Tasmanian Institute of Agriculture

A study of key meat spoilage bacteria

By

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Declaration of originality

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Publications and communications arising from this research

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- **Stanborough T.**, Fegan N., Powell S.M., Singh T., Tamplin M., and Chandry P.S., 2018. Genomic and metabolic characterisation of spoilage-associated *Pseudomonas* species. International Journal of Food Microbiology, 268: 61-72.
- **Stanborough T.**, Fegan N., Powell S.M., Tamplin M., and Chandry P.S., 2018. Vibrioferrin production by the food spoilage bacterium *Pseudomonas fragi*. FEMS Microbiology Letters, 365(6), fnx279..

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- **Stanborough T.**, Suryadinata R., Fegan N., Powell S.M., Tamplin M., Nuttall S.D. and Chandry P.S., 2016. Characterisation of a putative *Brochothrix thermosphacta* sortase enzyme. Australian Society of Microbiology 2016 Victoria/Tasmania Bi-state Conference, Launceston, Tasmania.
- **Stanborough T.**, Chandry P.S., Powell S.M., Tamplin M. and Fegan N., 2017. A putative siderophore locus of *Pseudomonas fragi*: Solving an iron problem. International Association of Food Protection 2017 Annual Meeting, Tampa, USA.

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Abstract

Fresh meat is a highly nutritious commodity, conducive to the rapid growth of microorganisms and spoilage. Despite the economic and social importance associated with preventing meat spoilage and minimising food waste, the physiology of many microorganisms involved in meat spoilage remains poorly studied. Such fundamental knowledge is of great importance to enable further improvements in the control of meat contamination and spoilage, and to facilitate shelf-life extension. In this context, the broad objective of this thesis was to improve current knowledge of the biology of important meat spoilage bacteria, *Brochothrix thermosphacta* and psychrotrophic pseudomonads *Pseudomonas fragi* and *Pseudomonas lundensis*.

In contrast to the well-studied and phylogenetically related pathogen *Listeria monocytogenes*, genome analyses of *B. thermosphacta* were lacking. Thus, draft genomes of 12 *B. thermosphacta* strains were analysed and compared to genomes of *Brochothrix campestris* and *L. monocytogenes* to identify genes that play a role in spoilage and persistence of *B. thermosphacta* throughout the meat production chain. The 12 strains shared a high degree of genomic similarity. Genes/pathways likely involved in the production of organoleptically unpleasant compounds such as acetoin, butanediol and isobutyric, isovaleric and 2-methylbutyric acid were identified in both *Brochothrix* species, while amino acid decarboxylase genes were not found, and phenotypic testing confirmed their absence. Orthologues of key *Listeria* virulence genes were absent from the *Brochothrix* genomes, however auxiliary virulence genes such as factors involved in surface protein anchoring (e.g. sortase A), and key stress response regulatory genes were identified, establishing parallels to and differences from this related foodborne pathogen.

Gram-positive bacteria utilise class A sortases to attach a diversity of proteins to the cell wall, including factors involved in the adhesion of pathogens to host cells and tissues. As a starting point for understanding how *B. thermosphacta* interacts with its environment, a truncated and tagged variant of the *B. thermosphacta* sortase A (His_6-BtSrtA) was generated and catalytic activity of His_6-BtSrtA was investigated. His_6-BtSrtA recognised and cleaved LPXTG sorting motifs and attached SrtA pseudo-substrates to rhodamine-labelled tri-glycine, demonstrating *in vitro* SrtA bioconjugation activity. Genome examination identified 11 potential SrtA substrate proteins, two of which contained protein domains associated with adherence of pathogens to host extracellular matrix proteins and cells, suggesting the *B. thermosphacta* SrtA may be indirectly involved in its attachment to meat surfaces.

Despite their importance as aerobic meat spoilers, genomic studies of the species *P. fragi* and *P. lundensis* were missing, and limited knowledge existed of their metabolic potential at the strain level. Thus, the genomes and metabolic activity of 13 *P. fragi* and seven *P. lundensis* were analysed. Genome investigations showed that the 20 isolates may belong to more than two species with possible spoilage potential; they revealed a high degree of diversity among the *P. fragi* and indicated that genetic flexibility and diversity may be traits of both species. Growth of the *P. lundensis* isolates on a beef paste was characterised by the production of large amounts of 1-undecene, 5-methyl-2-hexanone and methyl-2-butenoic acid, while *P. fragi* isolates produced extensive amounts of methyl and ethyl acetate. Some of the *P. fragi* produced extremely low levels of volatile organic compounds, suggesting not all strains have the same spoilage potential.

It is not understood how *P. fragi* competes so successfully with other bacteria in foods. As little is known about iron uptake systems of *P. fragi* and iron is a limiting factor in many

environments, the possibility of siderophore-mediated iron uptake as an iron acquisition system for *P. fragi* was explored. A vibrioferrin siderophore gene cluster was identified in the 13 *P. fragi* and experiments were conducted with a representative strain of this group (F1801). Chromeazurol S assays showed *P. fragi* F1801 produced siderophores under iron limitation. Disruption of the vibrioferrin receptor (*pvuA*) caused polar effects on downstream vibrioferrin biosynthetic genes. This led to impaired siderophore production of the Δ*pvuA* mutant and growth defects under severe iron-restriction, demonstrating that the identified vibrioferrin-mediated iron acquisition system is required for growth of this bacterium under iron-starvation.

Meat tenderness is an important quality attribute and considerable interest exists for the development of novel technologies to improve meat tenderness. Low field strength pulsed electric field (PEF) has come under investigation for its potential to tenderise meat due to its ability to cause limited muscle cell disruption and enhanced proteolysis, which may also promote meat spoilage owing to an increased availability of precursor metabolites of microbial spoilage. A brief study was conducted to determine the effects of a low field strength PEF (0.25 kV/cm, 3000 pulses) on the aerobic microbiota of beef. The microbial load of naturally contaminated PEF-treated and -untreated samples was compared and potential damage to muscle cells and enhanced proteolysis was assessed with reversed phase-high performance liquid chromatography (RP-HPLC). Until three days post-treatment, little difference was detected in the mean log cfu/g of treated and untreated samples. Differences in the sample means were observed at later time points, and on day-eight and -nine these differences were statistically significant ($P<0.05$), indicating that PEF may promote microbial growth. RP-HPLC showed no differences in the profiles of phosphate buffered saline-soluble compounds derived from the meat surface of treated and untreated

samples, demonstrating evidence for muscle cell leakage and enhanced proteolysis was not obtained with this method.

In summary, the genomic and physiological studies of key meat spoilage bacteria that form this thesis enhance knowledge of these poorly studied bacteria by: 1) providing insight into genomic and metabolic diversity of strains of these bacteria 2) demonstrating how strains of these bacteria may or may not contribute to spoilage by the production of various malodourous compounds 3) providing a starting point for further studies on surface protein attachment of *B. thermosphacta* and understanding mechanisms with which this bacterium interacts with its environment and 4) by revealing a siderophore-mediated iron acquisition system of *P. fragi.*

Chapter 1

Literature review and thesis objectives

INTRODUCTION

Australia's red meat industry, comprising the beef, sheep and goat meat sectors, produces $15 billion in earnings through the export of product to 100 countries and contributes $18 billion to Gross Domestic Product (Ernst and Young Global Limited, 2017). In 2016, Australia was the world's largest exporter of beef, the second largest exporter of sheep meat and the third largest exporter of livestock (Ernst and Young Global Limited, 2017). Australia was also the largest global exporter of goat meat in 2013 (most recent available data) (Ernst and Young Global Limited, 2017). These accomplishments are notable considering the domestic beef herd represents only 2%, and the domestic sheep flock 6%, of the global beef herd and sheep flock sizes (Ernst and Young Global Limited, 2017).

Australia is also a leader in domestic consumption of meat (Ernst and Young Global Limited, 2017). In 2014, Australia was the largest consumer of meat (defined as beef, pork, chicken and lamb) worldwide, with an estimated 90.21 kg of meat consumed per capita, and in 2015 and 2016, Australia's estimated consumption of meat was only surpassed by the USA (OECD, 2017). The success of the red meat industry in Australia is dependent on efficient production and supply chains, understanding and meeting customer and consumer demands and capitalising on market opportunities. However central to the success is the sustainable production of safe, high quality meat.

Although meat quality can be a subjective topic, research has shown that flavour, colour, texture, tenderness, juiciness and nutritive value of meat are considered important attributes, but meat must also be safe (free from food-borne pathogens) and easy for the consumer to handle (Aymerich et al., 2008, Font-I-Furnols and Guerrero, 2014, Troy and Kerry, 2010). Because of its unique chemical and biological properties, fresh meat is highly susceptible to spoilage, which is associated with organoleptic changes in meat colour, flavour and texture, and the development of undesirable odours (Borch et al., 1996, Ingram and Dainty, 1971). In addition to food safety requirements, these deteriorative changes determine the shelf-life of meat, and can have a significant economic impact on both the meat industry and the retail marketplace, cause food wastage and limit valuable food sources for human consumption.

Spoilage of meat is influenced by a number of interrelated factors including microorganisms, post-mortem activity of endogenous enzymes, storage temperature of the meat, levels of atmospheric oxygen, light and dehydration of the meat (Zhou et al., 2010). The predominant cause of spoilage is the propagation of spoilage bacteria and the accumulation of their metabolites (Kakouri and Nychas, 1994, Nychas and Arkoudelos, 1990, Braun et al., 1999, Stutz et al., 1991, Borch et al., 1996, Doulgeraki et al., 2012). Despite the economic and social importance associated with preventing meat spoilage and minimising food waste, basic aspects of the physiology of many of the bacteria that cause meat spoilage remain poorly studied. Such fundamental knowledge is of great importance to enable further improvements in the control of meat contamination and spoilage and ultimately to facilitate shelf-life extension.

The broad focus of this literature review is microbial spoilage of fresh red meat. As the nutrient source for spoilage bacteria, this review begins with the composition of red meat followed by pre-slaughter aspects and meat preservation and processing technologies, which play a crucial role in the control of contamination and growth of spoilage bacteria on meat. The novel, non-thermal processing technology pulsed electric field, which has come under investigation for its potential to improve quality attributes of meat, is discussed in more detail. Thereafter, the review focuses on microbial activity leading to meat spoilage, the complexity of microbial spoilage communities and major meat spoilage microorganisms, in particular the key meat spoilage bacteria *Brochothrix thermosphacta* and psychrotrophic pseudomonads *Pseudomonas fragi and Pseudomonas lundensis*.

COMPOSITION OF FRESH RED MEAT

Red muscle meat, defined as "the voluntary striated skeletal muscular tissue of red meat animals", is a nutrient-rich product (Roberts et al., 2005). Chemically the three main components are water, protein and lipids, with lean muscle tissues comprising 71-74% water (water activity 0.99), 20-22% protein and 3-8% lipids (Lambert et al., 1991). Total muscle proteins are composed of contractile myofibrillar proteins actin, myosin, troponin and tropomyosin; soluble sarcoplasmic proteins including myoglobin, haemoglobin and glycolytic enzymes; and the stromal proteins collagen, elastin and reticulin (Smulders, 1986). Lipids of both muscle and adipose tissue contain combinations of saturated, mono-unsaturated and polyunsaturated fatty acids (Wood et al., 2008). Abundant fatty acids of red meats include the saturated fatty acids palmitic and stearic acid, the mono-unsaturated fatty acid oleic acid, and linoleic acid is one of the most abundant polyunsaturated fatty acids (Wood et al., 2004). Low molecular weight soluble constituents of red meat include

glucose and glycolytic intermediates, glycogen, lactic acid, inosine monophosphate, creatine, nucleotides, dipeptides and amino acids (Roberts et al., 2005). Typical concentrations of these low molecular weight components in beef muscle of normal pH are shown in Table 1.1.

Table 1.1: Concentrations of low-molecular weight soluble components in beef muscle of normal pH[a]

Component	Concentration (mg/g)
Lactic acid	9.0
Creatine	5.5
Amino acids	3.5
Dipeptides	3.0
Inosine monophosphate	3.0
Nucleotides	1.0
Glycogen	1.0
Glucose-6-phosphate	0.2
Glucose	0.1

[a] (Jensen et al., 2004)

As a dietary component, red meat is an excellent source of protein, but it is also a valuable source of iron, complex B vitamins (particularly vitamin B12), zinc, selenium and phosphorus (Pereira and Vicente, 2013). Haem iron, which has a higher and more uniform absorption in the gut than non-haem iron, represents the majority of total iron in meat, with haem iron constituting 60 to 90% of total iron in beef (Ramos et al., 2009, Lombardi-Boccia et al., 2002, Hurrell and Egli, 2010).

The oxygenation and oxidation status of the sarcoplasmic haem protein myoglobin plays an important role in the appearance of red meat. Due to the anaerobic condition of uncut muscle, myoglobin is present as non-oxygenated deoxymyoglobin, which contains iron in

the ferrous state (Fe^{2+}), rendering the meat a purplish-red colour (Mancini and Hunt, 2005). Upon cutting and air exposure, deoxymyoglobin is rapidly oxygenated in a process known as blooming to oxymyoglobin (Mancini and Hunt, 2005). The iron atom in oxymyoglobin remains in the ferrous form, but oxygen is attached, giving the meat the desired cherry red colour (Mancini and Hunt, 2005). Oxymyoglobin is oxidised over time to metmyoglobin (iron atom in the ferric form, Fe^{3+}), resulting in brown discolouration of the meat, which is generally associated with a lack of freshness (Faustman and Cassens, 1990).

PRE-SLAUGHTER HANDLING OF LIVESTOCK

Pre-slaughter handling of livestock can have a marked impact on microbial spoilage of meat, as stressed or exercised animals prior to slaughter have reduced glycogen levels in muscles (Miller, 2002). Post-mortem anaerobic glycolysis of glycogen to lactic acid generally results in pH levels of fresh meats between 5.5 and 5.9 (Roberts et al., 2005). With reduced glycogen in muscles, pH levels may only reach 6.4–6.8, resulting in Dark, Firm, Dry (DFD) meat (Dave and Ghaly, 2011). DFD meat is not only characterised by high pH (>6.0), but also by deficiencies in glucose and glycolytic intermediates, which ultimately results in premature spoilage (Newton and Gill, 1981). The elevated pH of DFD meat facilitates growth of bacteria with high spoilage potential such as *Shewanella putrefaciens*, which are otherwise inhibited in growth at the normal ultimate pH of fresh meat (Samelis, 2006). In addition, reduced glucose levels and deficiencies in alternative low molecular weight compounds, results in early utilisation of amino acids, which are precursors of many offensive metabolic end-products responsible for spoilage (Newton and Gill, 1981).

SLAUGHTER PROCESS AND CARCASS DRESSING AS INITIAL CONTAMINATION POINTS

It is generally accepted that muscle meat from healthy animals is a sterile product and contamination of meat begins during the slaughter process (Samelis, 2006). Potential contamination sources include the abattoir air, hides or fleeces of the animals, abiotic surfaces or equipment within the plant, and the worker's hands and clothes. Thus, as with downstream steps in meat processing, good hygiene during the slaughter process is imperative to minimise microbial contamination.

The hides or fleeces of the animals have a high abundance and diversity of microorganisms including the normal hide or fleece microflora (e.g. micrococci, staphylococci and yeasts), faecal microorganisms and microbes derived from soil and other environmental sources (Roberts et al., 2005, Chandry, 2013, Chandry, 2016). The hide/fleece is separated from underlying tissue, during which direct contamination of the carcass is possible and considerable air-borne dissemination occurs (Bell, 1997). A recent study showed that 86% of the bacteria in air samples derived from a bovine abattoir taken close to the hide puller, in the chiller and just outside the slaughter floor were from the hides of the processed animals (Chandry, 2016). More importantly, results of this work suggested that air-contamination was a major source of carcass contamination with an average of 25% of the bacteria on the carcasses derived from the air.

Following skinning of the animals, evisceration must be carefully performed to ensure the organs, particularly the rumen and intestines, are not perforated, as the gastrointestinal tract of ruminants can be a reservoir for human pathogenic *Salmonella*, shiga toxin-producing *Escherichia coli*, *Campylobacter* and *Listeria monocytogenes* (Roberts et al., 2005, Fegan et al., 2004, Fegan, 2011, Nightingale et al., 2004). The carcasses are then trimmed

and washed to remove physical debris and associated microorganisms. Cold water carcass washing was shown to be relatively ineffective in removing microbial contamination and mainly resulted in redistribution of microorganisms on the carcasses (Bell, 1997). However, the antimicrobial potential of washing is improved when acid solutions such as lactic acid or acetic acid solutions or high water temperatures are applied (Castillo et al., 1998, Castillo et al., 1999, Brustolin et al., 2014, Carranza et al., 2013).

Following slaughter and dressing, the carcasses are chilled to prevent growth of mesophilic bacteria, which include many of the pathogens, and to retard growth of psychrotrophic microorganisms. This is considered the start of the cool chain (Roberts et al., 2005). Due to the number of possible sources of microbial contamination, marked differences in the microbial ecology of farms and abattoirs, and seasonal changes in the microbial communities, the abundance and diversity of microorganisms on carcasses can vary significantly (Samelis, 2006). Typically at this point, cattle and sheep carcasses carry between 10^2-10^4 mesophile cfu/cm^2, while the pyschrotrophic population of microorganisms is a variable percentage of the mesophilic count (0.2–10%) (Roberts et al., 2005). The next steps in meat processing include deboning, trimming and packaging, during which knives, surfaces, equipment and the hands of the workers are further sources of microbial contamination (Huynh et al., 2016).

MEAT PRESERVATION METHODS

To maximise the shelf-life of fresh meat, preservation measures such as product cooling and the use of packaging systems are applied. Fresh meat can also be preserved by interventions such as chemical treatments (salts, chlorine or 1-3% organic acid solutions) and irradiation. Under investigation for meat preservation potential are various biological treatments (e.g.

bacteriocins, bacteriophages, plant extracts and essential oils), as well as novel technologies like high pressure processing (Lambert et al., 1991, Zhou et al., 2010, Sohaib et al., 2016). Meat processors may also employ multiple interventions sequentially, known as a "hurdle approach", to obtain microbiologically cleaner carcasses (Samelis, 2006). The following section focuses on two of the main meat preservation methods, cooling and packaging.

Cooling

The most important and effective measure to preserve meat is the use of storage temperatures below the optimum range of growth of many microorganisms. For example, at 0 °C to 1 °C the growth rates of most microorganisms are reduced to half the rate observed at 5 °C, and they can be further reduced at lower temperatures (Egan et al., 1988). Meat freezes at around -1.5 °C to -2 °C and is commonly stored at temperatures both above (-1.5 °C to 5 °C) and below the freezing point (-18 °C) (Zhou et al., 2010, Coombs et al., 2017). For vacuum-packed primals that are transported to distant markets, -1.5 °C was recommended as the optimum storage temperature (Gill et al., 1988), while fresh meats close to retail outlets are generally stored at higher temperatures (around 2 °C), with temperatures above 5 °C considered as abusive (Mills et al., 2014). To ensure fresh meat is a safe commodity and to prevent early spoilage and shelf-life reduction, it is imperative that the cool chain is maintained during distribution and retail storage through to cooking and consumption.

Packaging systems

Packaging of meat also serves to extend its shelf-life. Commonly used packaging systems include overwrap packaging for short term storage and/or retail display, vacuum-packaging (VP) for primary cuts of red meat and modified atmosphere packaging (MAP) for longer

term storage of retail displays of meat (Chen et al., 2012). More recently, active packaging (AP) and intelligent packaging (IP) systems have emerged (Fang et al., 2017). VP and MAP of meat can inhibit growth of dominant aerobic spoilage bacteria such as Gram-negative psychrotrophic *Pseudomonas* species, which have fast growth rates and high spoilage potential (Chen et al., 2012). It is the lack of oxygen in vacuum-packaged meat that prevents growth of these organisms, while the carbon dioxide present in the head space of modified atmosphere packaged meat inhibits their growth (Gill and Jones, 1996). MAP can encompass both aerobic and anaerobic conditions depending on the gas composition. In order to minimise the oxidation of oxymyoglobin to metmyoglobin, oxygen levels are kept either below 0.05% or at saturating levels (Faustman and Cassens, 1990). Cooked meat is often stored in 70% nitrogen and 30% carbon dioxide (Smiddy et al., 2002), while fresh red meat is commonly stored in MAP with 80% oxygen and 20% carbon dioxide (Chen et al., 2012).

AP technology involves the use of specific compounds incorporated into the packaging system that can interact with the products themselves or the environment surrounding the food to extend the shelf-life of these products (Biji et al., 2015). These substances may serve as oxygen-scavengers or -producers, carbon dioxide controllers, moisture controllers, antimicrobials or odour regulators (Brody et al., 2008, Brody, 2009). IP systems use sensors that monitor the environment of the packaged food and provide the consumer with information on the condition of the meat product (Kerry et al., 2006, Yam et al., 2005). For example, colour-changing sensors can accurately measure in real-time volatile amines produced by spoilage microorganisms in the package head-space (Pacquit et al., 2007, Pacquit et al., 2006).

MEAT PROCESSING INTERVENTIONS

Among important meat eating qualities, tenderness was determined as the most important, due its influence on consumer choice of a particular meat cut and the re-purchase intent of consumers (Miller et al., 2001, Troy and Kerry, 2010). Post-mortem aging is commonly used to improve meat tenderness, but it is slow and expensive. Alternative interventions for increasing meat tenderness include electrical stimulation, aitchbone hanging and blade/needle tenderisation (Bolumar et al., 2013). More recently the novel technologies high pressure processing and pulsed electric field (PEF) have come under investigation for their potential to improve meat tenderness (Chen et al., 2012, Niemira, 2012, Jayasena et al., 2015, Sohaib et al., 2016).

Two components influence meat tenderness: 1) background toughness owing to the connective tissue in meat and 2) post-mortem shortening of the contractile apparatus of the muscle (sarcomere) during rigor mortis (Bekhit et al., 2014a). While little can be done to improve background toughness, shortening-induced toughness can be improved by activating endogenous proteases (Bekhit et al., 2014a). During post mortem aging, proteases such as cathepsins and calpains begin to degrade myofibrillar proteins (Huynh et al., 2016). Their activity is dependent upon calcium levels and other enzymes, thus processes that enhance (Ca^{2+} induction), activate (temperature and pH control) or extend the length of activity (aging) of endogenous proteases can improve meat tenderness (Bekhit et al., 2014a).

