Research in Microbiology 166 (2015) 384–392

www.elsevier.com/locate/resmic

Patho-genetics of *Clostridium chauvoei*

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Received 30 June 2014; accepted 26 October 2014
Available online 4 November 2014

Abstract

The genomic sequence of *Clostridium chauvoei*, the etiological agent of blackleg, a severe disease of ruminants with high mortality specified by a myonecrosis reveals a chromosome of 2.8 million base-pairs and a cryptic plasmid of 5.5 kilo base-pairs. The chromosome contains the main pathways like glycolysis/gluconeogenesis, sugar metabolism, purine and pyrimidine metabolisms, but the notable absence of genes of the citric acid cycle and deficient or partially deficient amino acid metabolism for Histidine, Tyrosine, Phenylalanine, and Tryptophan. These essential amino acids might be acquired from host tissue damage caused by various toxins and by protein metabolism that includes 57 genes for peptidases, and several ABC transporters for amino acids import.

Keywords: Blackleg; Virulence; Toxins; Metabolic pathway; Host–pathogen interactions; Mode of replication

1. Introduction

Clostridium chauvoei is a highly pathogenic, histotoxic, anaerobic, endospore forming Gram-positive bacterium causing blackleg, a severe disease of cattle, sheep and other domestic animals. Blackleg is globally spread among ruminants specified primarily as a myonecrosis with high mortality causing significant losses in livestock production [15,18,22,29]. *C. chauvoei* is one of the most pathogenic *Clostridium* species. Although *C. chauvoei* is mainly considered to be specific to ruminants, rare fatal cases of fulminant human gas gangrene and neutropenic enterocolitis caused by *C. chauvoei* have been reported and it is assumed that prevalence of *C. chauvoei* causing disease in humans may be higher than currently diagnosed [38,26]. Infection of ruminants by *C. chauvoei* is caused by exposure of the animals to the pathogen present in form of spores in the soil of "poisoned" pastures. However, *C. chauvoei* has been detected in manure which also represents a source of infection and can lead to contamination of pastures [2]. The infection by *C. chauvoei* leads to myonecrosis, oedemic lesions and fever, followed by lameness and death [18,34]. Pathology of blackleg is mostly found in muscular tissue of infected animals from where the pathogen generally is isolated. Blackleg in cattle and sheep is controlled worldwide by commercial vaccines that consist of whole, inactivated bacteria and chemically toxoided culture supernatants [35]. Furthermore, outer membrane proteins and flagella have been proposed as immunogens against *C. chauvoei* infections [5,6,24,25,31,32]. The molecular mechanisms of pathogenicity of *C. chauvoei* in particular the spreading of this pathogen from the digestive tract where it is taken up to the muscle tissue where lesions are most abundant and where the pathogen is found at high amounts assumingly due to replication, is largely unknown.

Current knowledge reveals that toxins, DNAse, hyaluronidase, neuraminidase and flagella seem to make a major contribution to pathogenicity of *C. chauvoei* [5,13,17,24,25,30,32,33,36]. Among the postulated toxins of *C. chauvoei*, including an oxygen stable haemolysin, an oxygen labile haemolysin and 27 kDa not further specified haemolytic protein. "*Clostridium chauvoei* toxin A" (CctA) a

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http://dx.doi.org/10.1016/j.resmic.2014.10.013

32 kDa protein β-barrel pore forming toxin belonging to the leucocidin superfamily of bacterial toxins has recently been reported and was analysed in detail both genetically and biochemically [13]. This leucocidin CctA represents the main haemolytic and cytotoxic activity of *C. chauvoei*. Recombinant polyhistidine tailed CctA produced in *Escherichia coli* and purified after solubilisation in 6 M guanidine hydrochloride, by Ni^{2+} chelate affinity chromatography, is strongly haemolytic [13] and does not lose haemolytic activity after exposure to atmospheric oxygen [13]. Furthermore, guinea pigs vaccinated with inactivated recombinant CctA were protected against challenge with virulent *C. chauvoei*, thus showing its central role in virulence and its potential in immune protection. Besides CctA, the genetic basis for the sialidase NanA has been determined [36]. Besides these two specific virulence genes and genes encoding flagellar antigens, little is known on the genetics of *C. chauvoei*. Very recently, we have determined the genome sequence of a virulent strain of *C. chauvoei* (JF4335) isolated from a cattle that succumbed of blackleg in 2004 [11]. This is currently the only comprehensive genomic dataset of *C. chauvoei* (GenBank/EMBL accession number CBML0000000000.1). The current study represents a comprehensive genomic analysis of *C. chauvoei* aiming in enhancing knowledge about virulence and adaptation of this clostridium to infect via ingestion and induce myonecrosis in muscle tissue.

