Comparison of virulence genes found in draft genomes of *F. necrophorum*

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Introduction

Fusobacterium necrophorum is a causative agent of persistent sore throat syndrome, tonsillar abscesses and Lemierre's syndrome (LS) in humans.[1] LS is characterised by thrombophlebitis of the jugular vein and bacteraemia.[2] It is a Gram-negative, anaerobic bacterium which to date has no available reference genome. Draft genomes suggest it to be a single circular chromosome of approximately 2.1Mb.[3]

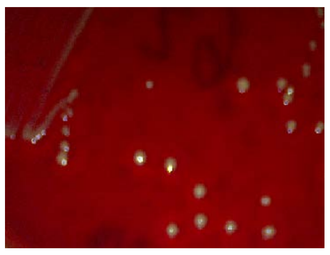

Figure 1: *F. necrophorum* culture on a fastidious anaerobe agar plate.

F. necrophorum is divided into two subspecies known as *F. necrophorum* ssp. *necrophorum* (FNN) and *F. necrophorum* ssp. *funduliforme* (FNF).[4] It is reported that FNN is the most virulent type. This is believed to be due to a greater production of a leukotoxin which is considered to be the organism's primary virulence factor.[5]

The aim was to *de novo* sequence three genomes, search for putative virulence factors and make comparisons between strains.

Methods

A reference strain of each *F. necrophorum* subspecies and a clinical isolate from a LS patient were kindly donated by the Anaerobe Reference Unit, Cardiff. These were commercially sequenced on a Roche 454 GS-FLX+ and genomes were assembled using Roche GS Assembler. The resulting contigs were provided in FASTA and FASTQ formats. These were annotated using xBASE for open reading frame (ORF) prediction and gene annotation using *F. nucleatum* as a reference. ORFs not assigned a gene were manually annotated with BLAST and Pfam. Annotation data was mined for gene products associated with virulence.

Leukotoxin presence was confirmed by PCR in a collection of 55 isolates. Primers were from previous studies[6,7] and specially designed. Artemis Comparison Tool (ACT) was used for sequence comparisons.

Clustal Omega was used to compare motifs within the amino acid sequence of a putative Type V two-partner secretion (TPS) system.

Results

Table 1: Data from 454 *de novo* sequencing and assembly with Roche Assembler.

	JCM 3718 (FNN)	JCM 3724 (FNF)	Clinical LS strain
Genome coverage	11.2X	8.4X	11.6X
Number of contigs from Roche Assembler	812	629	436
Average contig size	2,835	3,097	4,715
Largest contig	28,070	40,328	41,389
Total number of bases in contigs	2,301,868	1,947,814	2,055,836

Table 2: Number of genes annotated by xBASE.

	JCM 3718 (FNN)	JCM 3724 (FNF)	Clinical LS strain
Total number of open reading frames predicted	1991	1754	1918
Number assigned gene product	1194 (60%)	1112 (63%)	1167 (61%)
Number unlabelled or labelled as hypothetical	797 (40%)	642 (37%)	751 (39%)
Number remaining annotated with BLAST	502	408	425

The genome sizes were close to 2.1Mb. Putative virulence genes found in all strains include a leukotoxin, haemolysin, adhesin, filamentous haemagglutinnin, hemin receptor, phage genes, CRISPR-associated proteins, ecotin and a putative type V secretion system.

The leukotoxin gene was present in 100% of isolates. A cytotoxicity assay confirmed leukotoxic activity of the three strains against human donor white blood cells and HL60 cells (data not shown) before the leukotoxin operons were Sanger sequenced and aligned using Artemis Comparison Tool (Figure 2).

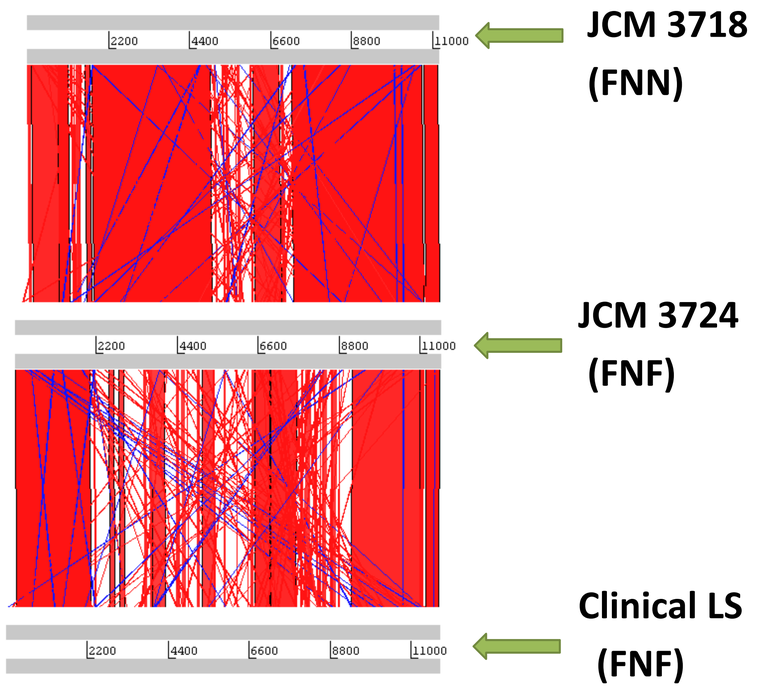

Figure 2: Artemis Comparison Tool shows comparison of leukotoxin operon between the FNN and FNF reference strains, and between the FNF reference strain and the clinical FNF strain. Grey horizontal bars represent the sequences, red blocks show high similarity and blue lines show reversed sections of similarity. White sections (or breaks) show unique regions.