PEF

PEF-processing is the application of very short pulses (µs to ms range) of low to high electric field intensity (0.1-50 kV/cm) to foods, which are either passed through or placed between

two electrodes (Buckow et al., 2013, Toepfl et al., 2014). The external electric fields cause an increase in electric potential across cell membranes leading to membrane thinning and eventually electroporation of cells, disruption of cell organelles and further structural changes to foods (Zimmermann et al., 1976, Toepfl et al., 2014, Gudmundsson and Hafsteinsson, 2001). An important parameter is the electric field strength (E), which can be approximated by dividing the voltage (U) that is applied by the distance (d) between the electrodes (E = U/d) (Buckow et al., 2013, Toepfl et al., 2007). While high field strength PEF (20-50 kV/cm) is effective for microbial inactivation in fruit juices owing to the disruption of microbial cell membranes (Buckow et al., 2013), it is not suitable as a decontamination method for muscle foods. The absence of a protective cell wall and larger size renders muscle cells highly susceptible to PEF-induced damage (Hülsheger et al., 1981, Grahl and Märkl, 1996). Low field strength PEF is however considered to have potential to improve meat tenderness due to limited muscle cell disruption, potential accelerated release of Ca^{2+} and proteases post-mortem and stimulation of the glycolysis process, all of which could contribute to enhanced post-mortem proteolysis (Suwandy et al., 2015, Bekhit and Hopkins, 2014). An advantage of PEF over other available technologies is the ability to modify the parameters of the technology to treat different cuts of meat, potentially increasing the quality of low value cuts (Bekhit et al., 2014b). However, damage to muscle fibres and enhanced proteolysis may promote microbial growth due to an increased availability of nutrients and precursor metabolites of microbial spoilage. Despite the potential for spoilage promotion, literature searches failed to identify studies addressing the impact of low field strength PEF on the microorganisms that grow on red meat during storage.

MEAT SPOILAGE

Meat spoilage is primarily caused by the growth of microbial spoilage communities and the production of their metabolites such as esters, organic acids, amines, sulphur compounds, ketones and aldehydes (Ercolini et al., 2009, Montel et al., 1998, Samelis, 2006). The high water activity and abundance of nutrients in fresh red meat enables microorganisms to grow on its surface to ~10^9 cfu/cm^2 (Ellis and Goodacre, 2001). When microbial loads reach ~10^7 cfu/cm^2, off-odours can be detected due to microbial production of volatile metabolites, and slime can be observed when numbers reach ~10^8 cfu/cm^2 owing to extensive bacterial production of extracellular polymeric substances (Nychas and Drosinos, 2014, Ingram and Dainty, 1971, Wang et al., 2017a).

Meat spoilage begins with the attachment of spoilage microorganisms to the meat surface. Knowledge of the molecular aspects of this step remain largely unknown. Various factors may influence the adhesion of microorganisms to the meat surface such as cell motility, cell surface charge, temperature and length of contact time, as well as treatments to the surface of meat such as salt treatments and chemical rinses (Piette and Idziak, 1992, Li and McLandsborough, 1999, Fratamico et al., 1996, Rivas et al., 2006, Zulfakar et al., 2012, Zulfakar et al., 2013a). Spoilage bacteria have a range of factors that could be involved in specific and non-specific adhesion to meat tissue such as pili, flagella and adhesins; however, evidence for the involvement of specific appendages or surface molecules in attachment is missing. Studies using various model systems have shown that bacteria can attach to muscle cells, as well as muscle extracellular matrix (ECM) proteins, and that attachment is strain-dependent (Zulfakar et al., 2013b, Zulfakar et al., 2012, Frank, 2001, Chagnot et al., 2013). However, bacterial attachment properties to actual meat may differ.

Recent work that investigated colonisation of skeletal muscle types by the pathogen *E. coli* O157:H7, showed that at a cellular level bacterial adhesion occurred at the ECM (Chagnot et al., 2017), suggesting that bacterial factors involved in adherence to ECM components could play an important role in this process.

Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) mediate the attachment of pathogens to host tissues by interaction of MSCRAMMs with ECM components such as collagen, fibronectin, laminin and elastin (Chagnot et al., 2012). In Gram-positive bacteria, MSCRAMMs are covalently ligated to the peptidoglycan by sortase enzymes (Heilmann, 2011). Both MSCRAMMs and sortase enzymes are not restricted to pathogens (Muñoz-Provencio et al., 2012, Yu et al., 2016). Thus, molecular mechanisms of adhesion of spoilage bacteria to ECM components of carcasses may occur via interactions between MSCRAMMs and ECM components. Further, sortase enzymes in Gram-positive spoilage bacteria may also play an important role by attaching MSCRAMMs to the surface of these bacteria.

The important physicochemical alterations that take place during spoilage occur within the aqueous phase of meat, which contains glucose, lactic acid and amino acids (Nychas and Drosinos, 2014). Although the protein in meat is a major potential nutrient for microorganisms, most bacteria do not express enzymes to break down complex substrates when simpler compounds are present, and concentrations of low molecular weight soluble compounds in meat are sufficient to support extensive microbial growth (Nychas and Drosinos, 2014, Jensen et al., 2004). The majority of spoilage microorganisms preferentially utilise glucose under both aerobic and anaerobic conditions (Nychas et al., 1988, Nychas and Arkoudelos, 1990). The meat surface will begin to feel tacky and then slimy due to the

growth of microorganisms and their production of extracellular polymeric substances (Ellis and Goodacre, 2001). When glucose levels sink and the diffusion gradient from the underlying tissue to the surface is insufficient to meet microbial demands, other low molecular weight substrates such as lactic acid and amino acids are attacked (Nychas et al., 1988, Drosinos and Board, 1994, Ellis and Goodacre, 2001). The catabolism of amino acids and other simple nitrogenous compounds such as urea and nucleotides is associated with the production of particularly offensive metabolites such as ammonia, sulphur compounds, amines, indole and scatole (Ellis and Goodacre, 2001). Degradation of meat proteins only takes place at very late stages of spoilage (Nychas and Drosinos, 2014).

Spoilage of meat can occur on lean and adipose tissue and was shown in one study to be essentially the same, with bacteria preferentially utilising low molecular weight compounds on the surface of adipose tissue, which are likely derived from the serum of cut blood vessels (Gill and Newton, 1980). Gill and Newton (1980) showed that when glucose is exhausted from the surfaces of fat, amino acids are attacked and malodourous compounds produced, demonstrating that lipolytic activity is not necessarily required for bacterial spoilage of adipose tissue.

The specific characteristics of spoiled meat (discoloration, off-odours, slime and gas production) depend on the microbes present (Remenant et al., 2015, De Filippis et al., 2013). For example, greening of meat and drip is sometimes observed when lactic acid bacteria (LAB) or Enterobacteriaceae are present due to their ability to produce H_2S or H_2O_2, as these molecules react with myoglobin to produce sulphmyoglobin and cholemyoglobin, respectively (Borch et al., 1996, Remenant et al., 2015). Off-odours detected in spoiled meat can be described as dairy, fatty, cheesy, sweet, fruity, putrid or sulphurous and are

attributable to the various volatile compounds produced by the microorganisms present such as organic acids, fatty acids, esters, sulphur compounds, ketones, aldehydes, alcohols and ammonia (Casaburi et al., 2015). For example, dairy or buttery aromas are associated with pyruvate catabolism and the production of ketones such as diacetyl and acetoin by *B. thermosphacta* (Smit et al., 2005). Fruity aromas can be linked to ester production and the presence of *Pseudomonas fragi*, and putrid aromas to sulphur compounds and biogenic amines (BA), which pseudomonads, Enterobacteriaceae and other members of spoilage communities can produce (Casaburi et al., 2015). BA are primarily the consequence of enzymatic decarboxylation of amino acids and their presence is considered a freshness marker in meat (Vinci and Antonelli, 2002). Not only do BA such as putrescine and cadaverine contribute to spoilage due their putrid aromas, but high levels of BA are toxic and can cause headaches, rashes, palpitations, hypertension and nausea (Vinci and Antonelli, 2002, Shalaby, 1996). Amino acid decarboxylases are found in the meat spoilage bacteria *Pseudomonas*, *Lactobacillus*, Enterobacteriaceae and *Clostridium* species (Shalaby, 1996).

Gas production is another characteristic of meat spoilage and can be observed when vacuum-packaged meat is contaminated with *Clostridium estertheticum*, Enterobacteriaceae (*Hafnia alvei*, *Serratia liquefaciens* and *Enterobacter aerogenes*) and psychrotolerant LAB species (Húngaro et al., 2016). These organisms cause blown pack spoilage (BPS), which is characterised by putrid odours and the production of large amounts of gas (mostly hydrogen and carbon dioxide) that causes gross distension of the packaging (Clemens et al., 2010, Silva et al., 2011, Chaves et al., 2012, Húngaro et al., 2016).

COMPLEXITY OF SPOILAGE COMMUNITIES

Previously, the term specific spoilage organism was used to describe the single species responsible for spoilage (Casaburi et al., 2015). Little was known about the diversity of microbial communities and their dynamics on meat, as studies relied on culture-based techniques with limited access to whole communities (Kiermeier et al., 2013). The development of advanced molecular techniques enabling metagenomic and metabolomic approaches has allowed a more complete description of microbial spoilage and led to the understanding that spoilage activities can be strain, species or group specific and are dynamic in nature (Nieminen et al., 2012, Ercolini et al., 2009, Ercolini et al., 2011, Kiermeier et al., 2013, Ercolini et al., 2010). Furthermore, they are strongly influenced by initial contamination numbers, the specific meat matrix, product packaging and storage conditions, which select for the growth of a small fraction of the initial microbial load (Remenant et al., 2015, De Filippis et al., 2013).

An example of the dynamic nature of microbial populations during meat storage was shown by Kiermeier et al. (2013) with a study that investigated microbial communities and population shifts on vacuum- and modified atmosphere packaged (100% CO_2) lamb over an 85 day period. Gram-positive and Gram-negative aerobic bacteria were initially predominant on vacuum-packaged lamb, but at later stages of storage a shift to Gram-positive facultative anaerobic LAB was observed. In addition, the authors observed an overall reduction in microbial community diversity during these later stages, as populations of LAB became more dominant. Less microbial diversity was observed on modified atmosphere packaged lamb samples, likely due to the strict anaerobic selective pressure. Although the microbial community of modified atmosphere packaged lamb samples did not

shift as much during shelf-life as the communities on vacuum-packaged lamb, facultative anaerobes also became dominant at late stages of storage.

Interactions between microorganisms, which may include competition, antagonism, metabiosis and cell-to-cell communication (quorum sensing), are also important in influencing the development of microbial spoilage communities (Gram et al., 2002). The competition for nutrients and limiting compounds such as iron, acidification of the media or the production of antimicrobials such as bacteriocins are all ways for microorganisms to antagonise other microorganisms and gain a competitive advantage (Gram et al., 2002). For example, members of the fluorescent group of *Pseudomonas* can not only compete very efficiently for available iron, but these bacteria also produce both antimicrobial and antifungal compounds (Ellis et al., 2000). LAB can also inhibit the growth of other microorganisms by producing large amounts of lactic acid (resulting in acidification of the meat medium) as well as bacteriocins (Jones et al., 2009, Casaburi et al., 2011).

Metabiosis is defined as "the reliance of one organism on another to produce a favourable environment" (Gram et al., 2002). An example of metabiosis relevant to the development of meat spoilage communities is the removal of headspace oxygen by Gram-negative aerobic bacteria, enabling growth of anaerobic bacteria such as *Clostridium* spp., which can cause BPS (Gram et al., 2002). Metabiosis can also be a situation where one microorganism provides nutrients, thereby enhancing growth of another species (Gram et al., 2002). For example proteolytic activity of pseudomonads in milk was suggested to be the cause of enhanced growth and acid production of lactic acid bacteria (Cousin and Marth, 1977).

Cell-to-cell communication among bacteria is termed quorum sensing (QS) and involves self-produced extracellular signal molecules that accumulate in the local environment to levels

required to activate transcription of specific genes (Whiteley et al., 2017). Via detection of these signalling molecules, bacteria respond to changes in the surrounding bacterial population and coordinate group behaviour (Ball et al., 2017). QS is omnipresent and despite limited studies on QS in food and in particular meat environments, QS likely participates in regulating processes involved in meat spoilage. Studies have shown that various signalling molecules such as autoinducer-1, autoinducer-2 and N-acyl-homoserine lactones can be detected in foods (milk, meat and vegetables) and that their concentrations increased during storage (Liu et al., 2006, Pinto et al., 2007, Lu et al., 2004, Bruhn et al., 2004, Skandamis and Nychas, 2012). Their production has been attributed to certain members of spoilage communities including pseudomonads, Enterobacteriaceae and LAB (Skandamis and Nychas, 2012). In addition, QS regulates spoilage traits such as attachment and biofilm production, and the production of extracellular proteases, and the regulation of these traits has been shown for spoilage species (Bai A and Rai Vittal, 2014, Liu et al., 2007, Christensen et al., 2003). Elucidation of the role of QS signal molecules in meat spoilage will be an important area for future research. Thus, knowledge of the microbiota present on carcasses and understanding that these communities are variable, that their growth is complex and governed not only by processing and storage conditions, but also influenced by other microorganisms present through interactive behaviour, is necessary to understand the spoilage process (Powell and Tamplin, 2012).

MEAT SPOILAGE MICROORGANISMS

Spoilage of fresh meat is mainly caused by Gram-negative (*Pseudomonas*, *Shewanella* and Enterobacteriaceae) and some Gram-positive bacteria (LAB, *B. thermosphacta* and clostridia) that dominate under different selective pressures (Pothakos et al., 2015). The

growth rates of yeast are generally considered too slow to give them a competitive advantage against psychrotrophic bacteria on fresh meat, however they can dominate the meat microbiota under conditions where the water activity has been reduced, and may be of importance when antibacterial substances are used as preservation methods (Samelis, 2006, Nychas and Drosinos, 2014). Like yeasts, moulds can also be present on carcasses, however they are usually not a spoilage concern for packaged, refrigerated fresh meat (Lambert et al., 1991).

Table 1.2 shows gaseous atmosphere requirements of the main groups of spoilage bacteria as well as information about their spoilage potential, the characteristic alterations they cause to meat and pH requirements relevant to their growth on fresh red meat. Determining these factors and attributes collectively for a group is problematic as the manifestation of spoilage is species and strain-dependent, and likely to depend on bacterial interactions, participation of bacteria that don't directly contribute to spoilage and the storage conditions that can influence the metabolic activity of the bacteria. Thus the information in this table must be considered as a generalisation.

Table 1.2: Spoilage bacteria found in red meat[a].

Bacteria	O_2-requirement	pH-requirement	CO_2 sensitivity	Spoilage potential	Spoilage characteristics	General remarks
Pseudomonas spp.	Aerobes	Progressively slower growth at lower pH values (Koutsoumanis et al., 2006).	High	High	Fruity, sulphurous and putrid off-odours	Dominant spoilage bacteria of aerobically stored meat
Shewanella putrefaciens	Facultative anaerobe	No growth below pH 6	Moderate	Very high	Sulphurous off-odours, some discolouration of meat	Spoilage of vacuum-packaged meat with high pH
Brochothrix thermosphacta	Facultative anaerobe	Poor anaerobic growth below pH 5.8	Moderate	High	Green drip, meat discolouration, pungent cheesy, dairy odours	Spoilage of vacuum- and MAP-packaged meat when sufficient oxygen is present
Enterobacteriaceae	Facultative anaerobes	Some anaerobic growth below pH 5.8	Moderate	High	Green drip, meat discolouration, production of sulphurous, putrid odours	Spoilage of high pH, vacuum-packaged meat, BPS
Lactic acid bacteria	Aerotolerant anaerobes	Can be the dominant bacteria on low and high pH meat stored anaerobically (Borch et al., 1996).	Low	Low	Greening of meat, slime production, cheesy, malty, acidic off-odours	Usually dominant bacteria of vacuum-packaged meat
Clostridium spp.	Anaerobes, but highly resistant spores	Growth inhibition below pH 5.8 (Huynh et al., 2016).	Low	High	Production of large amounts of gas and putrid off-odours	Causative agents of BPS

[a] Table adapted from Huynh et al. (2016).

LAB that typically dominate the meat microbiota under anaerobic conditions include the aciduric species *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc carnosum*, *Leuconostoc mesenteroides*, *Leuconostoc gelidum, Lactococcus raffinolyticus* and non-aciduric species *Carnobacterium divergens* and *Carnobacterium maltaromaticum* (Dainty and Mackey, 1992, Samelis, 2006, Doulgeraki et al., 2012). LAB are generally considered to have low spoilage potential due to slow growth rates and the production of less offensive

odours than other spoilage bacteria (Pothakos et al., 2015). However, they are associated with slime production, known to cause greening of meat and produce cheesy, malty, acidic off-odours (Nychas and Drosinos, 2014). Some LAB may contribute quite substantially to spoilage, while others may play a negligible role and have a bio-protective function (Pothakos et al., 2015).

Psychrotrophic *Clostridium* species are associated with spoilage of vacuum-packaged red meat. *Cl. estertheticum* is a common causative agent of BPS (Silva et al., 2011, Yang and Badoni, 2013, Broda et al., 1999, Broda et al., 2000) and *Clostridium putrefaciens* and *Clostridium algidicarni*s have been implicated in bone-taint spoilage, which is characterised by putrid odours from the internal sections of the muscle that are in contact with bone (Doulgeraki et al., 2012). *B. thermosphacta* is also a Gram-positive meat spoilage bacterium that produces malodourous compounds when sufficient oxygen is present (Nychas and Drosinos, 2014), and will be discussed in more detail in the following section.

Enterobacteriaceae usually only account for a small proportion of the spoilage microbiota (Nychas and Drosinos, 2014). Important meat spoilage members of this family include *H. alvei*, which can be found in modified atmosphere and vacuum-packaged beef and is associated with BPS (Chaves et al., 2012); *S. liquefaciens*, which is only found on high pH meat (Dainty and Mackey, 1992); and *Enterobacter agglomerans*, which is found on aerobically stored meat and meat in MAP (Samelis, 2006). Spoilage characteristics of members of this family include greening of the meat and the production of putrid, sulphurous odours (Doulgeraki et al., 2012, Nychas and Drosinos, 2014). Another frequently mentioned Gram-negative meat spoilage bacterium is *S. putrefaciens*. This bacterium is unable to grow at pH values under 6 at refrigeration temperatures, however on meat with

pH values greater than 6, *S. putrefaciens* can degrade cysteine and cystine resulting in H_2S production, even in the presence of glucose (Samelis, 2006).

Psychrotrophic *Pseudomonas* are the most important spoilage bacteria of aerobically stored meat. Dominant meat spoilage members of this genus include *Pseudomonas fluorescens* as well as the lesser studied species *P. fragi* and *P. lundensis* (Casaburi et al., 2011, Doulgeraki et al., 2012), both of which are discussed in a later section. *Moraxella* and *Acinetobacter* spp. can also constitute a major proportion of the aerobic bacteria on meat (Nychas and Drosinos, 2014). However despite utilising amino acids as growth substrates they do not produce malodourous volatile compounds and likely contribute indirectly to spoilage by limiting the availability of oxygen and thereby enhancing spoilage activities of other bacteria (Nychas and Drosinos, 2014, Samelis, 2006). For example, the facultative anaerobic bacterium *S. putrefaciens* has a strictly respiratory metabolism and under oxygen limitation will reduce alternative terminal electron acceptors, which can result in H_2S production, sulphmyoglobin formation and greening of meat (Nychas and Drosinos, 2014, Dawood et al., 1998).

As some of the most important microorganisms associated with the spoilage of fresh meat, but also some of the least studied, the following sections focus more closely on the bacteria *B. thermosphacta* and psychrotrophic pseudomonads *P. fragi* and *P. lundensis*.

***Brochothrix* spp.**

The *Brochothrix* genus comprises only two known species, *B. thermosphacta* and *B. campestris*. *Brochothrix* are Gram-positive, non-motile, facultative anaerobic, fermentative, non-spore forming rods (Stackebrandt and Jones, 2006). Based on 16S phylogeny, *Brochothrix* has been assigned to the Listeriaceae family and *Brochothrix* and *Listeria* species

share many common features such as the same main fatty acids and menaquinones, meso-diamino pimelic acid in the cell wall, a similar GC-content and catalase production (Stackebrandt and Jones, 2006). Despite these similarities, there is no data to suggest that *B. thermosphacta* or *B. campestris* have pathogenic potential.

B. thermosphacta was first isolated in 1951 from pork sausages and trimmings (Sulzbacher and Mclean, 1951) and has since been identified as a common meat spoilage bacterium that is ubiquitous throughout the meat production chain (Holley, 2014, Nychas et al., 2008, Patterson and Gibbs, 1978, Stellato et al., 2016). *B. thermosphacta* tolerates low pH (down to pH 5.5) and high salt concentrations (~10%), can grow at temperatures as low as -1.5 °C, and can become the dominant spoilage microorganism when sufficient oxygen is present (Gribble and Brightwell, 2013, Nowak et al., 2012). While glucose appears to be the preferred substrate, this bacterium can ferment a wide array of substrates including ribose, maltose, fructose, saccharose, trehalose, gentiobiose and N-acetylglucosamine (Nowak et al., 2012, Samelis, 2006). Under anaerobic conditions, *B. thermosphacta* ferments glucose, mainly producing lactic acid and ethanol as end-products (Labadie, 1999). Under aerobic conditions *B. thermosphacta* metabolises various substrates and produces numerous end-products including lactic acid, acetoin, acetic acid, isobutyric acid, isovaleric acid, 2,3-butanediol, diacetyl, 3-methylbutanal, 2-methylpropanol and 3-methylbutanol (Nychas and Drosinos, 2014). Thus, *B. thermosphacta* is capable of utilising different metabolic pathways depending on the growth conditions, and anaerobic storage conditions prevent more offensive metabolic activity of this bacterium (Hernández-Macedo et al., 2011a).

There is scarce and ambiguous data available regarding the proteolytic and lipolytic activity of *B. thermosphacta.* These data indicate *B. thermosphacta* is a non-proteolytic bacterium

with limited lipolytic activity at cold temperatures (Labadie, 1999, Nowak et al., 2012, Casaburi et al., 2014).

B. campestris has only ever been isolated from soil and grass (Gribble and Brightwell, 2013). Like *B. thermosphacta*, this bacterium will generally not grow above 30 °C and its biochemical activity profile is similar to that of *B. thermosphacta* (Talon et al., 1988). *B. campestris* produces Brochocin-C, a bacteriocin effective against *B. thermosphacta*, *Listeria monocytogenes* and other Gram-positive bacteria, which suggests it may be an interesting candidate for the bio-preservation of meat and other foods (Siragusa and Cutter, 1993). Very little data is published on this organism and its spoilage potential was only relatively recently examined (Gribble and Brightwell, 2013). This work demonstrated that *B. campestris* grows on low pH meat and can cause spoilage characterised by off-odours and pronounced green discolouration of drip in vacuum-packaged lamb. The authors suggested it is possible that *B. campestris* was falsely identified as *B. thermosphacta* in the past as sufficient differential testing may not always have been carried out.

***Pseudomonas* spp.**

Psychrotrophic pseudomonads are the most important meat spoilage bacteria under chilled, aerobic conditions (Nychas and Drosinos, 2014). Historically their identification has been difficult because of a lack of distinguishing bacteriological tests and unequivocal identification has remained difficult due to limited heterogeneity of the 16S rRNA gene sequences (Moore et al., 1996, Ercolini et al., 2007, Anzai et al., 2000, Yamamoto et al., 2000, Arnaut-Rollier et al., 1999). Thus the exact nature and diversity of aerobic meat spoilage communities has remained unclear. The well-studied bacterium *P. fluorescens* and

the lesser known members of the *P. fragi* subgroup, *P. lundensis* and *P. fragi* (Gomila et al., 2015) are all considered important meat spoilage organisms.