2. Results and discussion

2.1. C. chauvoei *general genome statistics overview and comparative genomics*

The genome of *C. chauvoei* strain JF4335 is estimated to consist of a single circular chromosome of 2.8 million base-pairs (Mbp). The currently available draft genome sequence is composed of 12 contigs covering a genome size of 2′825′126 base-pairs (bp) [11]. Moreover it contains a cryptic plasmid of 5.5 kilo base-pairs (kbp), which is present as medium copies per chromosome equivalent as estimated from total genomic DNA extraction (Fig. 1). The genome of the most frequently encountered pathogenic and also commensal *Clostridium* species, *Clostridium perfringens*, varies between 2.9 and 3.4 Mbp and contains various plasmids, that of *Clostridium botulinum* between 3.2 and 4.2 MBp and that of the metabolically diverse species *Clostridium acetobutylicum* is 4.1 Mbp. The relatively small genome of *C. chauvoei* as compared to other *Clostridium* sp. reflects its restricted environment, the bovine, caprine and ovine host, to which it is adapted and where the bacterium is able to replicate. The draft genome of *C. chauvoei* JF4335 contains 2630 predicted open reading frames (ORFs) of which 1935 protein sequences could be assigned and 632 ORFs represent hypothetical proteins that could not be assigned (Table 1). Of the assigned proteins most (259) are devoted to carbohydrate biosynthesis as revealed by RAST analysis (http://rast.nmpdr.org/) [28], showing the importance of carbohydrates for this pathogen both in the capsulated vegetative form during infection and in the sporulated form during rest and persistence in the environment (Fig. 2). Next frequent, 158 genes are involved in biosynthesis of cofactors, vitamins and prosthetic groups, 138 represent cell wall and capsule formation genes, 69 are directly involved in dormancy and sporulation, 65 are devoted to motility and chemotaxis, 60 represent stress protein genes, 44 are involved in antibiotic and metal resistance and 13 encode confirmed or

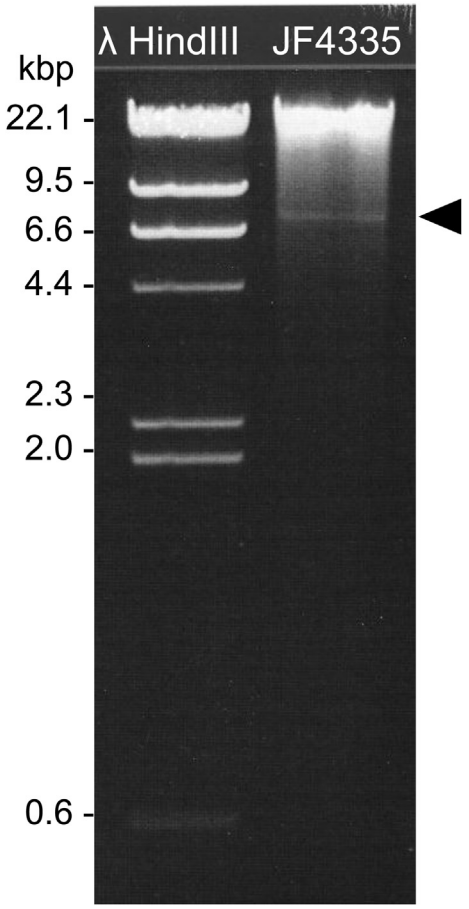

Fig. 1. Agarose gel analysis of total genomic DNA extracted from *C. chauvoei* JF4335 revealing the presence of a medium copy number plasmid marked by arrow.

Table 1
General genomic features predicted for *C. chauvoei* JF4335.

General feature	Number or % of the genome
Genome	
Number of ORFs	2630
Chromosome	
Size (bp)	2′825′126
G + C content	28.17
Number of ORFs	2′628
CDS numbers	2′567
Assigned function	1′935
Ribosomal RNA operons	5–10
Transfer RNA	50
Bacteriophage or phage-like 2	1
Plasmid	1
Plasmid size (bp)	5′566
Plasmid G + C content	25.28