TPS systems are often used for secreting large virulence proteins, such as adhesins. They contain conserved motifs with amino acid sequences NPNL and NPNGI within the secretion domain.[8] The JCM genomes were found to contain TPS secretion domains, with motifs NPNL and NENGI, 35 bases apart within a haemolysin gene (see Figure 3). The Clinical LS strain contained a haemolysin gene with NDNGI 39 bases upstream of NKNL.

```
fhaB        GALTKNPNLTRQASAILAEVTDTSPSRLAGTLEVYGKGADLIIANPNGISVNGL
3718haem    GEVSANPNLTNSASVILNEIQGNSASELNGGLEVFGKRADLVIANENGINVNGA
3724haem    GEVSANPNLTNSASVILNEIQGNSASELNGGLEVFGKRADLVIANENGINVNGA
            * :: ***** ..**.** *:  .* *.* * ***:** ***:*** ***.***
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Figure 3: Clustal Omega alignment of motif-containing segment of the haemolysin gene from JCM 3718 and JCM 3724. For comparison, a filamentous haemagglutinin from *Bordetella pertussis* was included (*fhaB*) as a sequence already characterised as being part of a TPS system.[8]

The motifs were not found as part of the filamentous haemagglutinin, nor adhesin. The adhesins contained domains known to be associated with type V autotransporters.

The three genomes contained the CRISPR-associated proteins Cas1, Cas5, Csx8 and DevR. The FNN strain additionally contained Cst1.

Ecotin, a serine protease inhibitor, was found to be highly conserved in the three strains. A recombinant ecotin was shown to inhibit clotting in human donor plasma (see poster S19/31 for results and further analysis).

Discussion

The ACT alignment (Figure 2) suggests variability within the middle sections of the leukotoxin operon sequence. This could indicate regions of unconserved repeats or membrane spanning regions. This should be taken into consideration when designing primers. Aligning further sequences may help highlight the active toxin region, which is likely to be conserved. The mechanism for this toxin is still unknown.

The motifs within the haemolysin gene of the JCM strains suggest the protein is secreted via a two-partner system. This has not previously been described in *F. necrophorum*. The motifs within the clinical strain are fairly conserved, but in the reverse order. Not all TPS proteins need both motifs[8] so secretion may be unaffected. Comparing secretion efficiencies *in vitro* would be of interest.

The three genomes appear to share most genes. FNN's greater virulence is likely to result from higher production of shared virulence genes, rather than additional genes not present in FNF. Small sequence variations may also make FNN virulence proteins more potent.

References

1. Batty, A., Wren, M. W. D., *et al*. (2005) "Fusobacterium necrophorum as the cause of recurrent sore throat: comparison of isolates from persistent sore throat syndrome and Lemierre's disease." *Journal of Infection* 51(4): 299-306.
2. Kuppalli, K., Livorsi, D., *et al*. (2012) "Lemierre's syndrome due to Fusobacterium necrophorum." *The Lancet Infectious Diseases* 12(10): 808-815.
3. Calcutt, M. J., Foecking, M. F., *et al*. (2014) Draft Genome Sequence of *Fusobacterium necrophorum* subsp. *funduliforme* Bovine Liver Abscess Isolate B35. *Genome Announcements* 2, 412-14.
4. Shinjo, T., Fujisawa, T., *et al*. (1991) "Proposal of Two Subspecies of Fusobacterium necrophorum (Flügge) Moore and Holdeman: Fusobacterium necrophorum subsp. necrophorum subsp. nov., nom. rev. (ex Flügge 1886), and Fusobacterium necrophorum subsp. funduliforme subsp. nov., nom. rev. (ex Hallé 1898)." International Journal of Systematic and Evolutionary Microbiology 41(3): 395-397.
5. Smith, G. R., Wallace, L. M., *et al*. (1990). "Experimental observations on the pathogenesis of necrobacillosis." 104(01): 73-78.
6. Ludlam, H. A., Milner, N. J., *et al*. (2009) *lktA*-encoded leukotoxin is not a universal virulence factor in invasive *Fusobacterium necrophorum* infections in animals and man. *Journal of Medical Microbiology* 58, 529–530
7. Tadepalli, S., Stewart, G. C., *et al*. (2008). Leukotoxin operon and differential expressions of the leukotoxin gene in bovine Fusobacterium necrophorum subspecies. *Anaerobe,* 14, 13-18.
8. Jacob-Dubuisson, F., Locht, C., Antoine, R. (2001) Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. Molecular Microbiology 40(2), 306-313.