Pseudomonas are Gram-negative, aerobic, motile rods with a purely respiratory metabolism that can shorten the shelf-life of meat drastically due to their fast growth rates (Koutsoumanis et al., 2008, Nychas and Drosinos, 2014). This group of bacteria preferentially utilise glucose, which they metabolise via the Entner-Doudoroff pathway, an alternative pathway to the Emben-Meyerhof-Parnas glycolytic pathway, to metabolise glucose to pyruvate (Dainty and Mackey, 1992). Following glucose exhaustion, pseudomonads switch to lactic acid utilisation (Nychas and Drosinos, 2014). Under glucose-limiting conditions, *Pseudomonas* can also metabolise gluconate and 2-oxo-gluconate, both catabolic products of glucose conversion via the Entner-Doudoroff pathway, giving these bacteria an added competitive advantage because these substances are not readily used by other microorganisms present on meat (Nychas and Arkoudelos, 1990, Gill, 1976). *Pseudomonas* species are responsible for sweet, fruity odours early in spoilage (Ercolini et al., 2010). At later stages when surface levels of glucose and lactate are depleted and amino acids and other nitrogenous compounds are degraded, *Pseudomonas* generate putrid, sulphury odours (Koutsoumanis et al., 2006, Dainty et al., 1985). Members of this genus produce dimethyl sulphide during amino acid degradation but are unable to generate H_2S like Enterobacteriaceae and *S. putrefaciens* (Samelis, 2006). Pseudomonads also produce ammonia during amino acid degradation, which is the main cause of increasing pH-values at advanced stages of spoilage (Gill, 1976).

P. fragi is the most dominant and therefore important meat spoilage-related species of this genus (Ercolini et al., 2010). This bacterium is lipolytic and also well-known for its ability to

spoil milk and milk products (De Jonghe et al., 2011). Besides the role it plays in food spoilage, *P. fragi* has attracted attention as a source of lipases (Alquati et al., 2002, Hao Tran and H. G. Crout, 1998); esterases of this bacterium have been used for the production of fruity aromas for the development of food products (Lamer et al., 1996); and this microorganism has potential for bioremediation (Adelowo et al., 2006), corrosion inhibition of steel surfaces (Jayaraman et al., 1998) and plant growth promotion (Selvakumar et al., 2009).

Spoilage characteristics of *P. fragi* include fruity, sickly sweet aromas ascribable to the production of esters, and putrid, sulphur odours at late stages of spoilage associated with dimethyl sulphide production (Ercolini et al., 2010, Casaburi et al., 2015). The proteolytic activity of *P. fragi* may be strain-dependent (Ercolini et al., 2010). *P. fragi* is metabolically diverse and able to catabolise creatine and creatinine, which may explain its position as such a common meat spoiler (Drosinos and Board, 1994). Another explanation for the dominance of this species was suggested to be its ability to efficiently use the different sources of iron present in fresh meat (Labadie, 1999). Iron metabolism of *P. fragi* remains largely unstudied although available data suggests that this bacterium has high iron requirements, does not produce siderophores and may be capable of utilising foreign siderophore-iron complexes (Champomier-Vergès et al., 1996, Ercolini et al., 2010). It was suggested that *P. fragi* saves the energy otherwise required for siderophore expression, explaining its shorter generation time in a meat medium compared to *P. fluorescens* (Labadie, 1999).

Far less is known about the bacterium *P. lundensis* that has been frequently isolated from spoiled, refrigerated meat and was only defined as a bacterial species in 1986 (Molin et al., 1986). *P. lundensis* is known for its strong proteolytic activity and like *P. fragi*, this bacterium

is a common spoilage agent of milk (De Jonghe et al., 2011). Little is known about how *P. lundensis* contributes to meat spoilage although one strain in cheese was shown to produce significant quantities of ketones, in particular 2-pentanone, 2-heptanone and 2-nonanone, which are odour-active compounds that impart fruity, cheesy odours (Morales et al., 2005c).

Even though meat spoilage bacteria are responsible for substantial economic losses to the meat and retail industry, they remain quantitatively less studied than pathogens and species of biotechnological interest. As a result, less is known about the physiology of bacterial spoilage species, and as is the case for *P. lundensis*, often very little is understood about how these species contribute to spoilage.

THESIS OBJECTIVES

The goal of this thesis was to improve current knowledge of the biology of the important meat spoilage bacteria *B. thermosphacta* and psychrotrophic pseudomonads *P. fragi* and *P. lundensis*. Greater knowledge of the physiology of these key spoilage bacteria is important in ensuring improvements in the control of meat contamination and spoilage, and in shelf-life extension. In contrast to the well-studied meat pathogens, genomes of many of the meat spoilage bacteria, including *B. thermosphacta*, *P. fragi* and *P. lundensis*, remain largely unanalysed. Not only do genome sequences facilitate reliable identification of bacteria, but access to the genome can provide crucial insight into the evolution of bacteria, their metabolic potential, environmental adaptability, resistance mechanisms and more. Furthermore, little is known about cellular pathways of spoilage bacteria critical to their persistence in food processing environments such as stress response mechanisms, factors

involved in their colonisation of meat such as surface adhesion proteins, and fundamental elements of their biology such as iron acquisition systems.

With the aim of identifying pathways that could play a role in spoilage and the persistence of *B. thermosphacta* throughout the meat production chain, chapter 2 focused on analysing genomes of different strains of this bacterium and comparing these to genomes of *B. campestris* and *L. monocytogenes*. Physiological analyses of the different *B. thermosphacta* strains were conducted with the aim of linking gene inventory discovered during the genome analyses with phenotypic traits.

The foundation of chapter 3 was based on the identification of a putative sortase A gene identified in the *B. thermosphacta* genome in chapter 2. In other Gram-positive bacteria, sortase A plays a housekeeping role, attaching multiple proteins to the cell surface of these bacteria including MSCRAMMs involved in the adhesion of pathogens to host ECM, and thus facilitating interactions of these microbes with the human host. Knowledge of the housekeeping sortase of *B. thermosphacta* and its substrate proteins provides a starting point for understanding how this bacterium interacts with its environment. Therefore the aim of this work was to characterise biochemically the putative sortase A of *B. thermosphacta* and determine potential sortase A substrate proteins.

As a genus with considerable unresolved taxonomy, one of the aims of chapter 4 was to provide insight into genomes of the psychrotrophic *Pseudomonas* species *P. lundensis* and *P. fragi*, and investigate genomic diversity of different isolates of these species. Furthermore, limited information is available about the odour-active compounds produced by *P. lundensis*, and a thorough understanding of the metabolic potential of *P. fragi* at the strain level is missing. Thus the second aim of this chapter was to compare the metabolic profiles

of the *P. lundensis* and *P. fragi* isolates during their growth on a beef matrix and where possible link metabolic activity with gene repertoire.

P. fragi is one of the most dominant spoilage microorganisms, but how this bacterium competes so successfully with other bacteria is not understood. As little is known about iron uptake systems of *P. fragi* and iron is a limiting factor in many environments including meat, where it is tightly bound to iron-binding proteins, the aim of chapter 5 was to explore the possibility of siderophore-mediated iron uptake as an iron acquisition system of *P. fragi*.

Meat tenderness is an important quality attribute strongly influencing consumer choice. Therefore considerable interest exists for the development of novel technologies which can improve the tenderness of meat. As a processing technology with potential for meat tenderisation, but also with a potential to enhance meat spoilage, the research chapters of this thesis conclude with chapter 6, which aimed to determine the effects of a low field strength PEF on the aerobic microbiota of beef.

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Chapter 2

Insight into the genome of *Brochothrix thermosphacta*, a problematic meat spoilage bacterium

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ABSTRACT

Brochothrix thermosphacta is a dominant, but poorly studied meat spoilage organism. It is a close relative of the foodborne pathogen *Listeria monocytogenes* and constitutes the second genus in the Listeriaceae family. Here, the genomes of 12 *B. thermosphacta* strains were sequenced, assembled into draft genomes, characterised and compared with the genomes of *Brochothrix campestris* and *L. monocytogenes*. Phenotypic properties including biogenic amine production, and antibiotic and heavy metal susceptibility were tested. Comparative genomic analyses revealed a high degree of similarity among the *B. thermosphacta* strains, with bacteriophage genes constituting a significant proportion of the accessory genome. Genes for the production of malodourous compounds acetate, acetoin, butanediol and fatty acids were found, as were stress response regulatory genes, which likely play important roles in the spoilage process. Amino acid decarboxylases were not identified in the genomes and phenotypic testing confirmed their absence. Orthologues of *Listeria* virulence proteins involved in virulence regulation, intracellular survival and surface protein anchoring were found, however, key virulence genes were absent. Analysis of

antibiotic susceptibility showed strains were sensitive to the four tested antibiotics, except for one tetracycline resistant isolate with plasmid-mediated tetracycline resistance genes. Strains tolerated higher levels of copper and cobalt than of cadmium, although not at concentrations high enough to categorize the strains as resistant. This study provides insight into the *Brochothrix* genome, links previous phenotypic data and data provided here to gene inventory and identifies genes that may contribute to the persistence of this organism in the food chain.

Importance

Despite increasing knowledge and advances in food preservation techniques, microbial spoilage of foods causes substantial losses with negative social and economic consequences. To better control the contamination and microbial spoilage of foods, fundamental knowledge of the biology of key spoilage bacteria is crucial. As a common meat spoilage organism, *B. thermosphacta* contributes substantially to spoilage-associated losses. Nonetheless, this organism and in particular its genome, remain largely unstudied. This study contributes to improving our knowledge of the *Brochothrix* genus. Spoilage-relevant pathways and genes that may play a role in the survival of this organism in a food processing environment were identified, linking previous phenotypic data and data provided here to the gene inventory of *Brochothrix*, and establishing parallels and differences to the closely related foodborne pathogen *L. monocytogenes*.

INTRODUCTION

Brochothrix thermosphacta is one of the most abundant spoilage organisms of fresh and cured meats, fish, and fish products, due to its tolerance to high salt and low pH conditions,

its ability to grow at refrigeration temperatures and production of organoleptically unpleasant compounds (Borch et al., 1996, Holley, 2014, Mamlouk et al., 2012). This Gram-positive, fermentative bacterium can become the dominant spoilage species of modified atmosphere- (MAP) and vacuum-packaged meats when sufficient oxygen is present (Ercolini et al., 2011, Nowak et al., 2012, Mamlouk et al., 2012, Gribble and Brightwell, 2013). Although aerobic spoilage is largely dominated by Gram-negative pseudomonads, *B. thermosphacta* can also play an important role in shortening the shelf-life of aerobically stored meat, particularly when bacteriostatic agents such as sulphites are used (Prieto et al., 1993, Barlow and Kitchell, 1966, Grau et al., 1985, Banks et al., 1985, Stringer et al., 1995).

B. thermosphacta is considered a non-proteolytic spoilage organism and is associated with spoilage characterised by cheesy, buttery or sour odours rather than putrefaction (Holley, 2014, Nychas and Drosinos, 2014). Under anaerobic conditions or oxygen limitation, *B. thermosphacta* produces lactic acid and ethanol as primary end-products, with smaller amounts of formate and acetate also produced (Labadie, 1999, Blickstad and Molin, 1984). Under aerobic conditions, the end-products of its metabolism include lactic acid, acetic acid, acetoin, diacetyl, 2,3-butanediol, ethanol, isobutyric acid, isovaleric acid and 2-methylbutyric acid (Nychas and Drosinos, 2014, Blickstad and Molin, 1984). These fatty acids are thought to be formed from amino acids and not by lipolysis (Holley, 2014). Information on the lipolytic activity of *B. thermosphacta* strains is scarce and ambiguous, and the role of lipolysis in meat spoilage caused by *B. thermosphacta* remains unclear (Collins-Thompson et al., 1971, Papon and Talon, 1988, Nowak et al., 2012).

The *Brochothrix* genus comprises only two known species, *B. thermosphacta* and the lesser known species *B. campestris* (Talon et al., 1988). *Brochothrix* are low GC-content bacteria

(Firmicutes) that have been placed in the Listeriaceae family, and members of the *Listeria* and *Brochothrix* genera share many common features such as the same main fatty acids, menaquinones, meso-diamino pimelic acid in the peptidoglycan, a similar GC-content and catalase production (Stackebrandt and Jones, 2006). Their 16S rRNA gene sequences share 92.8 to 96.6% sequence similarity (Ludwig et al., 1984).

While *B. campestris* has only been isolated from soil and grass (Talon et al., 1988), *B. thermosphacta* is thought to be ubiquitous throughout the meat production chain (Patterson and Gibbs, 1978, Nychas et al., 2008, Holley, 2014). As a saprophytic organism capable of growth at cold temperatures, *B. thermosphacta* shares its environmental niche with a member of its sister taxon, *L. monocytogenes*, the foodborne pathogen and causative agent of listeriosis. The persistence of microorganisms like *B. thermosphacta* and *L. monocytogenes* in the food chain and other ecosystems reflects their ability to adapt to numerous stresses such as osmotic, pH and temperature stress (Ratani et al., 2012). Furthermore, the use of antibiotics in animal husbandry and the presence of heavy metals in the environment, provides selective pressure for the survival and dominance of resistant bacteria (Verraes et al., 2013). Even low levels of antibiotics and heavy metals are thought to be sufficient to select and enrich bacteria carrying multiresistance plasmids (Gullberg et al., 2014). Resistance to heavy metals appears to be one of the longest known environmental adaptations of *Listeria* and although it remains unclear whether heavy metal resistance contributes to overall fitness in food processing environments or foods, it is interesting that heavy metal resistant *Listeria* strains are more common among food isolates than those recovered sporadically (Ratani et al., 2012).

While genome characterization of *L. monocytogenes* and other *Listeria* species has been extensive, until now, genomic analyses have focused only on bacteriophages of *B. thermosphacta* (Kilcher et al., 2010). A recent study also used the genome sequences of *B. thermosphacta* DSM 20171^T and *B. campestris* DSM 4712^T to investigate the dynamics of genome evolution of the *Listeria* genus, but without any in-depth analysis of the *Brochothrix* genomes (Chiara et al., 2015). To better understand the physiology of this problematic spoilage organism, we have sequenced the genomes of 12 *B. thermosphacta* strains and conducted comparative genomic analyses of these strains with *B. campestris* DSM 4712^T. Emphasis was placed on identifying genes and pathways that may play an important role in food spoilage and persistence of this organism throughout the food chain. Numerous virulence genes of *Listeria* are involved in the adhesion to mammalian cells and transition from an environmental organism to human pathogen. As *Brochothrix* is a close relative of *Listeria* that is commonly found associated with meat, we conducted a search for *Listeria* virulence orthologues that may play a role in the adaptation of *B. thermosphacta* from a farm-to-carcass ecosystem. To link the gene inventory identified in this study with phenotypic properties, amino acid decarboxylase activity and antibiotic and heavy metal tolerance of the strains were investigated.

MATERIALS AND METHODS

Bacterial strains

In total, 12 *B. thermosphacta* strains were used in this study (Table 2.1). *B. campestris* DSM 4712^T (GenBank accession number AODH00000000.1) and *L. monocytogenes* 10403S (GenBank accession number CP002002) (Edman et al., 1968, Bishop and Hinrichs, 1987)

were included for genomic comparisons. Strains 7803, 7804, 7806, 7807, 7808, 7809 and 7810 were isolated in 1993 during a meat spoilage study (data not published) by cutting 25 cm^2 cores from the meat and placing them in sterile bags with 300 mL of Maximum Recovery Diluent (MRD). These samples were macerated for 1 min using a Seward Stomacher 400 set to 230 rpm and then stored in ice water. Samples of homogenate were serially diluted in MRD and 50 µL of appropriate dilutions were plated onto non-selective plate count agar (Oxoid).

Strains 7811, 7813, 7816 and 7818 were isolated for the purpose of this study. The respective meat sources were kept at 4 °C until their used by date and then the strains were isolated using the method of Gribble and Brightwell (Gribble and Brightwell, 2013).

Table 2.1: *B. thermosphacta* strains.

Strain	Meat source[a]	Genome size (bp)	No. of contigs	G+C content (%)	No. of protein-coding genes	No. of tRNAs	GenBank accession no.
DSM 20171[Tb]	Pork sausage	2,494,302	41	36.4	2,290	84	MDLK00000000
7803[c]	Beef (MAP)	2,575,404	61	36.3	2,348	80	MDLL00000000
7804[c]	Beef (MAP)	2,613,933	56	36.3	2,415	83	MDLU00000000
7806[c]	Beef (air)	2,539,264	41	36.3	2,328	80	MDLM00000000
7807[c]	Lamb (air)	2,482,452	31	36.4	2,277	91	MDLN00000000
7808[c]	Lamb (air)	2,557,615	68	36.3	2,328	86	MDLO00000000
7809[c]	Beef (air)	2,491,703	22	36.3	2,273	80	MDLV00000000
7810[c]	Beef (air)	2,471,990	22	36.4	2,244	80	MDLP00000000
7811[d]	Beef (air)	2,548,405	186	36.3	2,292	81	MDLT00000000
7813[d]	Veal mince (MAP)	2,642,200	166	36.2	2,417	90	MDLQ00000000
7816[d]	Lamb (air)	2,584,597	157	36.3	2,342	71	MDLR00000000
7818[d]	Beef (air)	2,587,934	130	36.3	2,364	82	MDLS00000000

[a] MAP, modified atmosphere packaged. Air indicates storage under aerobic conditions.

[b] Type strain (isolated during a study on the microflora of fresh pork sausage by Sulzbacher and Mclean (McLean and Sulzbacher, 1953, Sulzbacher and Mclean, 1951)).

[c] Strains were isolated in 1993 (meat sources obtained in Melbourne, Australia) during a meat spoilage study (H. M. Craven and N. Baxter-Keene, CSIRO Agriculture and Food, unpublished data).

[d] Strains were isolated for this study from meat sources obtained in Melbourne, Australia.

Strain identification

PCR was performed prior to whole genome sequencing to confirm all isolates were *Brochothrix* strains. Primers were designed to target the glyceraldehyde-3-phosphate dehydrogenase gene in *Brochothrix*, following alignment of this gene with *Listeria* orthologues and the determination of conserved bases present only in the *Brochothrix* gene.

The forward primer bound to bases 611 to 632 of the gene on the plus strand. The reverse primer targeted bases 107 to 125 of the gene on the minus strand.

Template preparation was performed by immersing a small single colony in 25 µL H_2O, heating the sample for 15 min at 95 °C, before centrifugation at 15,000xg for 2 min and using 2 µL of supernatant as template. The amplification mixture also contained 0.25 µM of each primer (gapdhfwd: 5'-GTAGTGTTTGGAACAATGTTAG-3', and gapdhrev: 5'-CTGATGCTTCACAATTAGC-3') and 1x HotstarTaq Master Mix (Qiagen). PCR amplification was performed as follows: Initial denaturation at 95 °C for 15 min, followed by 30 cycles; denaturation at 94 °C for 30 sec, annealing at 50 °C for 1 min, polymerization at 72 °C for 1 min; final extension at 72 °C for 4 min.

DNA isolation, whole genome sequencing and gene annotation

B. thermosphacta strains from Table 2.1 were grown for 18 h (+/- 2 h) in Tryptone Soya Broth (TSB, Oxoid) at 25 °C. Genomic DNA was isolated with the Qiagen DNeasy Blood and Tissue Kit according the manufacturer's protocol for Gram-positive bacteria. Library preparation and genome sequencing was carried out at The Ramaciotti Centre at the University of NSW using the Nextera XT DNA library 300 bp paired end preparation kit and was performed on the Illumina MiSeq platform (Illumina USA). A public Galaxy instance supported by the CSIRO Bioinformatics Core and IM&T (Goecks et al., 2010) was used to run the assembly software. FastQC:Read QC (version 0.10.1) was applied for quality control of sequence reads and adaptor sequences and trailing bases were removed using Trimmomatic (version 1.0.0) (Lohse et al., 2012). A minimum read length of 150 bases was chosen. Assembly of the sequencing reads was performed using the SPAdes genome assembler tool (version 3.5) with k-mer sizes of 21, 33, 55 and 77 (Bankevich et al., 2012). For annotation,

the genomes were submitted to RAST (version 2.0, FIGfam version: Release 70) (Overbeek et al., 2014) and NCBI's Prokaryotic Genome Automatic Annotation Pipeline (version 3.3).

Genome sequence similarity analyses

Overall genome relatedness indices, Orthologous Average Nucleotide Identity (OrthoANI) (Lee et al., 2016) and digital DNA:DNA hybridization (DDH) estimates (Meier-Kolthoff et al., 2013), were determined using the OrthoANI Tool (version 0.93, BLAST+) and Genome-to-Genome Distance Calculator (GGDC) (version 2, BLAST+) with the recommended formula 2 (identities/high scoring segment pair lengths).

BLASTn comparisons were performed with BRIG (the BLAST Ring Image Generator application) (Alikhan et al., 2011). *B. thermosphacta* DSM 20171^T was used as the central reference sequence, and 70 and 90% were chosen as the lower and upper identity thresholds. Mauve (version 2.3.1) (Darling et al., 2004) and PHAST (PHAge Search Tool) (Zhou et al., 2011) were used for identifying bacteriophage genes present in *B. thermosphacta* DSM 20171^T, but absent in the query genomes.

Pan-genome analysis

Pan-genome analysis was performed with USEARCH (version 8.1.1861) using a minimum identity of 0.6. Seed sequences were parsed into tabular form with a custom script (https://github.com/bioinformatics-deakin/gene-matrix-from-uclust3.py) and genes less than 153 nucleotides in length (protein sequences <50 amino acids in length) were eliminated from the analysis. The core genome of the 12 isolates was determined as the set of genes shared by all strains, while the accessory genome was the set of genes present in a subset of strains or in a single strain.

The accessory genome was split into high (genes shared by 10 or 11 of the isolates), medium (genes common to at least three and up to nine strains) and low frequency coding sequences (unique genes and genes shared by only two isolates). Functional annotation of the translated seed sequences of these accessory groups was conducted with NCBI's CD-Search Tool to identify conserved domains using a mirrored COG (Clusters of Orthologous Groups of proteins)-database with the default settings (e-value threshold of 0.01, maximum number of hits of 500 and composition-corrected scoring was turned on). As each COG can have one or more general category letter associations, the pool of functional categories was compared for each accessory group. Due to the anticipated high number of non-assignable (i.e. hypothetical proteins) bacteriophage gene products, PHAST was used to determine the prophage regions in each of the 12 *B. thermosphacta* genomes. All genes within these identified prophage regions were then classified as "bacteriophage" genes.

The abundance of general categories from COG-assignment and prophage genes determined by PHAST were normalized for high, medium and low frequency genes by dividing these values by the number of genes present in each frequency group. Statistical analysis of the lengths of the core and accessory proteins was conducted using BoxPlotR as implemented by http://boxplot.tyerslab.com/.