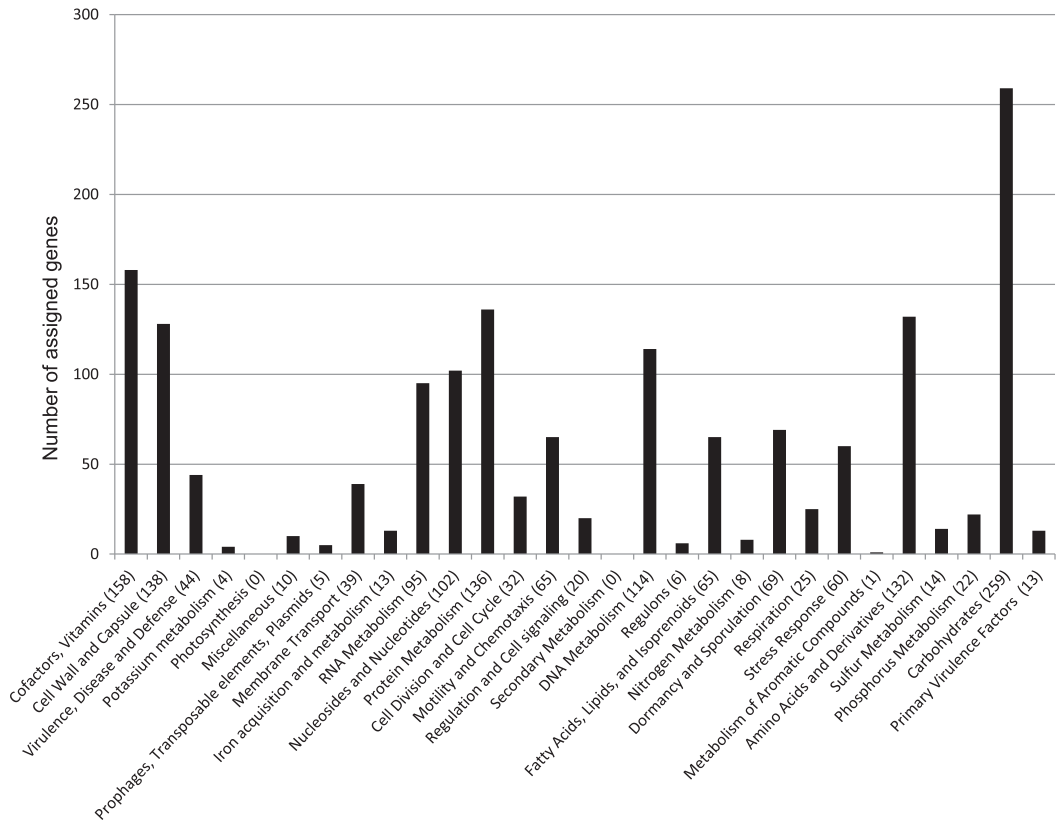

Fig. 2. Representation assigned proteins of the genome sequence of *C. chauvoei* arranged by the various functional groups.

primary virulence factors including 4 hemolysins/leucocidins (Table 2).

The putative origin of replication *oriC* consists of a DnaA binding domain including the nona-nucleotide consensus sequence TTATCCACA, followed by CDS comprising the replication initiation protein DnaA, DNA polymerase III beta-clamp DnaN, RecF GyrB and GyrA. The locus of the origin of replication was further supported by GC skew analysis calculated in 1000 bp windows across the genome. The scaffold containing the DnaA gene (NZ_CBML010000008.1 GI: 546551543) displays a characteristic peak near the position of this gene, adding more weight to the prediction (Fig. 3) (http://gcat.davidson.edu/DGPB/gc_skew/gc_skew.html).

The genome of *C. chauvoei* JF4335 reveals six genes encoding the RNA polymerase σ^{70} factor, and each a gene for alternative sigma factors σ^{54}, σ^{24} and σ^{H}. Furthermore there is a gene encoding an anti-sigma F factor and a gene for an anti-sigma F factor antagonist, which both are supposedly involved in the regulation of sporulation [10].

Analysis of the genome for the presence of prophages using the PHAST server [39] lead to the discovery of an intact prophage (26.2 kbp) similar to the *C. perfringens* bacteriophage ϕCP51 described recently [14] and having a similar gene organisation as bacteriophage ϕ3626 [40] showing in particular genes for small and large subunit terminase, portal protein, prohead protease, phage integrase, tail tape measure protein and major tail protein (Fig. 4).

The genome of *C. chauvoei* is very distant compared to other known clostridia genomes even to its closest available neighbour *C. perfringens str.13*. Comparing them using MAUVE [8] we confirmed the very low conservation as only few small co-linear blocks were identified (Fig. 5).

2.2. Metabolic genes

Comparing metabolic pathway with RAST, we found that the full presence of the main pathways like glycolysis/gluconeogenesis (Fig. 6), sugar metabolism, purine and pyrimidine metabolisms, as well as many amino acid metabolisms. Here we report the notable absence of genes of the citric acid cycle confirming similar discoveries in other anaerobic *Clostridium* species [1]. However, bypass genes like Si-citrate synthase or Re-citrate synthase [23] could not be identified indicating that *C. chauvoei* must use another bypass to the citric acid cycle.

Other pathways are deficient or partially deficient, mainly in the amino acid metabolism like Histidine, Tyrosine, Phenylalanine, and Tryptophan. These amino acids are essential and therefore it explains why RAST analysis revealed the presence of 136 functions involved in protein metabolism whereof 57 genes, mostly encoding for peptidases, play a role in protein degradation (Table 2). In addition several ABC transporters responsible for amino acids import are also identified. This reflects the protein rich environment, the

Table 2
Protein degradation genes.

