *S. pyogenes* subcutaneously on the back. Animals were weighed and monitored daily for survival for a maximum of 8 days. To determine bacterial loads and coagulation parameters mice were sacrificed 48 h after bacterial inoculation. Citrated blood was drawn by cardiac puncture and livers as well as spleens were removed. Blood and organ homogenates were plated onto blood agar in tenfold serial dilution. Bacterial loads were determined by counting colonies after 18 h of incubation at 37 °C.

2.3. Measurement of coagulation parameters

Plasma from uninfected and infected mice was prepared by centrifugation of citrated blood (ratio blood to sodium citrate 9:1) at 5000 rpm for 10 min and stored at −80 °C until further analysis. Activation of the intrinsic pathway of coagulation was determined by measuring activated partial thromboplastin time (aPTT) in a coagulometer (Merlin, Lemgo, Germany). Fifty microlitre of citrated plasma were incubated for 60 s at 37 °C, 50 μl kaolin (Dapttin®TC; Technoclone, Vienna, Austria) were added followed by another 60 s of incubation at 37 °C. The clotting time was measured after the addition of 50 μl $CaCl_2$ solution (25 mM).

2.4. DNA Isolation

Genomic DNA of *S. pyogenes* strain AP1 was isolated by the "CTAB" method (Ausubel et al., 1987) with the following modifications. Bacteria were grown overnight in Todd–Hewitt Broth supplemented with 0.5% yeast. Three mililitre were centrifuged and the pellet was resuspended in TE buffer containing 10% SDS, 20 mg/ml pronase E, 500 U/ml mutanolysin and 20 mg/ml lysozyme. After incubation at 37 °C for 1 h DNA was isolated as described in the protocol. Genomic DNA of *S. pyogenes* strain 5448 was isolated as described previously (Bergmann et al., 2014).

2.5. Genome sequencing and assembly

Genomic paired-end libraries (~530 bp insert size) were prepared according to the manufacturer's instructions using Illumina's TruSeq DNA LT Sample Prep Kit. Illumina sequencing was performed on a MiSeq platform with 250 cycles, resulting in 2.12 million raw reads for AP1 and 5.39 million for 5448. A kmer correction and clipping (quality- and length-filtering) were performed by fastq-mcf (Aronesty, 2013) and quake (Kelley et al., 2010).

The raw reads were assembled using both velvet 1.2.10 (Nawrocki et al., 2009) and SOAPdenovo 2.04 (Luo et al., 2012). Additionally, Opera (Optimal Paired-End Read Assembler) (Gao et al., 2011) was used for the assembly of 5448 sequences. The resulting draft genomes were merged by minimus2 aligner (Sommer et al., 2007). Remaining gaps were closed by manual editing and re-mapping approaches for SNP detection or by bridging PCR. The combined sequences provided a 238-fold (AP1) and 655-fold (5448) coverage of the genomes.

2.6. Genome annotation

Genes were identified using Prodigal (Hyatt et al., 2010) as part of the JGI (Joint Genome Institute) genome annotation pipeline. Translated CDSs were used to search the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGR-Fam, Pfam, PRIAM, KEGG, COG, and InterPro databases. RNA genes were identified using HMMER 3.0rc1 (Finn et al., 2011) (rRNAs) and tRNAscan-SE 1.23 (Lowe and Eddy, 1997) (tRNAs). Other non-coding genes were predicted using INFERNAL 1.0.2 (Nawrocki, 2014). Additional gene prediction analysis and functional annotation was performed using the Integrated Microbial Genomes – Expert Review (IMG-ER) platform (Markowitz et al., 2009). Putative prophage sequences were identified by PHAST (phage search tool) (Zhou et al., 2011). CRISPR elements were detected using PILER-CRT (Bland et al., 2007) and PILER-CR (Edgar, 2007).