Phylogeny of *Brochothrix* strains

Progressive Mauve (Darling et al., 2010) was used to create whole genome alignments of *B. thermosphacta* strains and *B. campestris* DSM 4712^{T}. StripSubset removed LCBs smaller than 2000 bases and Gblocks (version 0.91b) (Castresana, 2000, Talavera and Castresana, 2007) was run with the default settings to remove poorly aligned positions and divergent regions of the alignments prior to phylogenetic analysis. Tree building was performed using

FastTree (version 2.1.7) (Price et al., 2009, Price et al., 2010) with the GTR Gamma model of nucleotide evolution (determined by jModelTest (version 2.1.7) (Guindon and Gascuel, 2003, Darriba et al., 2012) as the most suitable model) and 1000 bootstrapping replicates. The tree was rooted on *B. campestris* DSM 4712^T.

Identification of genes involved in cell metabolism

The SEED Viewer was used to identify reactions for cellular processes in the *B. thermosphacta* strains and *B. campestris* DSM 4712^T. Pathway details were obtained using KEGG pathway reconstructions and comparisons were accessed via The SEED Viewer (Overbeek et al., 2005). Reconstructed KEGG pathways for which many reactions were present and only single or a few steps were missing, were manually controlled for gap filling. Genes not found within these models or gene candidates found to be missing critical protein domains are reported as not identified in the genomes.

Virulence factor search in *Brochothrix*

A *Brochothrix* protein database was generated using all protein sequences from the 12 *B. thermosphacta* strains and *B. campestris* DSM 4712^T. *Listeria* virulence factors included in this analysis were obtained from the table supplied for pathogenomic comparisons of *Listeria* spp. from the Virulence Factors of Pathogenic Bacteria (VFPB) database (http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Listeria). This list of 44 *Listeria* virulence proteins were searched against the *Brochothrix* database using the scoring matrix BLOSUM62 and the BLASTp algorithm. Proteins with statistically significant sequence similarity (BLASTp cutoff scores of E<10^{-5} and bit scores = or >50) were subjected to domain analysis using the InterproScanProgram and gene synteny was manually assessed.

Biochemical tests of strains

API 20E strips (BioMerieux) were used to test the following reactions: arginine, lysine and ornithine decarboxylase activity, Voges-Proskauer reaction and mannitol fermentation. Two strips per strain were inoculated as described by the manufacturer. One strip was incubated at 25 °C for 24 h. The second strip was incubated at 25 °C for 30 h. Reactions were read according to the product's instructions. Quality control of the strips was tested using the strain, *Escherichia coli* ATCC 25922.

Screening for biogenic amine production

An improved decarboxylase medium as described by Bover-Cid and Holzapfel, 1999 (Bover-Cid and Holzapfel, 1999), was used with one adjustment: The pH of the media was set to pH 5.6 to better mimic the pH of raw meat. Strains were grown at 25 °C for 18 h (+/- 2 h) in Nutrient Standard Broth (Oxoid) (with 1% precursor amino acids and 0.005% pyridoxal-5-phosphate added). Strains were then streaked on the decarboxylase medium containing the amino acids L-phenylalanine and L-tryptophan and the amino acid salts L-histidine monohydrochloride (Sigma), L-lysine hydrochloride (BDH) and DL-ornithine monohydrochloride (Sigma), and without amino acids (as controls) and were incubated for 7 d at 25 °C under aerobic and anaerobic conditions in parallel. Positive reactions increase the pH of the medium, changing the color of the media from a yellow/brown to purple. *E. coli* ATCC 25922 was used as a positive control for testing this system due to its known lysine and ornithine decarboxylase activity.

MIC testing of antibiotics

Minimal inhibitory concentrations of tetracycline, erythromycin, penicillin G and ciprofloxacin were established using Thermo Scientific Oxoid M.I.C.E Strips according to the

manufacturer's protocol for *Listeria* with one exception: The plate incubation temperature was 25 °C to allow the growth of *Brochothrix*.

MTC testing of heavy metals

The Maximum Tolerance Concentration (MTC) of three heavy metal salts ($CoCl_2$, CdCl, $CuSO_4$) was tested using a modified plate method (Hu and Zhao, 2007). *B. thermosphacta* strains were grown at 25 °C for 18 h (+/- 2 h) in TSB media. Subsequently, 100 µL of each cultured strain were spread onto TSA plates containing no heavy metal (control plates), 50, 100, 200, 300, 400, 450, 500 and 600 µg/mL of $CoCl_2$, 5, 10, 15, 30, 50, 70, 100 and 200 µg/mL of CdCl and 100, 200, 300, 350 and 400 µg/mL of $CuSO_4$. Plates were incubated at 25 °C and checked for growth at daily intervals for up to 5 d. The MTC was determined as the highest metal concentration at which the growth of bacteria was not altered compared to their growth on control plates. MTCs are lower than MICs by approximately a factor of two (Mergeay et al., 1985, Schmidt et al., 1991).

Accession numbers

Accession numbers of the 12 *B. thermosphacta* strains are listed in Table 2.1.

RESULTS

Genomic features and intergenomic sequence similarity of strains

Whole genome sequencing was undertaken for 12 *B. thermosphacta* isolates, including the type strain *B. thermosphacta* DSM 20171^T. Table 2.1 contains a summary of the assembly and annotation metrics of these draft genomes. The 12 draft genomes were highly similar in estimated size varying by approximately 1.7 kb and were between 2.47 and 2.64 Mb. Their

GC-content was between 36.2 and 36.4%, and the genomes harbored 2,244 to 2,417 predicted protein coding sequences.

A high degree of genomic sequence similarity is demonstrated among the *B. thermosphacta* strains by the two calculated genomic indices (Tables A1 and A2). Genomic comparisons of the *B. thermosphacta* isolates to *B. thermosphacta* DSM 20171^T revealed OrthoANI (Orthologous Average Nucleotide Identity) values between 98.99 and 99.54% (recommended cut-off range for species delimitation is 95-96%), while GGDC DDH (Genome-to-Genome Distance Calculator digital DNA:DNA hybridization) estimates, which correlate well with wet lab DNA:DNA hybridization percentages, were between 90.7 and 93.1%. For *B. campestris* DSM 4712^T and *B. thermosphacta* DSM 20171^T, OrthoANI and GGDC DDH values of 75.05 and 20.60% were obtained.

The genomic sequence similarity of *B. thermosphacta* isolates is also illustrated in a BLASTn ring diagram (Figure 2.1), where the 11 Australian strains and *B. campestris* DSM 4712^T were compared to the central reference genome of *B. thermosphacta* DSM 20171^T. The most prominent region absent in the 11 Australian isolates was an approximately 37 kb region of the type strain genome harboring phage genes.

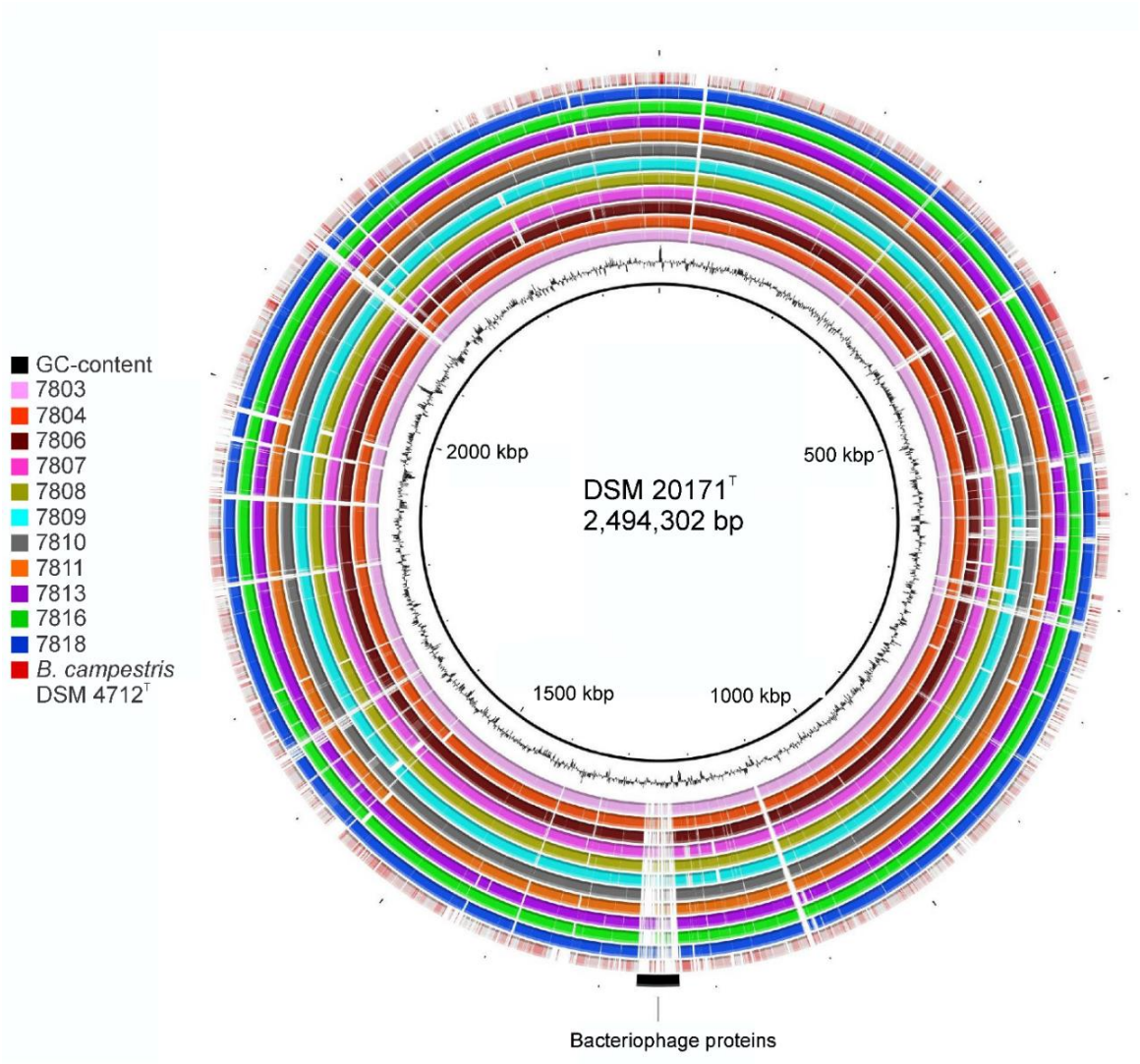

Figure 2.1: *B. thermosphacta* strains share a high degree of genomic similarity. Shown is a schematic of BLAST comparisons of nucleotide sequences of the draft genomes of *B. thermosphacta* strains and *B. campestris* DSM 4712^T (shown as concentric rings) to *B. thermosphacta* DSM 20171^T (central black ring). The GC-content of the genome of *B. thermosphacta* DSM 20171^T is shown in black outside of the inner ring. BLASTn matches are coloured on a sliding scale from 70 to 90%, demonstrating the degree of sequence similarity between the query strains (shown in the legend) and the *B. thermosphacta* type strain. Spaces in the rings indicate regions of the type strain genome that were absent in the query genomes. Bacteriophage proteins encoded in the type strain genome, but largely absent in the query genomes, are depicted with a black arc in the outer ring.

Pan-genome investigation

Strain diversity was investigated with a pan-genome analysis. The pan-genome of the 12 strains comprised 3012 protein coding sequences, with 2012 core genes (66.8% of the pan-genome) and 1000 accessory genes (30.8% of the pan-genome) (Figure 2.2A). With between 2,244 and 2,417 predicted protein coding genes identified in the draft genomes (Table 2.1), the 2012 core genes constituted between 83 and 90% of the predicted proteome of each isolate.

To investigate possible links between genes of the accessory genome and different evolutionary processes influencing their frequency of occurrence, this group was separated into high, medium and low frequency accessory genes. With 591 genes, the low frequency accessory group was the largest, followed by the medium frequency group with 344 genes and the high frequency group with 65 coding sequences (Figure 2.2A). Functional annotation of their gene products, assigned a pool of 46, 109 and 223 categories to the high, medium and low frequency accessory groups, respectively (Figure 2.2B). As prophage typically contain significant numbers of hypothetical genes to which no annotation can be assigned, bacteriophage-associated coding sequences were determined using PHAST. Of the high frequency accessory genes, 6.2% were bacteriophage coding sequences, while 28.8% and 33.5% of the medium and low frequency accessory genome were bacteriophage genes. Thus, prophage coding sequences constituted a significant proportion of the accessory genome, in particular the medium and low frequency groups.

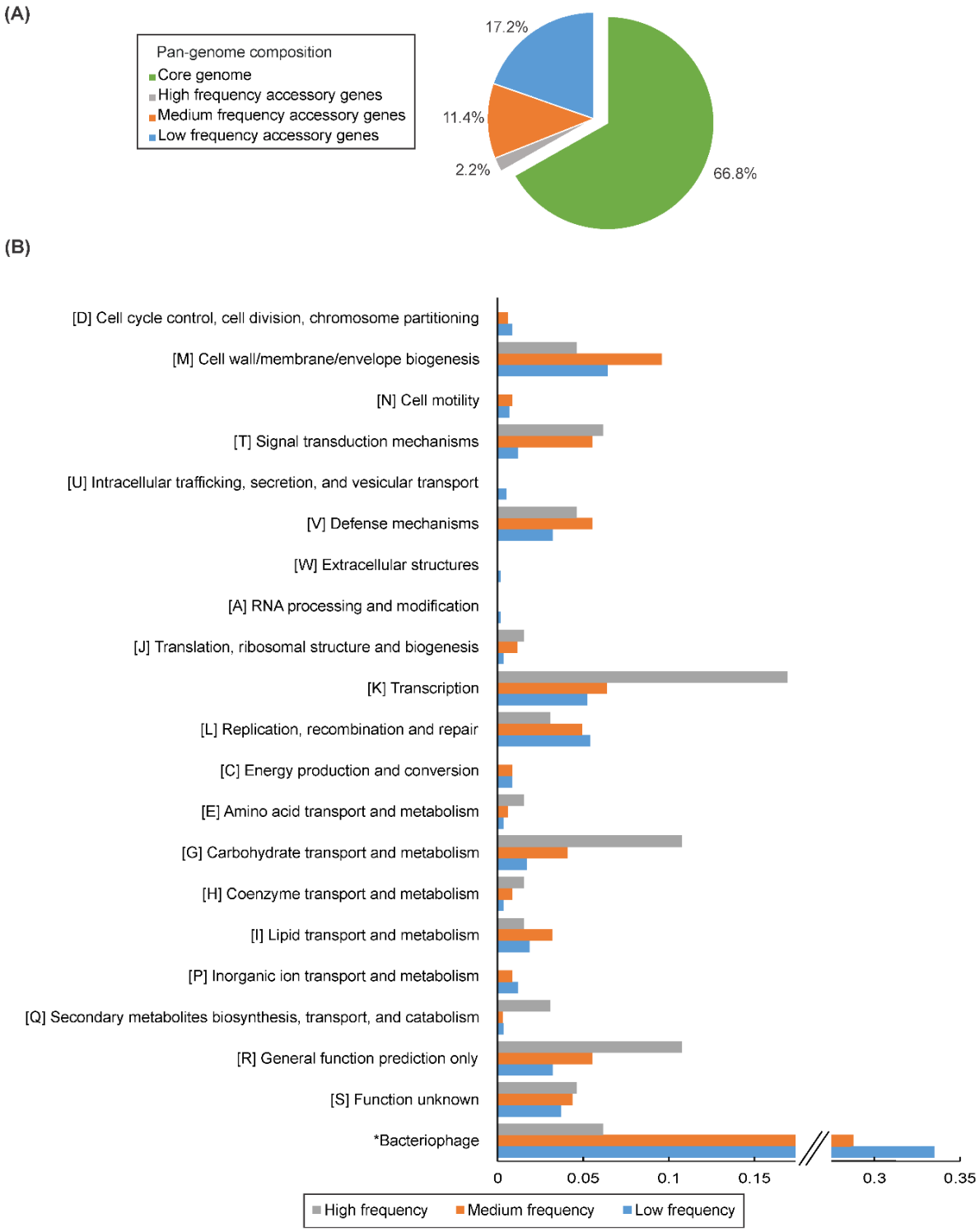

Figure 2.2: Pan-genome analysis shows that bacteriophage genes represent a large proportion of the accessory genome. (A) The pan-genome of the 12 *B. thermosphacta* strains comprises 3012 genes, with the core genome constituting 66.8% of the genes, the high frequency accessory group constituting 2.2%, the medium frequency accessory group representing 11.4%, and the low frequency accessory group representing 17.2%. **(B)** The relative abundance of COG functional categories for the high, medium and low frequency

accessory groups are shown. On the vertical axis, COG functional categories are depicted with their letter association, while the horizontal axis indicates their relative abundance. The relative abundance of prophage coding sequences is included at the bottom of the graph and the corresponding label on the vertical axis has been marked with an asterisk (*Bacteriophage) to highlight the different method by which these gene functions were determined. To aid visualisation, the x-axis and the low and medium frequency bars associated with bacteriophage coding sequences are truncated and the break points are marked with //.

In the high frequency pool, the highest relative number of coding sequences were found to be associated with transcription processes followed by carbohydrate transport and metabolism and signal transduction mechanisms. In addition to bacteriophage genes, which were notably the most abundant within the medium and low frequency accessory groups, genes involved in the biogenesis of the cell envelope, defence mechanisms such as CRISPR systems, transcription processes, and DNA replication, recombination and repair were also abundant in both groups. Furthermore, genes with functions in signal transduction, and carbohydrate and lipid transport and metabolism were well represented in the medium frequency group.

Annotation of the draft genomes involved a limited amount of manual curation. Thus, to help gauge the possible impact of miscalled or spurious open reading frames (ORFs) identified by the gene calling algorithm, the statistics of the lengths of the proteins belonging to the accessory group were determined (Figure A1: Appendix A). The median length of core and accessory proteins was 273 and 202 amino acids respectively, and individual groupings of accessory genes had median lengths of 214 (high frequency), 219

(medium frequency) and 189 (low frequency) residues, indicating possible miscalled ORFs in the accessory genome.

Phylogeny of *B. thermosphacta* strains

To better understand the relationship among the geographically and temporally diverse collection of strains, a phylogenetic tree of the 12 strains and *B. campestris* DSM 4712^T was inferred using alignments of orthologous genomic regions of the strains and an approximated maximum likelihood approach. The resulting tree shows four distinct phylogroups (groups 1 to 4) of strains (Figure 2.3). Genes specific to certain phylogroups, which were identified during the pan-genome analysis, are listed to the right of this tree and locus tags of these are given in Table 2.2.

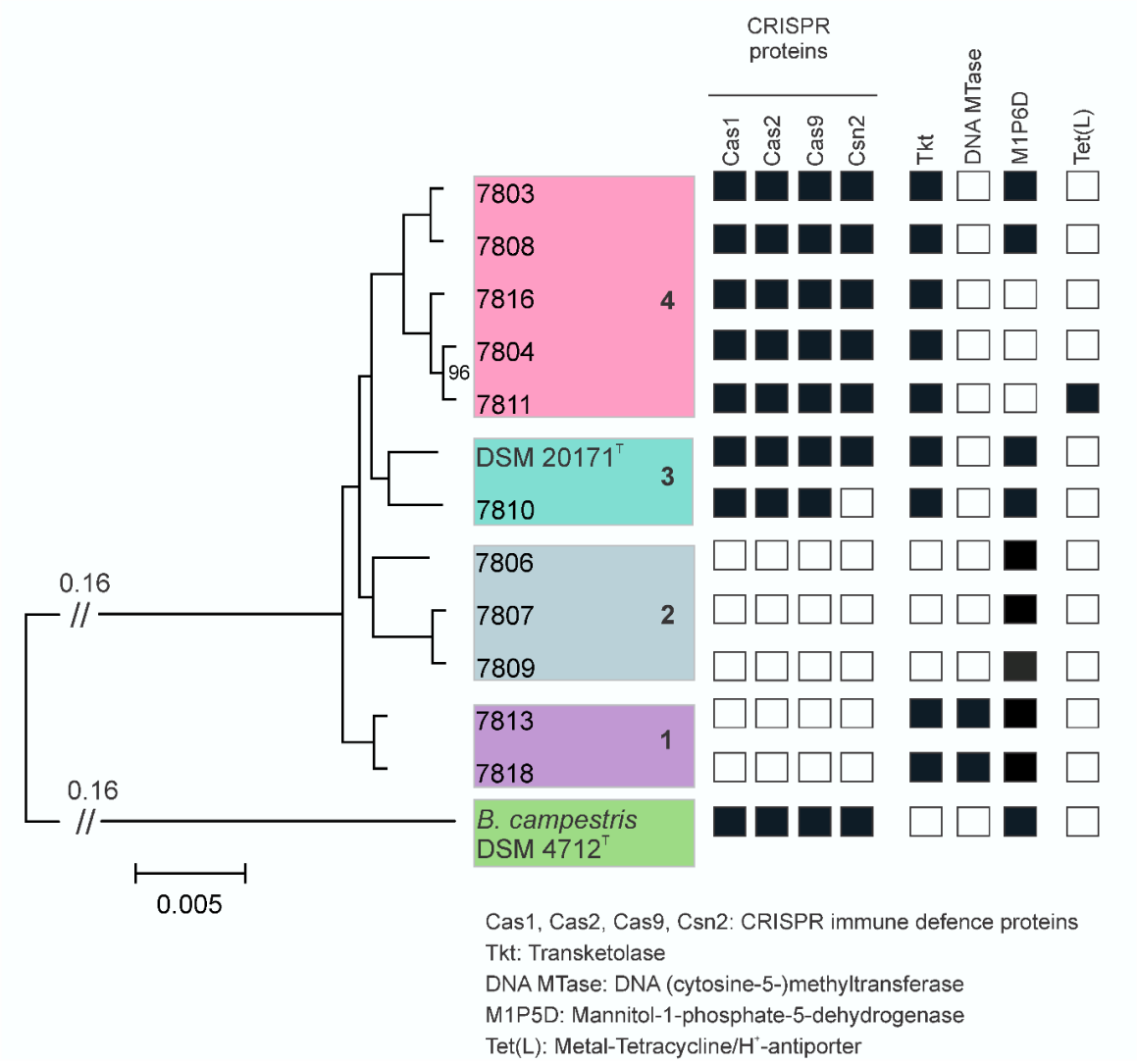

Figure 2.3: Phylogenetic tree showing the relationships of the 12 *B. thermosphacta* strains and accessory genes present or absent in these strains. The tree is based on alignments of orthologous regions of the genomes and was generated using an approximated maximum likelihood method. All interior node values are 100 or otherwise as marked and represent local support values from 1,000 bootstrap replicates. The scale bar denotes the number of substitutions per site. Branches that were truncated to aid visualisation are marked with //, and the original branch length is indicated above. The four phylogroups of *B. thermosphacta* strains are represented in coloured boxes, with the phylogroup number shown in boldface type at the right of the box. Accessory genes present or absent in strains are shown to the right of the tree and black boxes represent genes that are present, while white boxes represent the absence of genes from the genome.

Table 2.2: Locus tags of coding sequences[a].