ClpP	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)
ClpX	ATP-dependent Clp protease ATP-binding subunit ClpX
ClpS	ATP-dependent Clp protease adaptor protein ClpS
ClpA	ATP-dependent Clp protease ATP-binding subunit ClpA
ClpC	ATP-dependent Clp protease, ATP-binding subunit ClpC
ClpY	ATP-dependent hsl protease ATP-binding subunit HslU
ClpQ	ATP-dependent protease HslV (EC 3.4.25.-)
ClpB	ClpB protein
NERP	Nucleotide excision repair protein, with UvrB/UvrC motif
Lon	ATP-dependent protease La (EC 3.4.21.53)
LonI	ATP-dependent protease La (EC 3.4.21.53) Type I
LonII	ATP-dependent protease La (EC 3.4.21.53) Type II
DP	ATP-dependent protease domain protein (EC 3.4.21.-)
ClpXP	ClpXP protease specificity-enhancing factor
DegS	Outer membrane stress sensor protease DegS
DegQ	Outer membrane stress sensor protease DegQ, serine protease
AFG	ATPase, AFG1 family
CtsR	Transcriptional regulator CtsR
YacI	Putative ATP:guanido phosphotransferase YacI (EC 2.7.3.-)
PilT	Membrane-associated protein containing RNA-binding TRAM domain and ribonuclease PIN-domain
Col	Microbial collagenase (EC 3.4.24.3)
Col-Sec	Microbial collagenase, secreted (EC 3.4.24.3)
VibLysin	Vibriolysin, extracellular zinc protease (EC 3.4.24.25)
PseLysin	Pseudolysin, extracellular zinc protease (EC 3.4.24.26)
BacLys	Bacillolysin, extracellular neutral metalloprotease (EC 3.4.24.28)
LytM	Glycyl-glycine endopeptidase LytM precursor (EC
AcX	Acylamino-acid-releasing enzyme (EC 3.4.19.1)
PyroGlu	Pyrrolidone-carboxylate peptidase (EC 3.4.19.3)
IsoAsp-	Isoaspartyl aminopeptidase (EC 3.4.19.5)
gamma-Glu	Gamma-D-glutamyl-meso-diaminopimelate peptidase (EC 3.4.19.11)
PepD	Aminoacyl-histidine dipeptidase (Peptidase D) (EC 3.4.13.3)
PepQ	Xaa-Pro dipeptidase PepQ (EC 3.4.13.9)
PepE	Alpha-aspartyl dipeptidase Peptidase E (EC 3.4.13.21)
DacA	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)
GluCP	Glutamate carboxypeptidase (EC 3.4.17.11)
Mur4pep	Muramoyltetrapeptide carboxypeptidase (EC 3.4.17.13)
Zn-Dac	Zinc D-Ala-D-Ala carboxypeptidase (EC 3.4.17.14)
CarbP-T	Carboxypeptidase T (EC 3.4.17.18)
TaqCP	Thermostable carboxypeptidase 1 (EC 3.4.17.19)
PepC	Aminopeptidase C (EC 3.4.22.40)
YpdF	Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)
YpdE	Nondeblocking aminopeptidase YpdE (X-X-[PR]- specific)
DbAMP	Deblocking aminopeptidase (EC 3.4.11.-)
IadA	Isoaspartyl dipeptidase (EC 3.4.19.5)
Asp-X	Asp-X dipeptidase
Aat	Leucyl/phenylalanyl-tRNA-protein transferase (EC 2.3.2.6)
Ate	Arginine-tRNA-protein transferase (EC 2.3.2.8)
Dcp	Dipeptidyl carboxypeptidase Dcp (EC 3.4.15.5)
PrlC	Oligopeptidase A (EC 3.4.24.70)
Thimet	Thimet oligopeptidase (EC 3.4.24.15)
MIPEP	Mitochondrial intermediate peptidase, mitochondrial precursor (EC 3.4.24.59)
PepA	Cytosol aminopeptidase PepA (EC 3.4.11.1)
PepN	Membrane alanine aminopeptidase N (EC 3.4.11.2)
PepP	Xaa-Pro aminopeptidase (EC 3.4.11.9)
PepB	Peptidase B (EC 3.4.11.23)
AmpY	Aminopeptidase Y (Arg, Lys, Leu preference) (EC 3.4.11.15)
AmpS	Aminopeptidase S (Leu, Val, Phe, Tyr preference) (EC 3.4.11.24)