	Locus tag(s) (GenBank accession no.)	
Coding region	***B. thermosphacta*[b]**	***B. campestris* DSM 4712^T**
Cas1	BFR34_02990	BCAMP_RS03535
Cas2	BFR34_02995	BCAMP_RS03530
Cas9	BFR34_02985	–
Csn2	BFR34_03000	BCAMP_RS03525
Transketolase (N-terminal peptide) Transketolase (C-terminal peptide)	BFR34_08475, BFR34_08480	—
DNA (cytosine-5-)methyltransferase	BFR35_12330 (MDLQ00000000)	–
Mannitol-1-phosphate-5-dehydrogenase	BFR34_07330	BCAMP_RS09425
Glucokinase	BFR34_10620	BCAMP_RS05020
Fructokinases	BFR34_11880, BFR34_10080	BCAMP_RS00590
Pyruvate carboxylase	BFR34_02675	BCAMP_RS02425
Lactate dehydrogenase	BFR34_12075	BCAMP_RS09650
Pyruvate decarboxylase	BFR34_04065	–
Alcohol dehydrogenase	BFR34_11980	BCAMP_10295[c]
Acetaldehyde dehydrogenase	BFR34_12025	BCAMP_RS09610
Pyruvate formate-lyase	BFR34_00380	BCAMP_RS07680
Phosphate acetyltransferase	BFR34_05080	BCAMP_RS07720
Acetate kinase	BFR34_09255	BCAMP_RS05095
Acetolactate synthase	BFR34_08185	BCAMP_RS01540
Acetolactate decarboxylase	BFR34_08180	BCAMP_RS01545
Butanediol dehydrogenase	BFR34_03815	BCAMP_RS09100
Acyl-CoA thioesterase	BFR34_05700	BCAMP_RS02760
PotA PotB PotC PotD	BFR34_09935, BFR34_09930, BFR34_09925, BFR34_09920	BCAMP_RS06135, BCAMP_RS06130, BCAMP_RS06125, BCAMP_RS06120
Lipase	BFR34_09910	BCAMP_RS06110
Tet(L)	BFR45_11435 (MDLT00000000)	–
Alkylmercury lyase	BFR36_11230 (MDLR00000000)	–
Mercury(II)-responsive transcriptional regulator	BFR36_11260 (MDLR00000000)	–
RNA polymerase sigma factor SigmaB	BFR34_09145	BCAMP_RS05220

[a] – indicates that the gene was not identified.

[b] Gene locus tags are from *B. thermosphacta* DSM 20171^T (GenBank accession number

MDLK00000000) unless otherwise specified in parentheses (accession numbers of other *B. thermosphacta* genomes).

[c] This gene was identified in the sequence reported under the GenBank accession number AODH00000000, but is missing from the RefSeq version of the *B. campestris* DSM 4712^T draft genome under accession number NZ_AODH00000000.1.

Group 1 isolates 7813 and 7818 formed a clade separated from the other *B. thermosphacta* strains and these isolates were found to encode an immune defence restriction modification protein (DNA (cytosine-5-)methyltransferase) unique to this group. Group 2 comprised the three isolates 7806, 7807 and 7809. Absent in the genomes of group 1 and group 2 isolates were genes for the two universally conserved CRISPR-prokaryotic immune defence proteins Cas1 and Cas2, as well as genes for predicted Cas9 and Csn2 proteins. Group 2 strains were also lacking two genes (N- and C-terminal peptides) for an additional transketolase (Tkt) encoded in all of the other genomes. Group 3 comprised *B. thermosphacta* DSM 20171^T and a second isolate 7810, and group 4 contained the largest number of strains with five isolates (7803, 7808, 7816, 7804 and 7811). While all of the group 3 and 4 strains carried genes for the CRISPR proteins and Tkt peptide chains, a mannitol-1-phosphate-5-dehydrogenase (M1P5D) gene encoded in all of the other strains, was absent in the genomes of group 4 isolates 7811, 7804 and 7816. These three strains also encoded a truncated mannitol-specific phosphotransferase transporter and were missing an additional mannitol-uptake gene, both located upstream of the M1P5D gene, and tested negative for mannitol fermentation (Table 2.3).

Neither the meat environment nor the packaging type from which the strains were derived appeared to be congruent with the phylogrouping of the strains.

Table 2.3: Phenotypic properties of *B. thermosphacta* isolates[b].

Strain	Mannitol fermentation ability[a]	Antibiotic tolerance (MIC [µg/mL])[c]				Heavy metal tolerance (MTC [µg/mL])[d]		
		Pen G	Cip	Tet	Ery	CdCl	$CoCl_2$	$CuSO_4$
DSM 20171^T	+	0.12	0.12	0.25	0.02	10	200	200
7803	+	0.25	0.25	0.5	0.06	10	200	200
7804	–	0.5	0.25	0.5	0.06	15	200	200
7806	+	0.12	0.25	0.25	0.03	10	200	200
7807	+	0.5	0.25	0.25	0.06	10	200	200
7808	+	0.25	0.25	0.5	0.06	10	200	200
7809	+	0.25	0.12	0.25	0.06	15	200	200
7810	+	0.5	0.5	0.25	0.12	10	200	200
7811	–	0.5	0.25	32	0.12	10	200	200
7813	+	0.25	0.12	0.25	0.06	10	200	200
7816	–	0.25	0.25	0.5	0.06	10	200	200
7818	+	0.25	0.25	0.25	0.12	10	200	200

[a] + indicates an ability to ferment mannitol, and – indicates an inability to ferment mannitol.

[b] Voges-Proskauer tests were positive for all strains. Decarboxylase activity reactions were negative for all strains with lysine, arginine, ornithine, histidine, tryptophan and phenylalanine.

[c] Pen G, penicillin G; Cip, ciprofloxacin; Tet, tetracycline; Ery, erythromycin.

[d] The maximum tolerant concentrations (MTCs) are shown for the three heavy metal salts.

Cell metabolism

Inspection of the genomes of *B. thermosphacta* strains and *B. campestris* DSM 4712^T revealed a diverse repertoire of substrate-specific genes from the phosphotransferase system (PTS) including glucose, maltose, sucrose, fructose, mannose, trehalose, cellobiose, β-glucoside, mannitol, and N-acetylglucosamine specific EIIA, EIIB and EIIC genes. Ribose, glycerol-3-phosphate, maltose/maltodextrin and inositol transporter genes were also identified as well as lactate, gluconate and malate permease genes.

For both *Brochothrix* species, all genes required for glycolysis were found including genes encoding the sugar-specific kinases glucokinase and fructokinase (Table 2.2, and Figure A2: Appendix A). The full complement of genes required for the pentose-phosphate pathway were also found (Figure A3: Appendix A), while coding sequences for only four of the eight enzymes of the citrate cycle (tricarboxylic acid [TCA] cycle) were present (Figure A4: Appendix A). Genes identified as absent included α-ketoglutarate dehydrogenase, succinate dehydrogenase, succinate thiokinase and malate dehydrogenase genes. Furthermore, both species encoded a pyruvate carboxylase (Table 2.2), while a fumarate reductase was not found.

The *Brochothrix* genomes were examined for the presence of genes involved in the conversion of pyruvate to various end-products, particularly those considered as malodourous compounds, as described by pathways in KEGG (Figures A2 and A5: Appendix A). Genes involved in the production of lactate, ethanol, acetate and formate were present in both *Brochothrix* species and included genes for the enzymes lactate-dehydrogenase (EC 1.1.1.27), alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.3), acetaldehyde dehydrogenase (EC 1.2.1.10), pyruvate formate-lyase (EC 2.3.1.54), phosphate acetyltransferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) (Table 2.2). A pyruvate decarboxylase (EC 4.1.1.1) gene was identified only in the draft genomes of the *B. thermosphacta* strains (Table 2.2). Furthermore, in both species genes involved in butanediol fermentation were found including α-acetolactate synthase (EC 2.2.1.6) and acetolactate decarboxylase (EC 4.1.1.5) genes with contiguous locations for the production of acetoin from pyruvate, and an (S,S)-butanediol dehydrogenase gene required for the reduction of acetoin to 2,3-butanediol (EC 1.1.1.76) (Table 2.2, and Figure A5: Appendix A).

The production of acetoin, as determined by positive Voges-Proskauer reactions, was observed for all *B*. *thermosphacta* strains (Table 2.3).

Amino acid decarboxylase genes were not identified in the *B. thermosphacta* strains or *B. campestris*. Furthermore, lysine-, arginine, ornithine-, histidine-, phenylalanine- and tryptophan decarboxylase activity was not detected in the 12 strains using both API 20E strips and decarboxylase screening media (Table 2.3), while the control strain *E. coli* ATCC 25922 was clearly positive for lysine and ornithine decarboxylase reactions using both testing procedures. Both *Brochothrix* species were however found to possess a homologous gene cluster with conserved synteny in *L. monocytogenes* comprising spermidine/putrescine-specific uptake genes *potA*, *potB*, *potC* and *potD*. The translated products of these genes also have significant homology to the PotABCD components of the spermidine/putrescine ABC transporter in *Escherichia coli* K-12 (Table 2.2).

As it is thought that free fatty acids result from amino acid metabolism in *B. thermosphacta* (Holley, 2014), amino acid pathways were investigated. Genes were present in both *Brochothrix* species for the production of isovaleryl coenzyme A (CoA) (3-methylbutanoyl-CoA), isobutyryl-CoA and 2-methylbutanoyl-CoA from the degradation of valine, leucine and isoleucine, respectively (Figure A6: Appendix A). These pathways involve the common activity of a branched chain amino acid transaminase (EC 2.6.1.42), which catalyzes the reaction between the amino acids and their α-keto acids. Further steps involve the activity of a branched chain keto acid dehydrogenase (EC 1.2.4.4), a dihydrolipoyllysine-residue (2-methylpropanoyl)transferase (EC 2.3.1.168) and a dihydrolipoyl dehydrogenase (EC 1.8.1.4) for the conversion of the resulting 2-oxo acids to the respective branched chain fatty acyl-CoAs. Furthermore, genes of the distal pathways of valine and isoleucine degradation were

identified in *B. thermosphacta* strains for the synthesis of propanoyl-CoA (Figure A6: Appendix A). An acyl-CoA thioesterase gene was also identified in both *Brochothrix* species (Table 2.2) for the hydrolysis of the acyl-CoAs to free fatty acids and coenzyme A.

A search for lipase and esterase genes revealed the presence of a lipase in *B. thermosphacta* and *B. campestris* with 54% and 52% identity to lipases in *Listeria* species (Table 2.2), and multiple esterase genes in both *Brochothrix* species.

Resistance genes

Numerous multi-drug resistance efflux transporters were annotated in the draft genomes. As a close relative of *L. monocytogenes* and an organism that shares its environmental niche with this foodborne pathogen, we were interested in testing the MICs of the strains to antibiotics traditionally used to treat listeriosis such as penicillin, erythromycin and tetracycline, as well as the therapeutically important fluoroquinolone ciprofloxacin, to which *L. monocytogenes* shows decreased susceptibility (Allerberger and Wagner, 2010, Hof et al., 1997). *B. thermosphacta* strains were sensitive to all four tested antibiotics, with the exception of one isolate (7811) that displayed resistance to tetracycline (MIC was 32 μg/mL) (Table 2.3). Inspection of the draft genome of this strain revealed the presence of a tetracycline efflux transporter gene on a contig with notably high coverage (read coverage approximately 14-fold greater than the average genome read coverage) (Table 2.2 and Figure 2.3). This gene product was found to share more than 80% identity with known class L Tet determinants and greater identity with determinants from the plasmid subgroup (88% identity to Tet(L) protein from *Bacillus subtilis* plasmid pNS1981-Accession no. BAA00005) than those from the chromosomal subgroup (83% identity to *Bacillus subtilis* GSY908 Tet(L) protein Accession no. CAA30827) (Levy et al., 1999, Sakaguchi et al., 1988, Takayuki et al.,

1985). A *tetR* regulatory gene was also identified on this contig in close proximity to the efflux gene.

Subsystems of the SEED predicted numerous genes in both *Brochothrix* species to be involved in copper, arsenic, cobalt, zinc and cadmium homeostasis/resistance. Metal resistance is commonly defined based on the metal tolerance levels of other bacteria and until now data has been missing on metal tolerance levels of *B. thermosphacta*. The heavy metal tolerance of the isolates was analysed by determining the MTCs of $CoCl_2$, CdCl and $CuSO_4$ (Table 2.3). Overall, significantly higher concentrations of $CuSO_4$ and $CoCl_2$ were tolerated compared to tolerated CdCl levels, however none of the observed MTCs were at levels high enough to categorize the strains as resistant to these metals (Mullapudi et al., 2008, McLauchlin et al., 1997, Najiah et al., 2009, Dekker et al., 2014). An MTC of 200 µg/mL of $CoCl_2$ and $CuSO_4$ was observed for all of the strains. Although impaired, growth of the isolates was still observed in the range of 200–500 µg/mL $CoCl_2$, while 300-350 µg/mL was the upper limit for growth on media containing $CuSO_4$. For CdCl, the MTCs of the strains were between 10–15 µg/mL and although impaired, growth of all isolates was still observed at 50 µg/mL CdCl. At these higher concentrations, confluent growth was replaced by the emergence of single colonies.

Examination of the draft genomes of the strains revealed differences in the number of predicted copper, cobalt and cadmium resistance genes. The genomes of the strains encoded one or two genes predicted to be involved in cadmium transport, although no correlation was observed between the number of these genes and the tolerated cadmium concentrations. The strains were also found to encode two putative cobalt transporter

genes and three or four genes with a predicted function in copper transport and homeostasis.

Presence of *Listeria* virulence genes in *Brochothrix*

The genomes of *Brochothrix* were searched for orthologues of 44 well-characterised *Listeria* virulence factors with the aim of identifying gene candidates that may play a role in the contamination and growth on meat.

Key virulence genes *hly* (Listeriolysin O), *actA* (nucleation promoting factor), *plcA*, *plcB* (membrane damaging phospholipases), *inlA* (Internalin A) and *inlB* (Internalin B) were absent from the *Brochothrix* genomes. However, orthologues of other virulence factors involved in virulence regulation, intracellular survival and surface protein anchoring were identified (Table 2.4). Conserved synteny in the *Brochothrix* genomes was only observed for eight of the 22 identified virulence gene orthologues. These included two factors involved in adherence to mammalian cells (*dltA* and *fbpA*), one gene required for intracellular survival (*prsA2*), one factor with a function in iron uptake (*hbp2*), homologous *virR* and *virS* two-component regulatory genes, a threonine phosphatase (*stpA*) and the surface anchoring factor, *srtB* (Table 2.4).

Table 2.4: Orthologues of *L. monocytogenes* virulence factors present in *Brochothrix* genomes according to their functional category[a].

Function	Virulence factors	Related genes	Locus tag	
			***B. thermosphacta* DSM 20171^T**	***B. campestris* DSM 4712^T**
Adherence	D-alanine-phosphoribitol ligase	*dltA*	BFR34_01920	BCAMP_RS06480
	Fibronectin-binding protein	*fbpA*	BFR34_07800	BCAMP_RS01735
Bile resistance	Bile salt hydrolase	*bsh*	BFR34_01045	no hit
Enzyme	Threonine phosphatase	*stp*	BFR34_07765	BCAMP_RS01770
	Metalloprotease	*mpl*	no hit	BCAMP_RS07830
Intracellular survival	Lipoate protein ligase A1	*lplA1*	BFR34_04540	BCAMP_RS04420
	Oligopeptide-binding protein	*oppA*	BFR34_01280	BCAMP_RS03095
	Post-translation chaperone	*prsA2*	BFR34_04575	BCAMP_RS10760
	Sugar uptake system	*hpt*	BFR34_09035	BCAMP_RS05315
Iron uptake	Haemoglobin binding protein	*hbp2*	BFR34_05250	BCAMP_RS12765
Invasion	Cell wall teichoic acid glycosylation protein	*gtcA*	BFR34_03990	BCAMP_RS09170
	Promoting entry protein	*lpeA*	BFR34_01515	BCAMP_RS03260
Peptidoglycan modification	OatA	*oatA*	BFR34_04360	BCAMP_RS07115
Regulation	Positive regulatory factor	*prfA*	BFR34_00370	no hit
	LisR	*lisR*	BFR34_11190	BCAMP_RS07275
	LisK	*lisK*	BFR34_11195	BCAMP_RS07280
	VirR	*virR*	BFR34_09230	BCAMP_RS05130
	VirS	*virS*	BFR34_09225	BCAMP_RS05135
Surface protein anchoring	Diacylglyceryl transferase	*lgt*	BFR34_06385	BCAMP_RS05615
	Specific signal peptidase II	*lspA*	BFR34_07940	BCAMP_RS09260
	Sortase A	*srtA*	BFR34_10065	BCAMP_RS06070
	Sortase B	*srtB*	BFR34_05235	BCAMP_RS10940

[a] Rows containing orthologous genes with conserved synteny are highlighted in grey.

In *L. monocytogenes* the *srtB* gene (*lmo2181*) is located on a Fur-regulated operon, which also encodes the haem-binding virulence protein Hbp2 (*lmo2185*), the cell wall anchored haem-binding protein Hbp1 (*lmo2186*) and components of a ferric hydroxymate ABC

transporter (*lmo2182*, *lmo2183*, *lmo2184*) (Figure 2.4A) (Xiao et al., 2011). A conserved *srtB* locus was found in the *Brochothrix* genomes (Figure 2.4A) as well as a well conserved Fur box (Figure 2.4B) identified upstream of the first gene of this putative operon. In *B. thermosphacta* the *srtB* locus encoded five open-reading frames, while in *B. campestris* DSM 4712^{T} there were seven genes. Counterparts to the *L. monocytogenes* genes *lmo2182* and *lmo2180*, encoding a membrane ATPase of the ABC transporter and a soluble protein of unknown function, were missing. The first gene of this putative operon was predicted to carry a SrtB sorting signal (NPXTN) and encoded a protein in *B. thermosphacta* and *B. campestris* that shared 51 and 44% identity with its *L. monocytogenes* counterpart Hbp1. This gene was followed by a homologous *hbp2* gene, which showed striking variability in sequence and length among the *B. thermosphacta* strains and also between both species. The evolution of this gene in *B. campestris* appears to have led to three separate *hbp2* coding sequences. Downstream reading frames to *hbp2* encoded proteins with homology to Lmo2184 and Lmo2183, which are lipoprotein and permease components of a ferric hydroxymate ABC transporter. The *srtB* operon in *Brochothrix* lacked an orthologue for *lmo2180* and terminated with *srtB*.

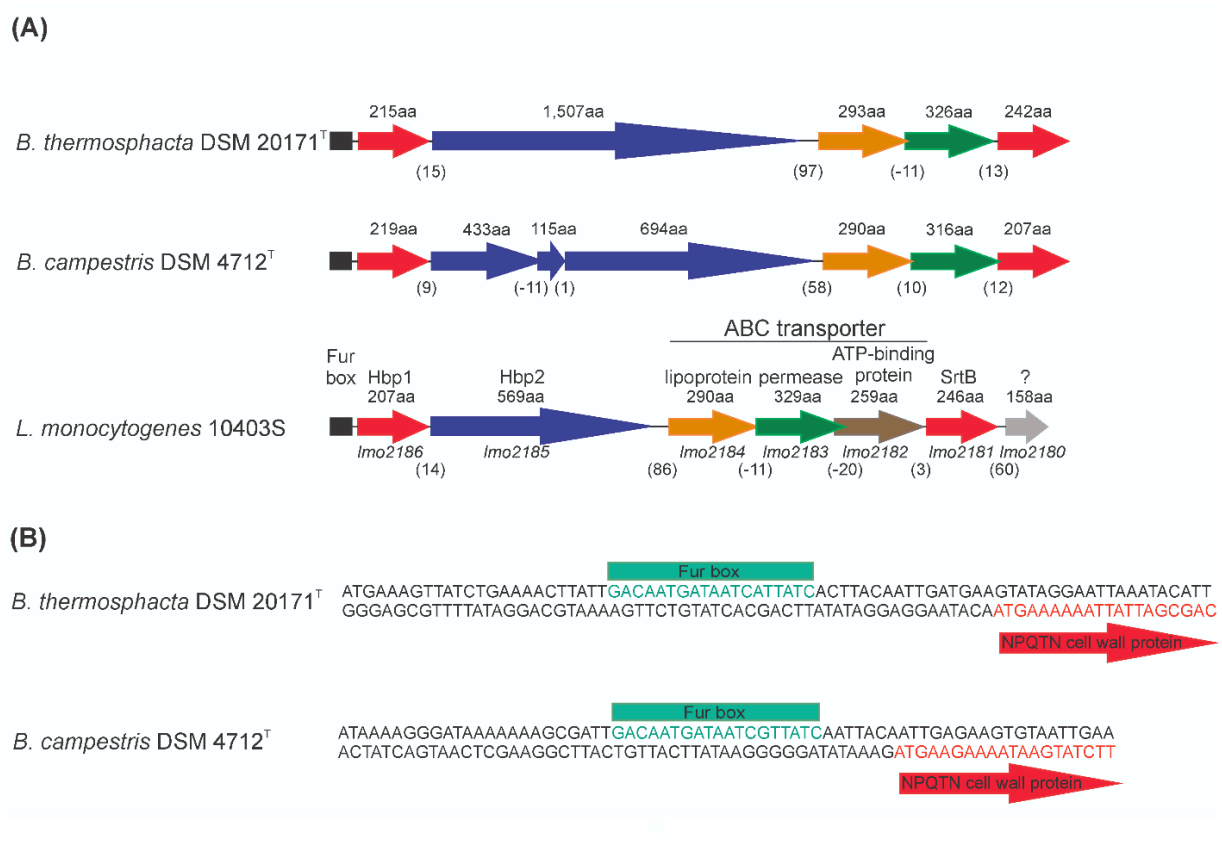

Figure 2.4: Schematic representation of the conserved synteny of the *srtB* locus in *Brochothrix*. (A) In *L. monocytogenes*, the *srtB* operon contains genes for cell wall-associated and secreted proteins (*lmo2186* and *lmo2185*), sortase B that ligates them to the peptidoglycan (*lmo2181*) and an ABC transporter (*lmo2182 to 2184*). Genes encoding an ATP-binding component of the putative ABC transporter were absent from this region in *Brochothrix* species. In *B. thermosphacta*, the length of the second gene of this locus varied considerably among the isolates, while in *B. campestris*, the evolution of this gene led to three separate coding regions. Numbers in parentheses indicate the number of spacer nucleotides between the genes or bases that overlap. aa, amino acids. **(B)** A Fur box was identified upstream of the first gene of the *srtB* locus in *Brochothrix* species.