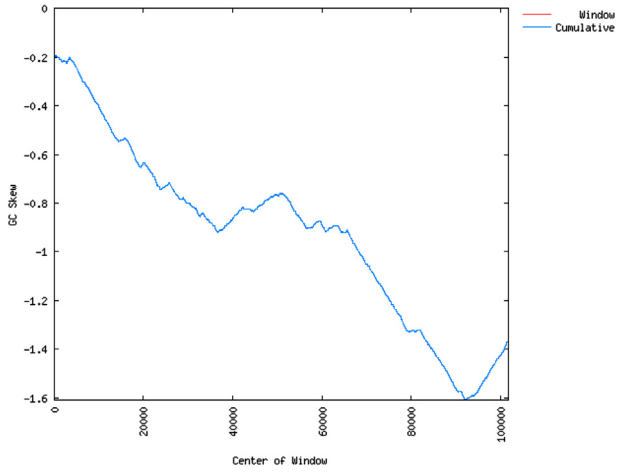

Fig. 3. Graphic representation of the GC skew of the *C. chauvoei* genome close in the origin of replication.

injured bovine tissues, where *C. chauvoei* performs its main replication and growth activities under natural conditions.

2.3. Haemolysins

C. chauvoei is a primary pathogen causing blackleg in ruminants. The complex pathogenicity of *C. chauvoei* is expected to require various virulence factors including potent toxins but also enzymes and organelles enabling the bacterium to reach its destination, the muscular tissue. It's very strong haemolytic appearance in cultures on blood agar plates supposes that one or several haemolysins of *C. chauvoei* represent the main toxins and virulence factors. The potential direct virulence genes found on the annotated genome of *C. chauvoei* JF4335 as listed in Table 3 include 4 haemolysins, a haemolysin belonging to the haemolysin III-superfamily, a haemolysin A, a haemolysin of the XhlA type and the haemolytic leucocidin CctA. The latter has been shown to represent the major haemolytic and cytotoxic activity secreted by *C. chauvoei*. This haemolytic activity is oxygen stable. Recombinant inactivated CctA used as antigen for vaccination showed nearly full protection in guinea pigs against challenge with virulent *C. chauvoei*, hence indicating that this toxin represent the main virulence factor of this pathogen [13]. The gene encoding *cctA* showed remarkable genetic stability. The *cctA* gene was found to be fully conserved within 7 *C. chauvoei* strains isolated over a period of 54 years from blackleg in 6 countries of 4 continents with a single strain showing 3 single nucleotide polymorphisms (SNP) all affecting the third nucleotide of the codon and not affecting the encoded amino acid [13].

The putative haemolysin III represents a protein of 220 amino acids (aa) with a molecular mass of 25 kDa and a calculated pI of 9.1 and characteristic domains of the haemolysin III-superfamily. This haemolysin could represent the 27 kDa haemolysin that was identified in *C. chauvoei* strain C6H by Hang'ombe and collaborators [16]. A gene encoding a highly similar Haemolysin III with 58% identical and 75%

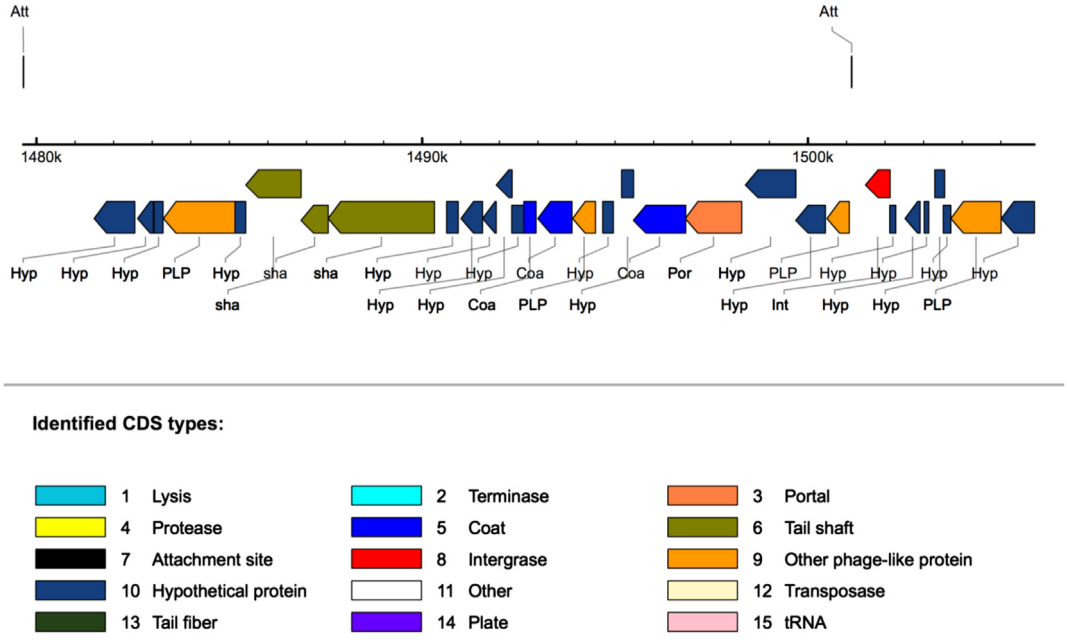

Fig. 4. Genetic structure of the prophage ϕJF4335 found integrated in the chromosome of *C. chauvoei*.

similar aa to that in *C. chauvoei* is found in nearly all *C. perfringens* strains that were analysed genetically in detail. Haemolysin III is referred as haemolysin D in *C. perfringens*. Putative haemolysins of the haemolysin III-superfamily are very widely spread in Gram-positive bacteria and are found in pathogenic as well as in commensal and environmental species such as *Flavibacterium* sp., *Bacillus* sp., *Stenotrophomonas* sp., *Paenibacillus* sp., *Photobacterium* sp., *Desulfitibacter* sp., *Desulfobacterium* sp. where it is named haemolysin III or alternatively haemolysin D. It has been described as one of the haemolysin-toxins of Bacillus cereus [3,4]. The role of haemolysin III in pathogenicity of *C. chauvoei* is still unclear. However in comparison with other bacterial species it seems to play a minor role in virulence.

The putative haemolysin A found in the annotated genome of *C. chauvoei* belongs to the FtsJ superfamily of RNA methyltransferases. Its putative haemolytic function could not be verified and it function in pathogenicity is questionable. The FtsJ protein is a well conserved heat shock protein that is found in all prokaryotes but also archaea and eukaryotes where it is responsible for methylation of the 23S rRNA supposedly in the late maturation phase of the ribosome. Hence *ftsJ* represents a house keeping gene and the designation *ftsJ* should be used in the future for all organisms.

A further haemolysin found on the annotated genome of *C. chauvoei* is a small 77 aa protein of 8.8 kDa belonging to the haemolysin XhlA superfamily. Genes encoding homologues to XhlA are found in many Gram-positive bacteria, in particular in insect pathogen species of *Paenibacillus*, *Bacillus*, *Clostridium*, but also in some *C. botulinum* and in *Clostridium glycolyticum*. This latter is a spore forming environmental bacterium also present in the natural gut microbiome of animals and humans. *C. glycolyticum* is sometimes found in wounds abscesses of animals and humans but its role in pathogenesis in not confirmed. Haemolysin XhlA was primarily shown as a major virulence factor of *Xenorhabdus nematophila* which is a pathogen for a variety of insects [7]. It is therefore questionable if XhlA plays a role in pathogenicity of blackleg by *C. chauvoei*, or if the gene is a requisite of evolution of *Clostridium* species.

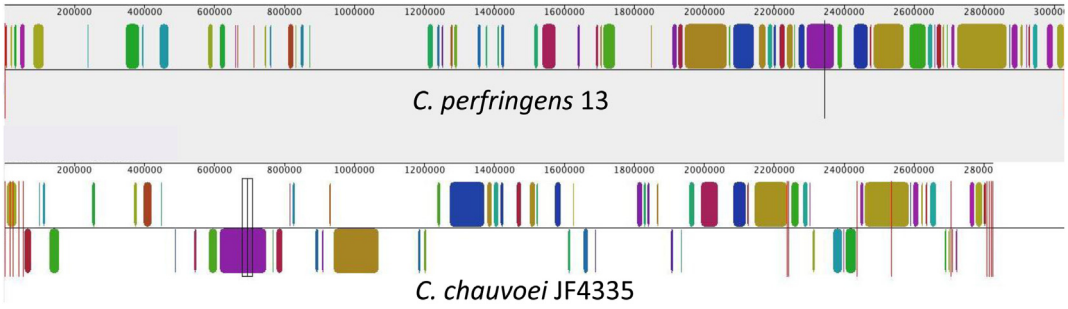

Fig. 5. Graphic representation of the similarity between the chromosomes of *C. chauvoei* JF4335 and *C. perfringens* strain 13, the closest related animal pathogen, by MAUVE analysis.