Stress response genes

Another region of notable synteny identified in the *Brochothrix* genomes, which extended beyond the Listeriaceae family to other Gram-positive organisms, was a locus encoding the key stress regulator in *L. monocytogenes*, the alternative sigma factor, σ^B (Figure 2.5 and

Table 2.2). Although the highest sequence similarity between amino acid sequences of σ^B and σ^B-related gene sequences from *Brochothrix* was shared with members of the *Listeria* genus, the gene components of this genomic region were most similar to the respective region in *Staphylococcus aureus* (Figure 2.5). In addition to the σ^B gene, a gene predicted to encode an anti-sigma factor (*rsbW*) was found, as were anti-sigma factor antagonist (*rsbV*) and RsbV-phosphatase (*rsbU*) genes. In contrast to *L. monocytogenes*, *B. subtilis* and *S. aureus*, where the *rsbU* gene was followed by a significant spacer sequence that holds a known σ^B-dependent promoter, the spacer region following the *rsbU* gene was notably shorter in *Brochothrix*. The length of this spacer in *B. thermosphacta* was only 18 bp and 15 bp in *B. campestris* (Figure 2.5). Consequently, a σ^B-dependent promoter is likely not present between these genes in *Brochothrix* due to the short length of the respective spacer regions. The *rsbU* gene was followed by *rsbV*, *rsbW* and *sigB* genes. As is the case in *S. aureus*, an *rsbX* gene was absent in *Brochothrix* genomes.

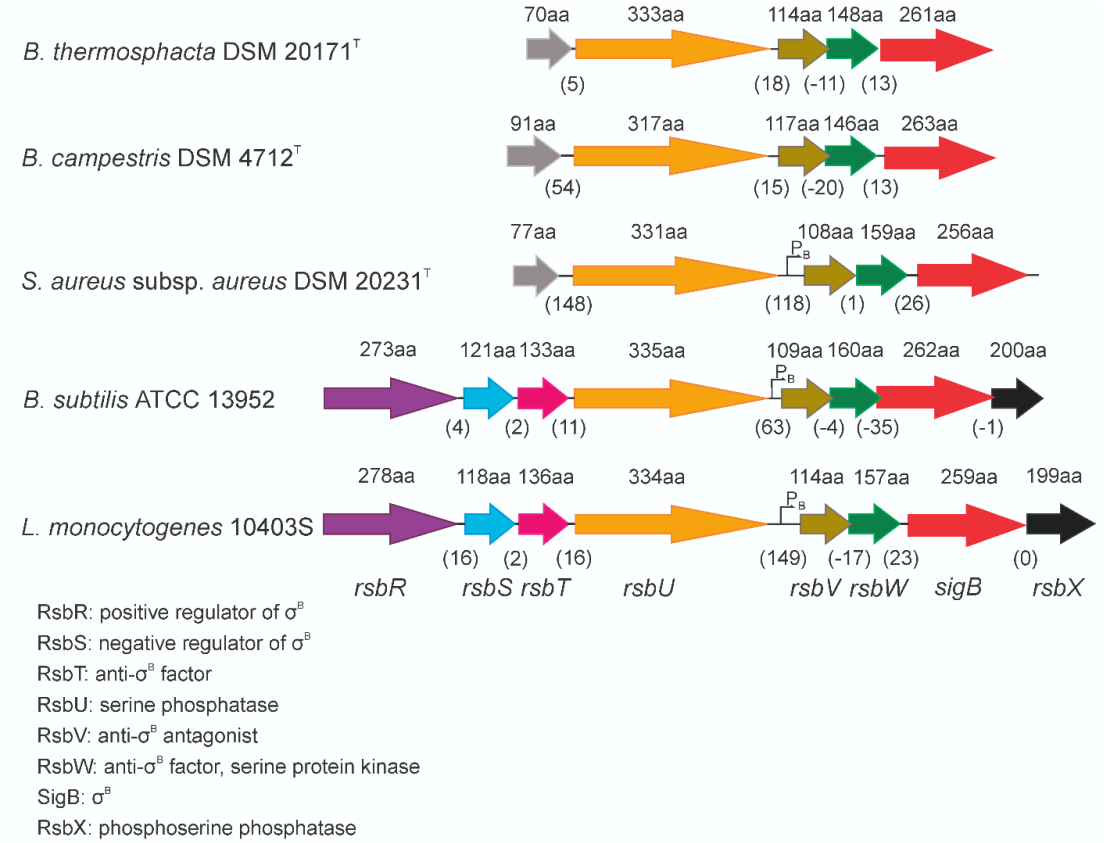

Figure 2.5: Conserved synteny of the *sigB* locus in Gram-positive bacteria. The conserved synteny of the *sigB* locus identified in the *Brochothrix* genomes extends beyond the Listeriaceae family to other Gram-positive organisms. In both *Brochothrix* species, this region harboured predicted *rsbU*, *rsbV*, *rsbW* and *sigB* genes. Large spacer regions between the *rsbU* and *rsbV* genes in *S. aureus*, *B. subtilis* and *L. monocytogenes* contain σ^B-dependent promotors, which are likely not present in this intergenic region in *Brochothrix* species due to the short length of the respective spacer regions. Grey arrows depict a hypothetical protein coding sequence.

DISCUSSION

Intergenomic sequence similarity of strains

A high degree of genomic similarity was observed for the 12 *B. thermosphacta* strains using genome-relatedness indices and BLASTn ring comparisons. Core genome phylogeny showed that several intraspecies clades exist and that strains 7813 and 7818 from the group 1

lineage were clearly separated from the remaining 10 strains. The high degree of similarity between the type strain and the 11 Australian isolates, and also among the Australian strains, was surprising considering their geographic and temporal differences and the different meat packaging types used for the meat sources of the isolates (Sulzbacher and Mclean, 1951). Although overall, the temporal and in particular the geographic diversity of the isolates was limited, the striking genomic similarity between the Australian isolates and the type strain could be indicative that a high degree of genomic similarity is a feature of this species. However, *B. thermosphacta* is an environmental organism and has been isolated from a variety of foods (Holley, 2014), and therefore, sequencing of isolates from alternative sources may shed more light of the overall intraspecies diversity.

Pan-genome investigation

The core genome of the strains contained an estimated 2012 coding sequences, which is similar in size to the predicted core genome of the *Listeria* genus (1,994 core genes) (den Bakker et al., 2010). Determination of the pan-genome from draft genomic data must be considered an estimate given the variable number of contig break points and difficulties in assembling large repeated regions. However, it can be anticipated that the number of genes in the core genome will decrease as the numbers of available *B. thermosphacta* genomes increase. The accessory genome was estimated to constitute 30.8% of the pan-genome of the strains. While this illustrates a relatively limited amount of strain diversity, this number was nonetheless higher than anticipated based on the BLASTn comparisons that we conducted. A notable reduction in median sequence length of accessory proteins was observed and may be the result of incorrect ORF assignments during the automated

annotation process. Hence, it is possible that the prediction of 30.8% accessory genome content of the 12 strains, and therefore their strain diversity, is an overestimate.

The accessory genome was categorized into high, medium and low frequency genes in the place of a typical binary analysis of core and accessory genomes, enabling a linkage of genes in these groups to different evolutionary processes as suggested by Cordero and Polz, 2014 (Cordero and Polz, 2014). High frequency genes are thought to be primarily maintained by vertical inheritance and homologous recombination, typically encode core metabolic functions and are potentially involved in habitat-specific tasks (Cordero and Polz, 2014). In line with these assumptions, the high frequency accessory pool in *B. thermosphacta* contained genes involved in signal transduction, transcription regulation, and carbohydrate uptake and metabolism. All of these processes may enable adaptation of this organism to new environments and the uptake and utilisation of different carbon sources, and can be explained by the saprophytic lifestyle of this bacterium. It is important to note that as the genomes are drafts, it cannot be excluded that some of the genes from this group may in fact belong to the core genome.

Bacteriophage genes represented the largest proportion of the medium and low frequency genes. Also identified among the medium and low frequency pools were bacterial defence systems including CRISPR and restriction modification elements. Interestingly, there was no noticeable correlation between the presence of these genes and the abundance of prophages in the isolates. Low frequency genes tend to be encoded in mobile regions of the genome, allowing a higher rate of DNA turnover (Cordero and Polz, 2014), and congruent with this hypothesis the low frequency group comprised genes conferring resistance to the antibiotic tetracycline that were located on a putative plasmid.

Cell metabolism

In line with previous observations, the draft genome sequences of the *B. thermosphacta* strains and *B. campestris* DSM 4712^T indicated that these organisms should be able to utilise a variety of carbon sources including C3-compounds such as glycerol (Stackebrandt and Jones, 2006). Additionally, work from Chiara et al. (2015), which identified ethanolamine utilisation genes (*eut* genes) in *Brochothrix*, as well as the annotation of the draft genomes presented here suggest that this organism may also be capable of utilising this compound (Chiara et al., 2015). Evidence suggests that the utilisation of ethanolamine, a major constituent of lipids in eukaryotic and bacterial cells, may play a role in the coexistence of bacteria like *L. monocytogenes* with other microorganisms that are incapable of using these substrates (Tang et al., 2015).

Genes encoding enzymes for the major catabolic pathways, the glycolysis and pentose phosphate pathways, were present in *Brochothrix*; however, four enzymes of the TCA cycle appear to be absent. Previous reports have shown that *Brochothrix* does not have an operational TCA cycle, and indeed, this organism must have alternate means to provide amino acid precursors such as oxaloacetate. Like *L. monocytogenes*, it is possible that pyruvate carboxylase-catalyzed carboxylation of pyruvate is the predominant reaction leading to oxaloacetate in *B. thermosphacta* (Joseph and Goebel, 2007). Furthermore, both the requirement of CO_2 for oxaloacetate production and the absence of a decarboxylating enzyme like malate dehydrogenase, which may be inhibited by elevated CO_2-concentrations, could help explain why CO_2 has only a limited inhibitory effect on *B. thermosphacta*.

B. thermosphacta has been shown to be capable of producing a number of organoleptically unpleasant compounds such as acetoin, butanediol, and a variety of fatty acids and alcohols, imparting cheesy, sour and malty odours that characterise its growth on meat (Casaburi et al., 2014, Nychas and Drosinos, 2014, Casaburi et al., 2015). One study also investigated the spoilage ability of *B. campestris* in vacuum-packaged lamb, demonstrating that this species has the potential to cause spoilage due to the production of off-odours and discoloration of drip in the packs (Gribble and Brightwell, 2013). Despite the multitude of studies demonstrating the ability of *B. thermosphacta* to produce these malodorous substances, the gene inventory responsible for their production has remained unknown. Under anaerobic conditions, *Brochothrix* species likely engage a pyruvate formate-lyase for the decarboxylation of pyruvate to acetyl-CoA and formate, which could initiate the transformation of pyruvate to acetate via the sequential activity of phosphate acetyltransferase and acetate kinase enzymes, and to ethanol via the sequential activity of acetaldehyde dehydrogenase and alcohol dehydrogenase enzymes. These enzymes, in addition to an identified lactate dehydrogenase, likely explain the production of lactate, acetate, formate and ethanol by *B. thermosphacta* under anaerobic conditions, as previously described (Grau, 1983). Under aerobic conditions pyruvate decarboxylase activity may initiate both ethanol and acetate production with the reduction of acetaldehyde to ethanol via alcohol dehydrogenase activity and the oxidation of acetaldehyde to acetate by aldehyde dehydrogenase activity. Genes were also found in both species for the production of key spoilage products acetoin and butanediol, which have been shown to be produced under aerobic conditions when glucose is metabolized (Dainty and Hibbard, 1983). Diacetyl production is also characteristic of spoilage caused by *B. thermosphacta* and it can be formed via the non-enzymatic oxidative decarboxylation of acetoin (Dainty and Hibbard,

1983). All 12 strains demonstrated acetoin production in a Voges-Proskauer test, further underlining the importance of these substances in spoilage caused by *Brochothrix*.

Previous analyses have shown conflicting results regarding the presence and activity of secreted lipases of *B. thermosphacta* (Papon and Talon, 1988, Nowak et al., 2012, Collins-Thompson et al., 1971). In both *Brochothrix* species, a lipase gene with significant homology to lipases in *Listeria* species as well as numerous esterase genes were identified. Hence, the gene inventory for the production of fatty acid end-products from lipolysis may be present in *Brochothrix*. It is also possible that lipid metabolism in the form of uncharacterised or poorly characterised lipid oxidation pathways may play a role in spoilage caused by this organism. However, there is strong evidence for the production of fatty acid moieties from amino acids (Dainty and Hofman, 1983, Dainty and Hibbard, 1983). In line with these observations, genes were present in both species for the conversion of branched chain amino acids leucine, valine and isoleucine to their branched chain acyl-CoA derivatives. These steps, which are common for all three amino acids, involve the conversion of the amino acids to their respective α-ketoacids and the subsequent conversion of the α-ketoacids to acyl-CoAs. In addition, *B. thermosphacta* strains may be able to produce propanoyl-CoA from distal leucine and valine degradation pathways. Thus, it is likely that together with the activity of acyl-CoA thioesterases present in both species, *Brochothrix* produce branched chain fatty acids isovaleric acid, isobutyric acid and 2-methylbutyric acid from leucine, valine and isoleucine degradation, while propionic acid production by *B. thermosphacta* may also be the result of valine and/or isoleucine metabolism.

The secretion of biogenic amines into the environment can contribute to spoilage due to their putrid aroma and can have serious health implications (Yoshida et al., 2004, Shalaby,

1996). Biogenic amines are primarily produced by the decarboxylation of amino acids via amino acid decarboxylase activity. A number of studies have demonstrated that different *B. thermosphacta* strains are capable of producing various biogenic amines such as histamine, tyramine, tryptamine, putrescine and cadaverine (Casaburi et al., 2014, Nowak and Czyzowska, 2011, Paleologos et al., 2004). Nonetheless, amino acid decarboxylase genes were not found in the genomes of the strains. Moreover, lysine-, arginine- and ornithine reactions were negative using API 20E strips and an amino acid decarboxylase screen also failed to detect decarboxylase activity of a wide range of target amino acid decarboxylases. The *B. thermosphacta* strains from this study and *B. campestris* DSM 4712^{T} may be reliant on the uptake of polyamines such as spermidine or putrescine, via the homologous *potA*, *B*, *C* and *D* genes, which enable spermidine-preferential uptake in *E. coli* (Igarashi and Kashiwagi, 1999).

Considering the similarity of the biochemical profiles of *B. thermosphacta* and *B. campestris*, it is not surprising that for the metabolic reactions analysed in this study the gene inventory of both organisms is so similar. The presence of all genes required for acetoin and butanediol formation also provides further evidence that *B. campestris* may be capable of spoilage.

Resistance genes

Although *B. thermosphacta* is not a pathogen, antibiotic resistance genes in spoilage bacteria increase the gene pool from which pathogens can acquire antibiotic resistance traits (Verraes et al., 2013). Previously, both *Brochothrix* species were described as being susceptible to a wide range of antibiotics (Stackebrandt and Jones, 2006). In this study, a tetracycline resistant isolate (7811) was identified and found to possess a *tetL* gene, which

encode transmembrane, tetracycline efflux pumps in known *tetL*-carrying organisms such as *B. subtilis* (Takayuki et al., 1985, Sakaguchi et al., 1988). This gene was located on a contig with an approximately 14-fold higher read coverage than the average read coverage of the genome, suggesting this contig may represent a high copy plasmid.

There is growing concern about bacterial cross-resistance to antibiotics, disinfectants and/or heavy metals in various environments (Chapman, 2003, Langsrud et al., 2003, Baker-Austin et al., 2006, Elhanafi et al., 2010). Until now, there has been no available data on the heavy metal tolerance of *B. thermosphacta* strains. The MTCs of the 12 isolates to the tested heavy metals were not at levels considered high enough to be resistant to these substances. However, growth, although impaired, was still observed at much higher concentrations of the metals and could be attributed to the presence of putative copper, cobalt and cadmium resistance genes identified in the draft genomes. Considering heavy metal resistant *Listeria* isolates are more common among food isolates than those recovered sporadically (Ratani et al., 2012), further studies on heavy metal resistance of *B. thermosphacta* and other spoilage-related organisms, which could also act as potential reservoirs for resistance determinants, may be warranted.

Presence of *Listeria* virulence genes in *Brochothrix*

Notable among the *Listeria* virulence orthologues detected in the *Brochothrix* genomes was the significant representation of factors involved in intracellular survival and surface protein anchoring, while the majority of factors with a function in adherence and invasion of mammalian host cells were not found. Homologs of virulence proteins involved in immune modulation were also missing, which, considering the non-pathogenic nature of this bacterium and specialized functions many of these proteins have, is not surprising.

Speculation can be made that some of the homologous virulence genes present in the *Brochothrix* genomes are required for the attachment to and growth on animal carcasses. For example, FbpA protein (fibronectin-binding protein) facilitates adhesion of *Listeria* to host fibronectin (Osanai et al., 2013), while OppA (Oligopeptide-binding protein) enables oligopeptide uptake with pleiotropic effects on growth at cold temperatures and PrsA2 (post-translocation secretion protein) contributes to the integrity of the cell wall and bacterial resistance to osmotic stress in *Listeria* (Borezee et al., 2000, Cahoon and Freitag, 2014). In addition, sortase B (SrtB) plays an indirect role in virulence in *L. monocytogenes* and haem/haemoglobin uptake by anchoring Hbp1 and Hbp2 to the peptidoglycan (Borezée et al., 2001, Xiao et al., 2011). Hbp2 promotes intracellular survival by facilitating bacterial escape from phagosomes in macrophages (Borezée et al., 2001) and while SrtB binds Hbp2 to the cell wall, a pool of Hbp2 is also secreted into the environment, where this protein operates as a haemophore (Xiao et al., 2011). Due to the conserved nature of the *srtB* locus and the finding of a Fur box upstream of this region in *Brochothrix*, it is likely that this locus is iron-regulated and may contribute to the uptake of haem, facilitating the use of the tightly bound iron present in animal carcasses.

The absence of both key *Listeria* virulence genes and conserved synteny for the majority of virulence gene orthologues identified in the *Brochothrix* genomes, highlights the degree of genetic divergence we detected between these organisms. A number of studies suggest that *Listeria* species have evolved from a pathogenic ancestor and that non-pathogenic *Listeria* members arose primarily via gene loss (den Bakker et al., 2010, Hain et al., 2006, Schmid et al., 2005). Hence, it is possible that *Brochothrix* species have evolved from a more recent non-pathogenic member of the *Listeria* genus, however, further phylogenetic studies would be required to answer this intriguing question.

Stress response genes

In *L. monocytogenes*, σ^B plays a crucial role in the growth and survival at cold temperatures, low pH, in oxidative and osmotic stress and carbon starvation (Becker et al., 1998, Becker et al., 2000, Ferreira et al., 2001, Wemekamp-Kamphuis et al., 2004). It is also involved in regulating a number of genes of virulence determinants including genes from the internalin family (Wiedmann et al., 1998). It is tempting to speculate that σ^B and σ^B-related genes may regulate gene expression in *B. thermosphacta*, facilitating survival and growth under high salt conditions and the adaptation to refrigeration temperatures. The practical importance of this key regulatory factor for the food industry has been previously recognised, as the activation of σ^B results in increased stress resistance of the bacterial strains (Wemekamp-Kamphuis et al., 2004). A better fundamental knowledge of the σ^B-activation process and σ^B-regulon will be crucial for the effective design of food processing steps to ensure the stress response is not activated, as its activation would have the consequence of enhanced resistance of the bacteria to inactivation and food preservation treatments.

Conclusion

This study provides a detailed analysis of the *B. thermosphacta* genome, identifying spoilage-relevant pathways and establishing parallels and differences to the closely related foodborne pathogen *L. monocytogenes*. New insights into the gene inventory of *Brochothrix* are provided and genes involved in meat spoilage pathways, which have been previously described in the literature, are discussed. The limited genetic diversity of the isolates in this study may reflect their niche specificity and therefore as more strains of *B. thermosphacta* are sequenced, it will be interesting to see if this is true of the species as a whole.

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Chapter 3

Characterisation of a putative *Brochothrix thermosphacta* sortase A enzyme

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ABSTRACT

Gram-positive bacteria utilise class A sortases to coat the surface of their cells with a diversity of proteins that facilitate interactions with their environment and play fundamental roles in cell physiology and virulence. A putative sortase A gene was identified in the genome of the poorly studied meat spoilage bacterium *Brochothrix thermosphacta*. To understand how this bacterium mediates interactions with its environment, an N-terminal truncated, His-tagged variant of this protein (His_6-BtSrtA) was expressed and purified in a two-step purification process. Catalytic activity of recombinant His_6-BtSrtA was investigated, including sorting motif recognition of sortase substrate proteins and bioconjugation activity. Furthermore, the *B. thermosphacta* genome was examined for the presence of sortase A (SrtA) protein substrates. Recombinant His_6-BtSrtA recognised and cleaved LPXTG sorting motifs and attached SrtA pseudo-substrates to rhodamine-labelled tri-glycine, demonstrating *in vitro* SrtA bioconjugation activity. Genome examination identified 11 potential SrtA substrates, two of which contained protein domains associated with adherence of pathogens to host extracellular matrix proteins and cells, suggesting the

B. thermosphacta SrtA may be indirectly involved in its attachment to meat surfaces. Thus, further work in this area could provide crucial insight into molecular mechanisms involved in the colonisation of meat by *B. thermosphacta*.

INTRODUCTION

Proteins present on the cell surface of microorganisms facilitate interaction of the microbes with their environment, and play fundamental roles in microbial physiology and virulence. Gram-positive bacteria employ sortase enzymes to coat the surface of their cells with proteins (Spirig et al., 2011). These cysteine transpeptidases have two distinct, but mechanistically related functions: 1) to directly attach proteins to the cell wall 2) to assemble pili (Jacobitz et al., 2017).

Based on primary sequence, most sortase homologs can be assigned to one of six classes (A-F) (Spirig et al., 2011). Sortase A (SrtA) proteins are considered to be versatile, capable of attaching relatively large numbers of functionally distinct proteins to the cell wall, which has led to the proposal that these enzymes fulfil a housekeeping role in the cell (Comfort and Clubb, 2004). The *Staphylococcus aureus* SrtA (SaSrtA) is the archetypal SrtA protein, responsible for the attachment of key virulence factors to the cell surface of this bacterium including microbial surface components recognising adhesive matrix molecules (MSCRAMMs) such as staphylococcal protein A (SpA), clumping factors A and B (ClfA and ClfB), fibronectin-binding proteins A and B (FnBPA and FnBPB) and collagen adhesin CNA (Schneewind et al., 1992, Mazmanian et al., 2000, Ghasemian et al., 2015, Kang et al., 2013). SaSrtA recognises substrate proteins with the five amino acid sorting motif Leu-Pro-X-Thr-Gly (LPXTG), where X is any amino acid (Kruger et al., 2004, Ton-That et al., 1999,

Mazmanian et al., 1999). Sorting motifs of sortase substrates are located at the C-terminal end of the proteins and are followed by a hydrophobic stretch and a positively charged cytoplasmic anchor to ensure these cell surface proteins are retained in the membrane (Schneewind et al., 1992).

The transpeptidation reaction of the SaSrtA can be seen as a two-step process (Ton-That et al., 1999, Jacobitz et al., 2017). In step one, SaSrtA cleaves the Thr-Gly bond of the LPXTG motif, resulting in the formation of a semi-stable thioacyl intermediate, whereby the SaSrtA is covalently attached to its protein substrate via its cysteine residue (Clancy et al., 2010). The second half of the transpeptidation reaction involves the recognition of the cell wall precursor, lipid II (Perry et al., 2002). Oligo-glycine of lipid II functions as a nucleophile to dissociate the thioacyl intermediate and promote SaSrtA-catalysed linkage between the N-terminal primary amino group of oligo-glycine and the Thr carboxyl group of the substrate (Perry et al., 2002). The resulting protein-lipid II product, in which the components are joined by a peptide bond, is then incorporated into the peptidoglycan via cell wall synthesising transpeptidation and transglycosylation reactions (Jacobitz et al., 2017). The chemistry of sortase-mediated ligation has been exploited for a range of biotechnological applications including the attachment of fluorophores to drugs or antibodies, the immobilisation of peptides to solid surfaces and the labelling of cell surface proteins on living cells (Antos et al., 2016, Ritzefeld, 2014).

Class B, C, D, E and F sortases are associated with more specialised functions such as iron acquisition (class B) (Maresso et al., 2006, Maresso and Schneewind, 2006, Mazmanian et al., 2002, Mariscotti et al., 2009, Bierne et al., 2004), pilus growth (class C) (Wu et al., 2012, Cozzi et al., 2012, Naziga and Wereszczynski, 2017) and sporulation (class D) (Marraffini and

Schneewind, 2006, Marraffini and Schneewind, 2007, Suryadinata et al., 2015). Differences between the different classes of enzymes include the sorting motifs that they cleave and nucleophile substrate specificity. For example, sortase B enzymes recognise an NP(Q/K)(T/S)(N/G/S)(D/A) sequence, which differs notably from the canonical LPXTG motif (Spirig et al., 2011). Class A and D sortases of *Bacillus anthracis* recognise the closely related motifs LP[A/N/K]TG and LPNTA, respectively, although interestingly the enzymes function non-redundantly (Marraffini and Schneewind, 2006, Spirig et al., 2011). Comparative genome analyses also indicate that class E enzymes target LAXTG signals (Comfort and Clubb, 2004), while class C and F sortases of *Clostridium diphtheriae* target the sorting motifs LPLTG and LAFTG, respectively (Spirig et al., 2011). Common features of all characterised sortases to date include the presence of a His-Cys-Arg catalytic triad and the ability to catalyse a transpeptidation reaction, resulting in the attachment of cell surface proteins carrying C-terminal sorting signals to an amino nucleophile (Jacobitz et al., 2017).

In a previous study (Stanborough et al., 2017a), a sortase gene predicted to encode a class A sortase was identified in the genome of the poorly studied, psychrotrophic, Gram-positive, meat spoilage bacterium *B. thermosphacta*. Knowledge of the housekeeping sortase of *B. thermosphacta* and its substrate proteins provides a starting point for understanding how this bacterium interacts with its environment. Therefore the aims of this study were to generate recombinant *B. thermosphacta* SrtA (BtSrtA), investigate the *in vitro* catalytic activity of this enzyme and to identify potential SrtA substrate proteins in the *B. thermosphacta* genome.

MATERIALS AND METHODS

Multiple sequence alignment of SrtA proteins

MAFFT multiple sequence alignment of SrtA sequences from *S. aureus* (GenBank accession number: WP_000759367), *Listeria monocytogenes* (GenBank accession number: WP_003722751), and *B. thermosphacta* Bth-7807 (GenBank accession number: ODJ55388.1) were performed in Geneious (Kearse et al., 2012) using MAFFT version 7.308 (Katoh and Standley, 2013) and the default settings.

Cloning, expression and purification of BtSrtA construct

DNA manipulations were performed using standard protocols (Sambrook and Russell, 2001). An N-terminal truncated *srtA* construct was generated by PCR amplification of the *B. thermosphacta* Bth-7807 *srtA* (locus tag: BFR41_06585 in GenBank assembly: GCA_001715635.1) starting from codon 31 (Figure 3.1) using the forward primer BtNdeISrtA_31fwd (5'-aaaaaCATATGGCAAATACCAGCAAGGTTG-3') and reverse primer BtBamHISrtArev (5'- aaaaaGGATCCTTATTTGTGCTTGGTAACTT-3'). An in-house pET43 expression vector (Amp^R) variant was used that was modified between the NdeI and XhoI restriction sites to contain from 5' to 3' a His_6-tag, a KpnI restriction site, a TEV-cleavage site, BamHI and NheI restriction sites and a stop codon. The *srtA* gene fragment was cloned into the vector using the BamHI and NheI sites to generate an N-terminally His_6-tagged recombinant protein (His_6-BtSrtA).

The expression of His_6-BtSrtA was performed in a BL21 (DE3) Rosetta *Escherichia coli* strain. Cultures were grown for 18 h +/- 2 h in 2YT media (50 µg/mL ampicillin and 2% glucose (w/v)) at 37 °C, diluted 1/100 in fresh 2YT media (50 µg/mL ampicillin and 2% glucose (w/v)) and cultured at 37 °C and 200 rpm until OD_{600} 0.4-0.5. Cultures were then shifted to 16 °C

and expression of recombinant protein was induced with 1 mM isopropyl-ß-D-1-thiogalactopyranoside for 20 h. Cells were harvested and resuspended in lysis buffer (50 mM HEPES pH 8, 300 mM NaCl, 2 mM $MgCl_2$, 0.0025 units/µL Benzonase (Novagen), 100 µg/mL lysozyme and protease-inhibitor cocktail (Roche)). Lysates were subjected to two freeze and thaw cycles (on dry ice and in a 22 °C water bath) followed by sonication on ice. Cell debris and insoluble proteins were removed by centrifugation and clarified lysate was run over a 5 mL His-Trap FF IMAC column (GE). Following extensive washes with Buffer A (50 mM Tris/HCl pH 8, 150 mM NaCl and 15 mM Imidazole), recombinant His_6-BtSrtA was eluted in a Buffer B (50 mM Tris/HCl pH 8, 150 mM NaCl, 250 mM Imidazole). The eluate was further purified on a Superdex 75 16/60 gel-filtration chromatography column (GE), resulting in the recovery of recombinant His_6-BtSrtA in 50 mM Tris/HCl and 150 mM NaCl. Aliquots of protein were kept at -80 °C.

***In vitro* thioacyl intermediate reactions**

Reaction mixes contained 12 µM His_6-BtSrtA and 4 µM of a peptide comprising the first 16 amino acids of the amyloid-β ($Aβ_{1-16}$) peptide fused at the C-terminus to a variety of sortase signal motifs (LPETG, LPQTGS, LPNTGS, LAETG). Reactions were performed in a HEPES buffer (50 mM HEPES pH 8, 150 mM NaCl and 10 mM β-Mercaptoethanol) at 22 °C and components were incubated for 5 h. Reaction products were separated by SDS-PAGE using 4-12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes for Western Blot analysis of thioacyl intermediates with an antibody against Aβ (WO2). Equal loading was determined using an anti-His_5 antibody (Qiagen).

In vitro bioconjugation

Bioconjugation reactions were performed as previously described (Baer et al., 2014) with some adjustments. Briefly, the reaction buffer consisted of 50 mM HEPES pH 8, 150 mM NaCl and 10 mM β-Mercaptoethanol. Reaction mixes contained 60 µM His_6-BtSrtA, 20 µM of substrate protein and 2 mM of rhodamine-labelled nucleophile. Two recombinant substrate proteins were used: 1) the Im7 immunity protein with the LPETGG peptide attached at its C-terminus followed by a His_6-tag. 2) 1E8 scFv fusion protein (variable heavy and variable light antibody chains against the amyloid-β protein joined by a (Gly4Ser)3 linker attached at its C-terminus to the LPETGG peptide. These substrate proteins and the rhodamine-labelled nucleophiles (GGG-EDA-TAMRA, cadaverine-TAMRA, lysine-EDA-TAMRA and EDA-TAMRA) were provided by CSIRO Manufacturing, and manufactured as previously described (Baer et al., 2014, Juraja et al., 2006, Nisbet et al., 2013). Reactions were incubated at 22 °C for 5 h and analysed by SDS-PAGE and Coomassie staining on 4-12% Bis-Tris gels (Invitrogen). Following subsequent removal of Coomassie dye from the gels, rhodamine fluorescence was measured at 605 nm with a VersaDoc MP Imaging System (Bio-Rad).

Genome mining for and characterization of BtSrtA substrate proteins

Genome mining for BtSrtA substrate proteins was performed with the CW-PRED tool (Fimereli et al., 2012) available on http://bioinformatics.biol.uoa.gr/CW-PRED/. MAFFT protein alignment with the default settings was used to determine percent identity between the substrate candidate orthologues in *B. thermosphacta* Bth-7807 and *B. thermosphacta* DSM 20171^T. InterproScan analysis of substrate candidates was applied for protein classification and to inform on predicted protein function.

RESULTS

The *srtA* gene of the meat spoilage bacterium *B. thermosphacta* consisted of 636 nucleotides encoding a 211 amino acid long protein with a calculated molecular weight of 23.4 kDa. Figure 3.1 shows a multiple sequence alignment of the BtSrtA with SrtA proteins from other Gram-positive firmicutes, *Listeria monocytogenes* and *S. aureus*. The alignment identified His127, Cys189 and Arg197 as the active site residues in the BtSrtA and the TMHMM Server (version 2.0) predicted amino acids 6-25 to form the transmembrane domain. The BtSrtA shared 47% sequence identity with the SrtA sequence of *L. monocytogenes*, as determined by MAFFT protein alignment, while sequence identity between the BtSrtA and SaSrtA was substantially lower (26%).

Figure 3.1: Multiple sequence alignment of SrtA proteins. Shown is a MAFFT multiple sequence alignment of SrtA proteins from *S. aureus*, *L. monocytogenes* and *B. thermosphacta*. Amino acids 6-25 of the BtSrtA, predicted to form transmembrane helices, are underlined in red. To highlight the residues of the N-terminal truncated construct used for recombinant expression (His_6-BtSrtA), amino acids 31-211 of the BtSrtA are marked below with a black line. Active site residues are highlighted in orange and the conserved TLXTC motif of sortases is marked with a red box.

For biochemical characterisation of BtSrtA, an N-terminal His-tagged and truncated variant of the enzyme, missing the transmembrane domain, was expressed (His_6-BtSrtA) (Figure 3.1). This truncated variant showed good solubility as demonstrated by SDS-PAGE and Western blot analysis of the clarified lysate (Figure 3.2A) and was purified using a two-step purification process (Figure 3.2A and 3.2B). The protein was readily concentrated to ~20 mg/mL, a yield of ~40 mg/L of recombinant His_6-BtSrtA from 500 mL of culture.

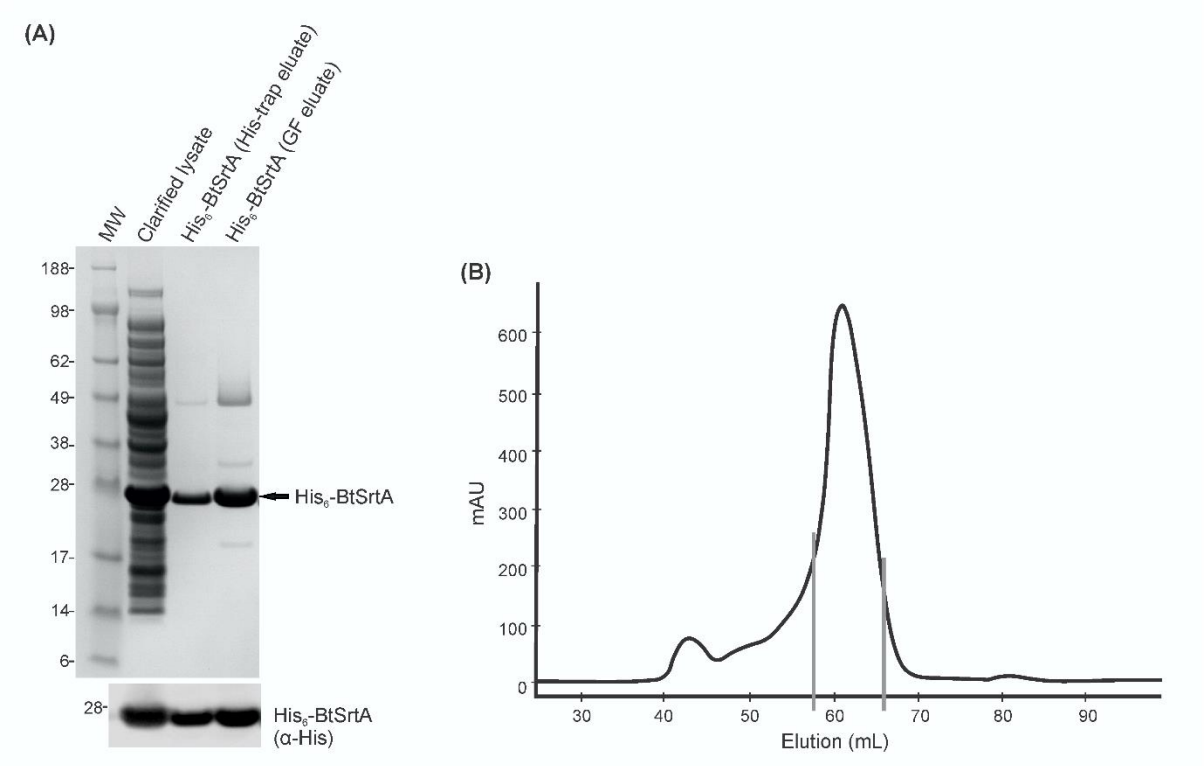

Figure 3.2: Expression and purification of recombinant His_6-BtSrtA. (A) SDS-PAGE (top panel) and Western blot analysis (bottom panel) of purification steps of His_6-BtSrtA. A faint dimeric species is visible at approximately 50 kDa, consistent with other purified recombinant SrtA proteins (Baer et al., 2014). Lane MW: protein marker. **(B)** Purification of His_6-BtSrtA on a Superdex 75 16/60 gel-filtration chromatography column. The grey vertical lines indicate the fractions that were collected and analysed in (A) in lane four.

To test catalytic activity and sorting motif recognition of His_6-BtSrtA, the recombinant enzyme was incubated with substrates consisting of the first 16 amino acids of the amyloid-β ($Aβ_{1-16}$) peptide fused at its C-terminus with the sorting signals LPETG, LPQTGS, LPNTGS and LAETG. His_6-BtSrtA-$Aβ_{1-16}$ thioacyl intermediates were detected by Western blot using an anti-Aβ antibody (Figure 3.3). Results of these experiments showed His_6-BtSrtA formed thioacyl intermediates with substrates containing the sorting signals LPETG, LPQTGS and LPNTGS, while catalytic activity of His_6-BtSrtA towards $Aβ_{1-16}$-LAETG was not detected.

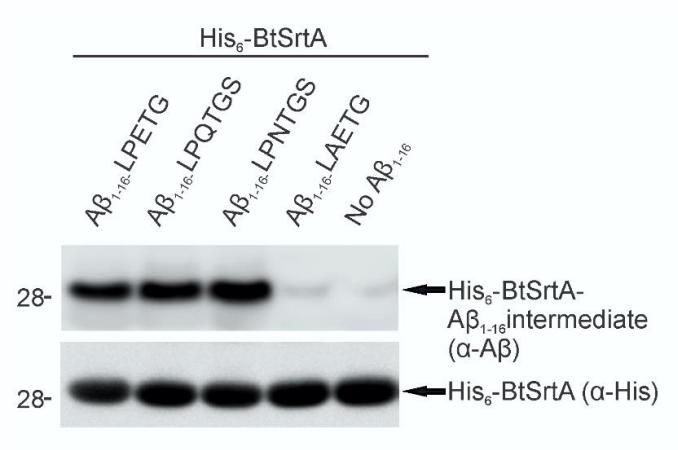

Figure 3.3: His_6-BtSrtA recognises and cleaves LPXTG sorting motifs. Western blot analysis of recombinant His_6-BtSrtA incubated in the absence (lane five) or presence of $Aβ_{1-16}$-peptides fused to LPETG (lane one), LPQTGS (lane two) LPNTGS (lane three) or LAETG (lane four) sorting signals. The Western blot was probed with an anti-Aβ antibody (top panel). Equal loading was determined with an anti-His antibody (bottom panel).

In vitro bioconjugation activity of His_6-BtSrtA was investigated with a previously described model system (Baer et al., 2014), utilising recombinant Im7 protein fused at its C-terminus with the LPETGG peptide followed by a His-tag (11.6 kDa), as a SrtA pseudo-substrate, and rhodamine-labelled tri-glycine (GGG-EDA-TAMRA) to mimic oligo-glycine in the cross-bridge

of lipid II. Successful attachment of the Im7 protein to rhodamine was detected by rhodamine fluorescence at 605 nm. Incubation of His_6-BtSrtA and Im7-LPETGG alone, resulted in a faint band visible at ~34 kDa (Figure 3.4), representing His_6-BtSrtA-Im7-LPET intermediates. More importantly, incubation of His_6-BtSrtA and Im7-LPETGG in the presence of GGG-EDA-TAMRA, resulted in dissociation of the His_6-BtSrtA-Im7-LPET intermediates and formation of the rhodamine-labelled Im7 end-products (Im7-LPET-TAMRA), as indicated by the fluorescent band in the bottom panel.

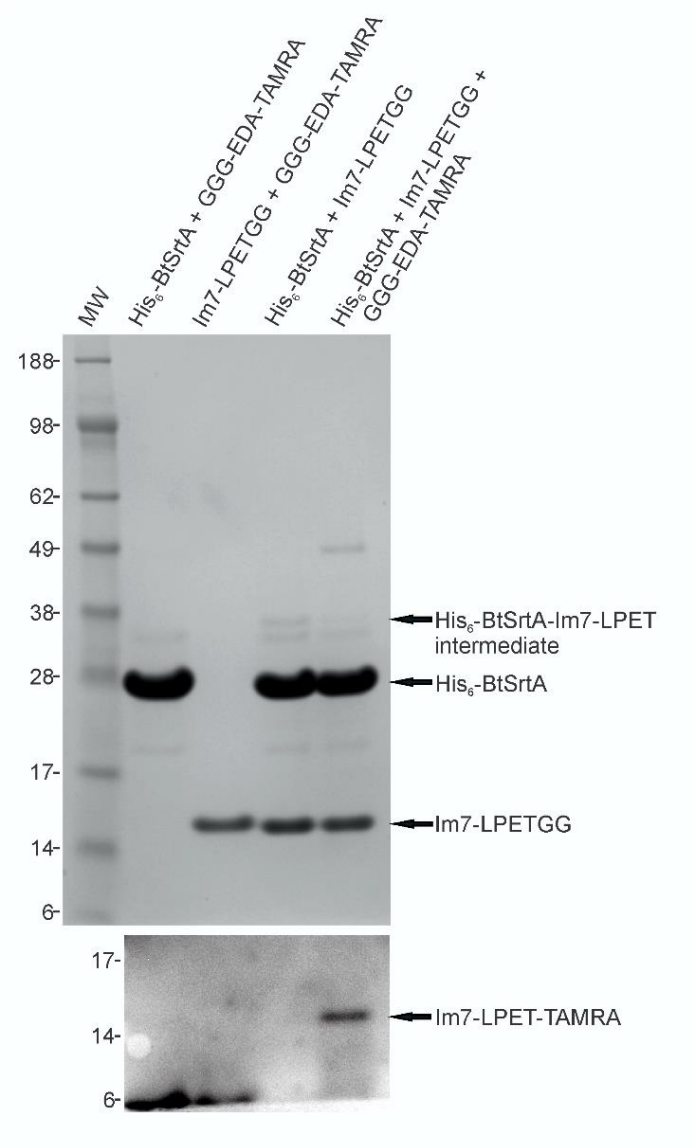

Figure 3.4: His_6-BtSrtA shows *in vitro* bioconjugation activity. His_6-BtSrtA was tested for its ability to catalyse conjugation between Im7-LPETGG and rhodamine-labelled tri-glycine (GGG-EDA-TAMRA). Coomassie stained SDS-PAGE analysis (top panel) shows incubation of His_6-BtSrtA and Im7-LPETGG in the absence of GGG-EDA-TAMRA resulted in the formation of His_6-BtSrtA-Im7-LPETG intermediates (lane four), which are decreased when GGG-EDA-TAMRA is added to the reaction (lane five). Together with this reduction of intermediate products, a fluorescent band, indicating Im7-LPET-TAMRA end-products (lane five in bottom panel, measured at 605 nm), was detected when all three reaction components were present. Lane MW: protein marker.

To demonstrate that bioconjugation activity was not limited to the Im7 system, His_6-BtSrtA was incubated with another C-terminal LPETGG-tagged acceptor protein, the 1E8 scFv (~30 kDa), and GGG-EDA-TAMRA. In addition, conjugation activity was tested with the alternative nucleophiles cadaverine, lysine and EDA. As seen with the Im7 system, formation of His_6-BtSrtA-1E8 scFv-LPET intermediates was observed in the absence of GGG-EDA-TAMRA (Figure 3.5). Addition of the nucleophile GGG-EDA-TAMRA to the reaction mix, resulted in the detection of fluorescent 1E8 scFv-LPET-TAMRA end-products and a concomitant reduction in His_6-BtSrtA-1E8 scFv-LPET intermediates. Although notably weaker, fluorescent bands were also observed for reactions containing cadaverine-TAMRA, lysine-EDA-TAMRA and EDA-TAMRA, indicating that these alternative nucleophiles also facilitated His_6-BtSrtA-mediated bioconjugation.