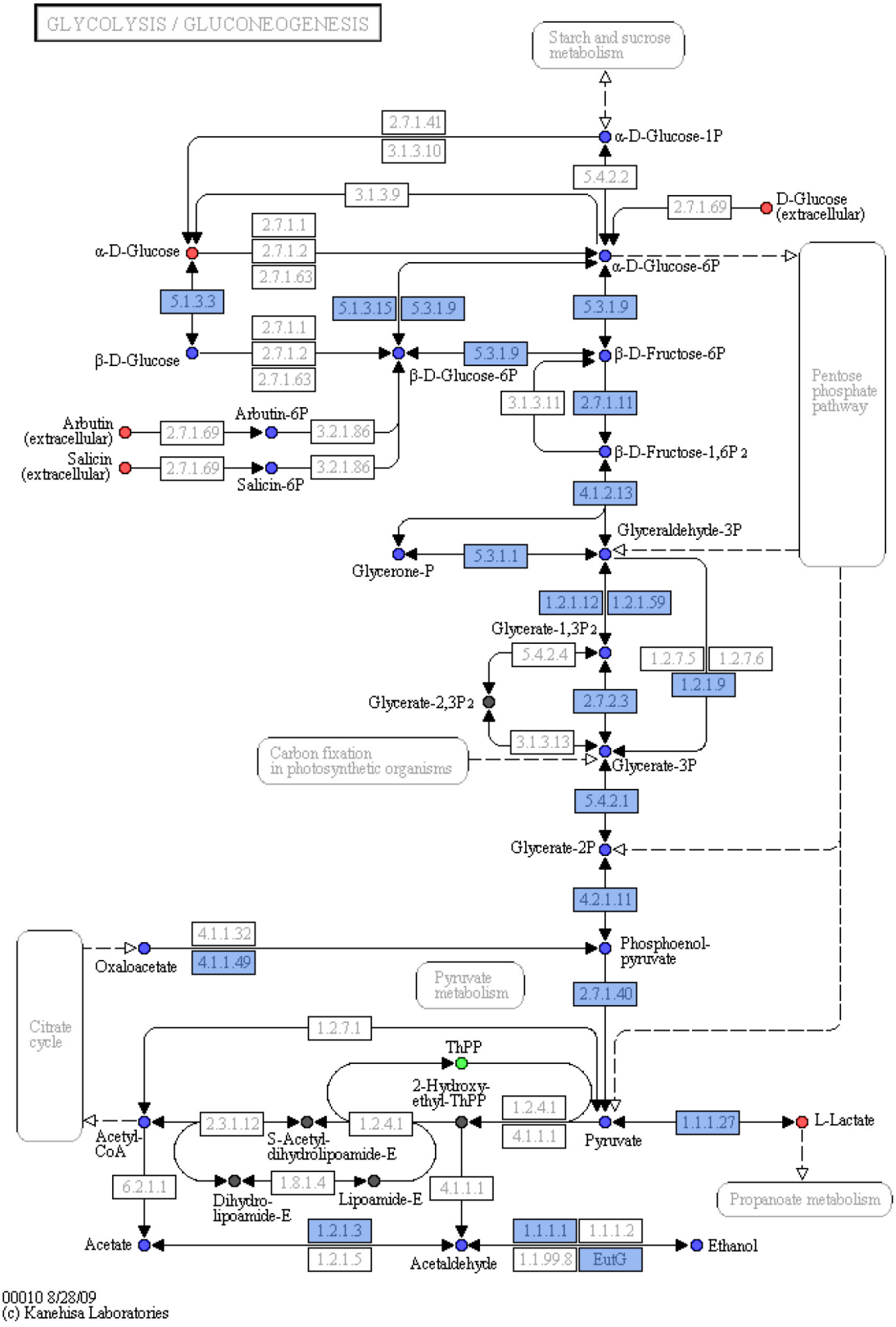

Fig. 6. Glycolysis/glycogenesis pathway. The blue boxed functions are annotated in the genome of *C. perfringens*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Primary virulence genes of *Clostridium chauvoei* JF4335.

Virulence factor	Gene designation	Protein nr	Comment
Panton Valentine leucocidin CctA	*cctA*	1863	Activity confirmed
Haemolysin III		276	Also named haemolysin D
Haemolysin XhlA		799	
(Haemolysin A)[a]	*ftsJ*	1036	RNA methyltransferase
Sialidase	*nanA*	1464	Activity confirmed
Hyaluronidase H	*nagH*	2049	Activity confirmed
Hyaluronidase J	*nagJ*	1919	
Patatine phospholipase		181	
Patatine phospholipase		1777	
Collagen binding protein		1915	
Collagen binding protein		1916	
Internalin A	*inlA1*	2016	
Internalin A	*inlA2*	2017	

[a] Correct annotation is FtsJ RNA methyltransferase.

2.4. Non-haemolysin virulence factors

Besides haemolysin- and putative haemolysin genes, *C. chauvoei* contains a gene, *nanA*, encoding a sialidase whose activity has been confirmed previously by expressing it in *E. coli*. Sialidase activity could be obtained from a recombinant protein expressed from a cloning vector containing the *C. chauvoei nanA* gene [36]. Sialidases or neuraminidases hydrolyse the α 2–3, α 2–6, α 2–8 glycosilic linkages of terminal sialic residues in oligosaccharides, glycosaccarides, glycoproteins and glycolipids and are known as virulence factor by affecting the intracellular matrix and by undercutting pathways in the host's immune regulation. Sialidase is supposed to underpin the rapid spread of *C. chauvoei* in the tissue of the infected host and has previously been suggested to play an important role in blackleg [33]. The *nanA* gene and secreted sialidase activity was found in all 7 *C. chauvoei* strains mentioned above. However genetic differences of the *nanA* gene was found among the various *C. chauvoei* strains, in contrast to the leucocidin gene *cctA* [36].

Hyaluronidase is further enzyme affecting the intracellular matrix of the host. The genome of *C. chauvoei* reveals two different hyaluronidase genes, *nagH* and *nagJ* (Table 3) of which the activity of *nagH* has been confirmed in our laboratory (Frey and Wüthrich unpublished). Hyaluronidase, similar to sialidase is thought to be involved in loosening the intracellular matrix and tissue connections, thus favouring the spread of *C. chauvoei* in the infected host.

The *C. chauvoei* genome furthermore reveals two genes encoding for potential patatine phospholipases (PPARs). PPARs belong to the ligand inducible nuclear receptors and transcription factors in the steroid superfamily [20]. PPARγ is suggested to be beneficial in hyper-inflammatory diseases, such as sepsis, by repressing expression of inflammatory genes (inducible nitric oxide synthase, TNFα, or IL-1β) [37]. However, it can also provoke apoptosis which in the hyper-inflammatory phase of sepsis might be helpful by reducing the number of immune cells that are involved in secreting high amounts of pro-inflammatory mediators [37]. PPARs are activated by ligands such as fatty acids, eicosanoids and oxidised fatty acids [9,12,21,27].

Furthermore, two genes encoding potential collagen binding proteins as well as two genes encoding homologues to internalin A, which are supposed to be involved in virulence were found on the chromosome of *C. chauvoei*.

Although flagella are often not directly involved in virulence, they might contribute to the infectious process by their potential to provide the bacterium mobility and hence to reach the target tissue where the bacterium causes tissue damage and disease. Furthermore flagellar antigens have been studied in detail in the view of their potential as valuable candidate antigens for vaccines [24,25,30–32]. Twenty flagellar biosynthesis genes were found on the coromosome of *C. chauvoei* in four clusters, probably arranged in operons: *fli DMNFGIJLPSQ*, *flg KBCDG*, *flb D*, the gene encoding the flagellar protein 3 and the genes for flagellar hook biosynthesis and hook length control proteins.

2.5. Antibiotic resistance

All above named strains of *Clostridium chauvoei* are sensitive to all antibiotics tested that are currently used against clostridial infections (Frey unpublished results). The minimal inhibitory concentrations for strain JF4335 as determined in Mueller Hinton II Broth using custom Sensititre™ susceptibility plates (Trek Diagnostics Systems, East-Grinstead, England, and MCS Diagnostics BV, JL Swalmen, The Netherlands) according to the CLSI guidelines [19] for *C. chauvoei* strain were: Cephalotin 2 μg/ml, Clindamycin 0.5 μg/ml, Enrofloxacin 0.25 μg/ml, Erythromycin 0.25 μg/ml, Penicillin <0.012 μg/ml, Vancomycin 1 μg/ml, Tetracyclin 1 μg/ml and Chloramphenicol <4 μg/ml. This reflects the fact that blackleg is not treated with antibiotics due to its virulent appearance leading to rapid death of the animal. It is therefore surprising that *C. chauvoei* strain JF4335 harbours each a gene potential for penicillin resistance, a β-lactamase (EC 3.5.2.6), as well as an elongator Factor EF G type tetracycline resistance gene potentially involved in protection of ribosomes from tetracycline and catalysis and release of tetracycline (*tetM*, *tetO* analogue) and a vancomycin B-type resistance protein gene *vanW*, but no further genes potentially involved in vancomycin resistance. Furthermore there are 9 genes encoding potential Multi antimicrobial extrusion proteins (Na^+/drug antiporters). However, these genes seem either not to be expressed in *C. chauvoei* JF4335, or are expressed as non-functional proteins or most probably have functions other than export of antibiotics.

In conclusion, the genomic sequence of a virulent strain of *C. chauvoei* gives insight in its particular lifestyle which implies the replication in damaged host tissue during infection and the various virulence genes that allow the pathogen to reach the host tissue and prepare it for its replication. Potential antibiotic resistance genes are present on the chromosome of

C. chauvoei, although this pathogen is sensitive to the corresponding antibiotics reflecting the fact that infected animals are not treated with antibiotics.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Research Foundation 3R, Reduction, Refinement and Replacement of Animal Experimentation grant nr. 136-13 to JF.

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