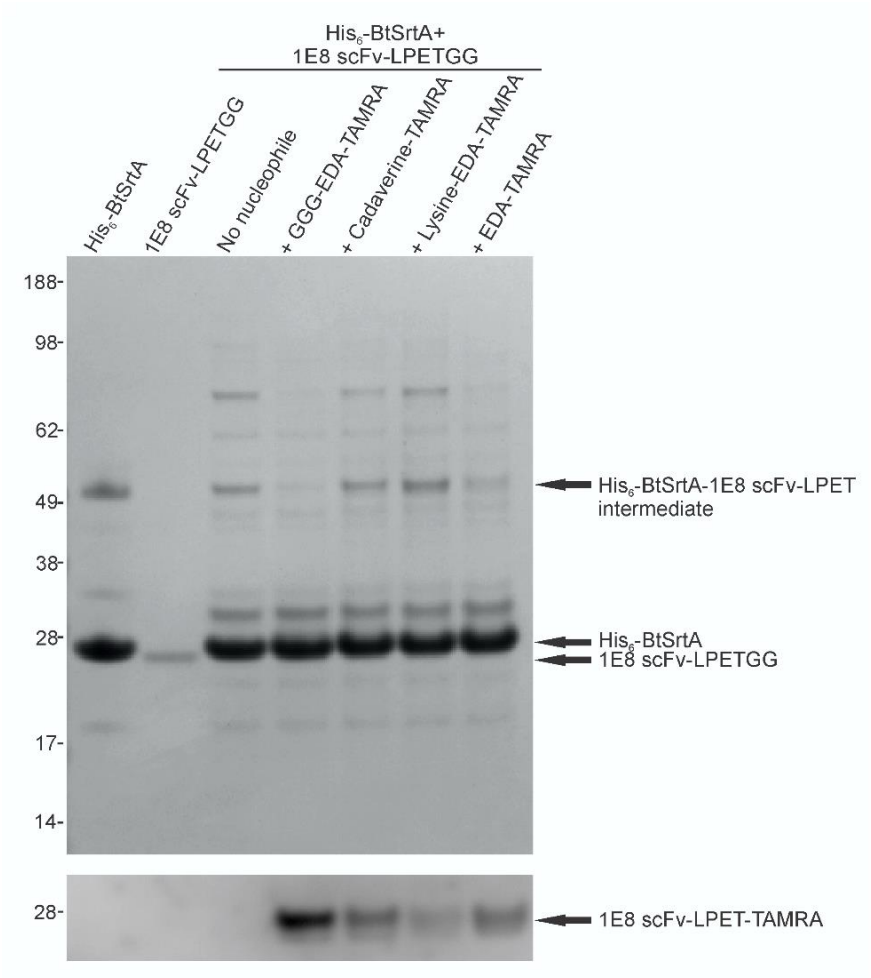

Figure 3.5: His_6-BtSrtA shows *in vitro* bioconjugation activity with 1E8 scFv-LPETGG and alternative nucleophiles. Bioconjugation activity of His_6-BtSrtA was tested with the 1E8 scFv-LPETGG pseudo-substrate and rhodamine-labelled tri-glycine (GGG-EDA-TAMRA), as well as with the alternative nucleophiles, cadaverine-TAMRA, lysine-EDA-TAMRA and EDA-TAMRA. Coomassie stained SDS-PAGE analysis (top panel) shows incubation of His_6-BtSrtA and 1E8 scFv-LPETGG in the absence of GGG-EDA-TAMRA resulted in the formation of His_6-BtSrtA-1E8 scFv-LPETG intermediates (lane three), which are decreased when GGG-EDA-TAMRA is added to the reaction (lane four). A fluorescent band indicating 1E8 scFv-LPET-TAMRA endproducts (lane four in bottom panel, measured at 605 nm), was detected when all three reaction components were present. Fluorescent bands were also detected when His_6-BtSrtA and 1E8 scFv-LPETGG were incubated together with cadaverine-TAMRA (lane five), lysine-EDA-TAMRA (lane six) and EDA-TAMRA (lane seven).

To determine potential BtSrtA substrates, the genome of *B. thermosphacta* Bth-7807 was mined for the presence of substrate proteins using a hidden Markov model-based tool for accurate prediction of LPXTG and LPXTG-like cell wall proteins of Gram-positive organisms (Fimereli et al., 2012). Results of this search revealed 11 SrtA substrate candidates, of which the respective GenBank accession numbers and sorting motifs are included in Table 3.1. The consensus sorting motif for the 11 proteins was LPXTG with this sequence of amino acids followed by a glutamic acid in six, an aspartic acid in four and a valine in one of the proteins. To investigate whether these substrate candidates are conserved among different strains of this organism, the genome of the geographically and temporally diverse type strain, *B. thermosphacta* DSM 20171^{T}, was searched for orthologues of the 11 proteins. Not only did this search show that the coding sequences of the 11 candidates were conserved in the type strain genome with protein sequences sharing between 70% and 100% identity based on MAFFT protein alignment, but 10 of the 11 sorting motifs of these proteins were identical to those of the Bth-7807 orthologues. The *B. thermosphacta* DSM 20171^{T} orthologue with the GenBank accession number ODJ47926.1 was an exception, with the sorting motif of this protein differing by one residue (LPKTGE). As the majority of these proteins were annotated as hypothetical proteins, InterProScan analysis was performed to determine whether protein family assignment and domains could be predicted that were missed in the automated annotation (Table 3.1).

Table 3.1: SrtA substrate candidates identified in *B. thermosphacta*.

Substrate candidates identified in Bth-7807[a]	Sorting motif[b]	Protein classification	Orthologues identified in DSM 20171[Tc]	Identity of orthologues (%)
ODJ52574.1	LPHTGE	Two adhesion domains including one fibrinogen binding domain (Go-term: cell adhesion)	ODJ50812.1	76
ODJ52759.1	LPSTGE	Terpenoid cyclase/prenyltransferase	ODJ51174.1	100
ODJ52782.1	LPQTGE	None predicted	ODJ51150.1	100
ODJ54194.1	LPVTGE	None predicted	ODJ48938.1	88
ODJ55230.1	LPKTGD	WD40/YVTN repeat-like domain	ODJ48437.1	85
ODJ55992.1	LPTTGD	None predicted	ODJ51226.1	70
ODJ56202.1	LPTTGD	Six adhesion domains including one fibrinogen and four collagen binding domains/12 immunoglobulin-like folds (Go-term: cell adhesion)	ODJ49110.1, ODJ49111.1, ODJ49112.1[d]	96 97 81
ODJ56210.1	LPETGE	Leucine-rich repeat domain	ODJ47926.1	96
ODJ57179.1	LPHTGD	ParB/Sulfiredoxin-like domain	ODJ50440.1	85
ODJ57328.1	LPKTGV	None predicted	ODJ48146.1	99
ODJ57540.1	LPKTGE	Five MucBP domains	ODJ47926.1	98

[a] Provided are the GenBank accession numbers of the identified proteins from *B. thermosphacta* Bth-7807 (GenBank accession number of genome assembly: GCA_001715635.1).

[b] Sorting motifs of the substrate candidates of *B. thermosphacta* Bth-7807.

[c] Provided are the GenBank accession numbers of the orthologues identified in *B. thermosphacta* DSM 20171^T (GenBank accession number of genome assembly: GCA_001715655.1).

[d] Blastp analyses showed that while the substrate protein with the GenBank accession number ODJ56202.1 in Bth-7807 is a large protein consisting of 2395 amino acids, and this sequence is largely conserved in DSM 20171^T, stop codons present in this genomic region in DSM 20171^T result in three coding sequences.

Functional domains for four of the 11 proteins were not found. Two proteins that were assigned the Go-term “cell adhesion” for biological processes contained multiple N-terminal

located domains belonging to the adhesion domain superfamily, including a fibrinogen binding domain in each, and four predicted collagen binding domains in one of these proteins (GenBank accession number: ODJ56202.1). This protein also comprised 12 C-terminal immunoglobulin-like folds. Other substrate candidates included a terpenoid cyclase/prenyltransferase domain protein, a WD/40YVTN repeat-like domain protein, a leucine-rich repeat domain protein, a ParB/Sulfiredoxin-like domain protein and a MucBP (MUCin binding protein) domain protein comprising five MucBP domains.

DISCUSSION

The BtSrtA shares only moderate sequence homology with the SrtA of the closely related food-borne pathogen *L. monocytogenes* and notably less sequence homology with the archetypal SrtA from *S. aureus*. Despite substantial primary sequence divergence, this work shows that the BtSrtA recognises and cleaves LPXTG motifs, which are sorting motifs targeted by class A sortases. In contrast, a non-canonical LAETG motif, targeted by class E sortases of Actinobacteria (Kattke et al., 2016), was not cleaved by the BtSrtA enzyme. Together with the observed *in vitro* thioacyl intermediate activity, analysis of the sorting motifs present in the 11 identified SrtA substrate candidates suggests that the BtSrtA targets substrate proteins with the LPXTG motif, which in most cases is followed by a conserved glutamic or aspartic acid residue.

Utilising a model system that was previously tested on SaSrtA (Baer et al., 2014), the BtSrtA demonstrated *in vitro* bioconjugation activity with two different SrtA pseudo-substrates and a variety of nucleophiles, although rhodamine-labelled tri-glycine was most effective. These *in vitro* results indicate that like other SrtA enzymes of Gram-positive bacteria, this enzyme

could be capable of covalently linking substrate proteins to the cross-bridge of lipid II. As genome examination identified 11 potential SrtA substrates that were conserved between strain Bth-7807 and the geographically and temporally diverse type strain of this bacterium, BtSrtA may also fulfil a housekeeping function, attaching multiple, functionally distinct proteins to the cell wall.

Domain analysis of the substrate proteins revealed two proteins with multiple adhesion domains including predicted fibrinogen and collagen binding domains. SaSrtA attaches a variety of adhesion domain containing virulence proteins (MSCRAMMs) to the cell wall, which are involved in the adherence to host extracellular matrix proteins and cells (Madani et al., 2017, Tsompanidou et al., 2012, Roche et al., 2004). These include proteins with fibrinogen, fibronectin and collagen binding domains such as ClfA, ClfB, FnBPA, FnBPB and CNA (Piroth et al., 2008, Deivanayagam et al., 1999, Jemima Beulin and Ponnuraj, 2017). As a non-pathogenic, meat spoilage bacterium, the two adhesion domain containing substrate proteins of *B. thermosphacta* could be involved in the attachment and colonisation of this bacterium to meat carcasses. Indeed, similar to the domain organisation of CNA, one of the candidates contained N-terminal collagen binding domains and C-terminal repetitive immunoglobulin-like folds (Jemima Beulin and Ponnuraj, 2017). A MucBP domain protein was also among the identified sortase substrate proteins. Interestingly, cell-surface MucBP-domain proteins are currently known to be present in *Listeria* and Lactobacillilaes species only, and these proteins are assumed to function as adhesins binding to mucus material, although few studies have been conducted to determine their genuine role (Nishiyama et al., 2016, Bierne and Cossart, 2007). Although it is tempting to speculate that the MucBP domain protein of *B. thermosphacta* may be involved in adherence of this bacterium to

animal mucus layers such as the gastrointestinal tract, *B. thermosphacta* is a psychrotrophic bacterium and clear evidence does not exist that it inhabits these niches.

Among the identified BtSrtA substrates were also three factors with domains that are found in a variety of different proteins and often implicated in protein-protein interactions (WD40 repeat-like domain, leucine-rich repeat domain and immunoglobulin-like fold), a terpenoid cyclase/prenyl transferase substrate protein, which are enzymes involved in the synthesis of terpenes, terpenoids and sterols, and a ParB/Sulfiredoxin-like domain containing protein, which constitute a largely uncharacterised family of proteins.

Enzymes from psychrotrophic and psychrophilic microorganisms may offer novel opportunities for biotechnological exploitation due to their high catalytic activity at low temperatures, low thermostability and other unusual specificities (Feller, 2013). Therefore, in addition to understanding more about the fundamental biology of a key meat spoilage bacterium, the continued study of the BtSrtA may prove fruitful for future biotechnological applications.

Together, the results of this study show that *B. thermosphacta* encodes a SrtA enzyme, which targets substrate proteins that contain C-terminal LPXTG sorting motifs. Protein homology with other SrtA proteins coupled with *in vitro* bioconjugation activity, suggests the enzyme is capable of attaching these substrates to the cross-bridge of lipid II. Genome examination identified 11 potential SrtA substrates, two of which contained protein domains associated with the adherence of pathogens to host extracellular matrix proteins and cells, suggesting the *B. thermosphacta* SrtA may be indirectly involved in its attachment to meat surfaces. Thus, further work in this area may lead to an understanding of molecular mechanisms involved in the colonisation of meat by *B. thermosphacta*.

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Chapter 4

Genomic and metabolic characterisation of spoilage-associated *Pseudomonas* species

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ABSTRACT

Pseudomonas are common spoilage agents of aerobically stored fresh foods. Their ability to cause spoilage is species- and may be strain-specific. To improve our understanding of the meat and milk spoilage agents *Pseudomonas fragi* and *Pseudomonas lundensis*, we sequenced the genomes of 12 *P. fragi* and seven *P. lundensis* isolates. These genomes provided a dataset for genomic analyses. Key volatile organic compounds (VOCs) produced or metabolised by the isolates were determined during their growth on a beef paste and where possible, metabolic activity was associated with gene repertoire.

Genome analyses showed that the isolates included in this work may belong to more than two *Pseudomonas* species with possible spoilage potential. Pan-genome analyses demonstrated a high degree of diversity among the *P. fragi* and genetic flexibility and diversity may be traits of both species. Growth of the *P. lundensis* isolates was characterised by the production of large amounts of 1-undecene, 5-methyl-2-hexanone and methyl-2-butenoic acid. *P. fragi* isolates produced extensive amounts of methyl and ethyl acetate and

the production of methyl esters predominated over ethyl esters. Some of the *P. fragi* produced extremely low levels of VOCs, highlighting the importance of strain-specific studies in food matrices.

INTRODUCTION

Psychrotrophic *Pseudomonas* play a major role in limiting the shelf-life of fresh foods stored aerobically at cold temperatures (Remenant et al., 2015). Members of the *Pseudomonas fragi* subgroup, *P. fragi* and *P. lundensis* (Gomila et al., 2015), are recognised as common agents contributing to the spoilage of fresh meat and fish, and pasteurised and ultra-high-temperature-treated milk (products) via the activity of heat-stable extracellular proteases and lipases (Ercolini et al., 2007, Quigley et al., 2013, Tryfinopoulou et al., 2002, De Jonghe et al., 2011). In the case of meat, their growth and utilisation of food nutrients such as sugars and amino acids, and their release of both volatile and non-volatile metabolites such as esters, ketones, alcohols, aldehydes organic acids, sulphur compounds and amines, cause organoleptic changes to the meat resulting in spoilage (Ercolini et al., 2006, Samelis, 2006, Montel et al., 1998).

P. fragi are associated with fruity off-odours due to the production of esters, in particular short chain ethyl esters (Casaburi et al., 2015, Morales et al., 2005a). However, a number of studies indicate that ester production of this bacterium is strain-dependent (Morales et al., 2005a, Edwards et al., 1987). Other volatile compounds commonly associated with *P. fragi* include ketones, alcohols, 1-undecene and sulphur compounds (Wang et al., 2017b, Ercolini et al., 2010). The production of volatile sulphurous compounds with putrid aromas is

characteristic for *Pseudomonas* and occurs when glucose in meat is depleted and the amino acid pool is attacked (Edwards et al., 1987).

Volatile analyses of pure cultures of *P. lundensis* are rare and thus little is known about the contribution of this species to off-odours in meat spoilage. One study, which looked at the volatile profile of a single strain of this species in cheese, showed that it produced significant quantities of ketones, but that the production of alcohols, butanoic acid and the hydrocarbons 1-undecene and cyclohexane were also noteworthy (Morales et al., 2005c).

Despite their biological and economic importance, the genomes of *P. fragi* and *P. lundensis* remain largely unstudied. Furthermore, thorough understanding of their metabolic potential at the strain level is missing, in particular for *P. lundensis*.

In this study, we sequenced the genomes of 17 red meat- and two raw milk-derived *P. fragi* and *P. lundensis* isolates. To understand intra-species genetic diversity and provide insight into the genomes, average nucleotide identity, phylogenetic and pan-genome analyses were conducted. To investigate the heterogeneity of the bacteria in relation to their meat spoilage potential, the main volatile organic compounds (VOCs) produced or metabolised by the isolates during growth on a beef paste were assessed, and where possible, metabolic activity was linked to gene repertoire.

MATERIALS AND METHODS

Bacterial isolates

Table 4.1 lists the isolates and type strains used in this study and provides their source information.

Bacteria were isolated from meat sources by aseptically removing 25 g pieces and placing them into sterile stomacher bags with 300 mL of maximum recovery diluent (MRD, Oxoid, Basingstoke, UK). The samples were macerated for 1 min using a Seward Stomacher 400 set to 230 rpm. Samples of homogenate were decimally diluted and 100 µL of appropriate dilutions were spread on tryptone soya agar (TSA, Oxoid, Basingstoke, UK) or plate count agar (PCA, Oxoid, Basingstoke, UK); plates were incubated at 22 °C for 24 h to 48 h. Isolation of bacteria from spoiled milk was achieved by decimally diluting milk samples in MRD, spreading 100 µL of appropriate dilutions on PCA and incubating the plates at 30 °C for 72 h.

Identification of the isolates was performed by a multiplex *carA* PCR as previously described (Ercolini et al., 2007).

Table 4.1: Source and genome information of bacteria used in this study.

Organisms	Species	Source[a]	Total sequence length (bp)	GC-content (%)	No. protein coding genes	No. of contigs >1000 bp in length	Genome coverage	GenBank accession no.
F1786[b]	*P. fragi*	Raw cow's milk[c]	4,976,899	59.7	4,441	23	120x	NQKV00000000
F1791[b]	*P. fragi*	Beef steak[d]	5,143,683	58.2	4,641	44	25x	NQKU00000000
F1792[b]	*P. fragi*	Beef steak[d]	5,013,810	58.3	4,488	45	20x	NQKT00000000
F1793[b]	*P. fragi*	Beef steak[d]	4,970,618	58.4	4,460	43	30x	NQKS00000000
F1794[b]	*P. fragi*	Lamb fillets (MAP)[d]	5,256,020	58.2	4,730	44	20x	NQKR00000000
F1801[b]	*P. fragi*	Beef strips[e]	5,051,439	58.0	4,538	63	80x	NQKQ00000000
F1813[b]	*P. fragi*	Beef topside roast[e]	5,210,542	58.1	4,658	42	78x	NQKP00000000
F1815[b]	*P. fragi*	Beef topside roast[e]	4,979,003	58.4	4,479	44	36x	NQKO00000000
F1816[b]	*P. fragi*	Lamb strips[e]	5,113,509	58.4	4,634	30	58x	NQKN00000000
F1818[b]	*P. fragi*	Beef steak[e]	5,298,866	59.2	4,797	93	45x	NQKM00000000
F1820[b]	*P. fragi*	Minced lamb[e]	5,215,977	58.1	4,745	53	70x	NQKL00000000
F1821[b]	*P. fragi*	Minced beef[e]	4,975,134	58.4	4,478	57	40x	NQKK00000000
L1788[b]	*P. lundensis*	Raw cow's milk[c]	4,931,932	58.7	4,403	135	40x	NQKJ00000000
L1802[b]	*P. lundensis*	Beef strips[e]	4,800,490	57.7	4,336	92	78x	NQKI00000000
L1810[b]	*P. lundensis*	Beef steak[e]	4,982,671	58.3	4,474	64	90x	NQKH00000000
L1814[b]	*P. lundensis*	Beef topside roast[e]	5,016,267	58.7	4,455	64	30x	NQKG00000000
L1817[b]	*P. lundensis*	Minced beef[e]	4,955,748	58.4	4,405	81	30x	NQKF00000000
L1819[b]	*P. lundensis*	Beef steak[e]	5,035,790	58.7	4,487	73	60x	NQKE00000000

Organisms	Species	Source[a]	Total sequence length (bp)	GC-content (%)	No. protein coding genes	No. of contigs >1000 bp in length	Genome coverage	GenBank accession no.
L1822[b]	*P. lundensis*	Minced beef[e]	4,966,160	58.6	4,484	48	77x	NQKD00000000
ATCC 4973[Tf]	*P. fragi*	Soil around the Arctic Ocean	5,072,304	59.3	4,516	1	105x	GCA_900105835.1
P121[g]	*P. fragi*	Arctic sediment	5,101,809	58.6	4,683	1	200x	GCA_001543265.1
ATCC 49968[T]	*P. lundensis*	Prepacked beef	4,992,502	58.5	4,576	78	70x	GCA_001042985.1

[a] Meat sources were stored aerobically unless indicated. MAP: Modified atmosphere packaging (30% CO_2/70% N_2).

[b] Genomes of isolates sequenced for this work.

[c] Milk was acquired in CSIRO Agriculture and Food laboratories Melbourne, VIC, Australia, and was stored at 4 °C (L1786) and 8 °C (L1788) for 15 days.

[d] Meat sources were acquired in Melbourne, VIC, Australia and stored at 1 °C (F1794) and 10 °C (F1790, F1791, F1793) until visible deterioration was observed (Baxter, 2000).

[e] Meat sources were purchased in Melbourne, VIC, Australia in 2015 and stored at 4 °C for 2 to 7 d until their labelled expiry date.

[f] Strain acquired from BioNovus Life Sciences, NSW, Australia.

[g] (Yanzhen et al., 2016)

DNA isolation, genome sequencing, assembly and annotation

Isolates were grown for 18 h in tryptone soya broth (TSB, Oxoid, Basingstoke, United Kingdom) at 25 °C. Genomic DNA was isolated with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according the manufacturer's protocol for Gram-negative bacteria. Library preparation and genome sequencing was carried out at The Ramaciotti Centre at the University of NSW using the Nextera XT DNA library 300 bp paired end preparation kit, and was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Genome assembly and annotation was performed as previously described (Stanborough et al., 2017a). GenBank accession numbers of the 19 genomes sequenced in this study are listed in Table 4.1.

Genomic analyses

Determination of average nucleotide identity

Orthologous Average Nucleotide Identity (OrthoANI) was determined using the OrthoANI Tool (version 0.93, Blast+) (Lee et al., 2016).

Single Nucleotide Polymorphism (SNP) analysis

SNP analysis was performed with the software kSNP3.0 (Gardner et al., 2015). Optimal k-mer length was determined by the auxiliary script kChooser and was 19 for the *P. lundensis* and 21 for the *P. fragi* genomes. The analysis of core SNPs (loci present in all input genomes), rather than "all SNPs" or "majority SNPs", was chosen as this method is better suited to remove recombination/horizontally transferred genomic elements from the analysis (Pettengill et al., 2016). Core SNP trees were estimated by parsimony and based on a consensus of up to 100 equally parsimonious trees. Trees were drawn with Dendroscope (version 3.5.9) (Huson et al., 2007). The *P. fragi* core SNP matrix was plotted as a

NeighborNet network using SplitsTree4 and uncorrected P-distances (version 4.14.2) (Huson and Bryant, 2006). SplitsTree4 was used to calculate the δ score and Q-residual score as measures of "treelikeness" of the phylogenetic distance data (Holland et al., 2002).

Pan-genome

Pan-genome analysis was performed with USEARCH (version 8.1.1861) using an all versus all comparison with a minimum identity cut-off score of 0.6. Seed sequences were parsed into tabular form with a custom script (https://github.com/bioinformatics-deakin/gene-matrix-from-uclust3.py) and genes less than 153 nucleotides in length (proteins <50 amino acids in length) were eliminated from the analysis. The core genome was determined as the set of genes shared by all organisms, while the accessory genome was the set of genes present in a subset of organisms or in a single organism. Functional annotation of the translated seed sequences of the accessory genome was conducted with the NCBI CD-Search tool to identify conserved domains using a mirrored COG (Clusters of Orthologous Groups of Proteins) database with the default settings (E-value threshold of 0.01, maximum number of hits of 500, and composition-corrected scoring turned on). As each COG can have one or more general category letter association, the pools of functional categories were compared. The relative abundances of COG functional categories were determined by dividing the number of genes assigned to each category by the total pool of functions determined for each accessory genome. Principal component analysis of the pan-genome matrix was conducted with a custom script (https://github.com/bioinformatics-deakin/PCA/blob/master/PCA.py).

Identification of homologous genes

A nucleotide database was generated in Geneious (version 9.0.5) (Kearse et al., 2012) containing the genome sequences of the isolates and type strains included in Table 4.1.

Query proteins were searched against the database using the scoring matrix BLOSUM62 and tblastn algorithm. Sequences with E-value scores of $<10^{-5}$ and bit scores of ≥50 were further subjected to protein domain analysis with the InterProScan tool. Query proteins and source organisms are included in Table 4.2.

Table 4.2: Locus tags of gene homologs

Query proteins (GenBank accession numbers)	Query organism	Locus tags of homologous genes[a]	
		P. fragi	***P. lundensis***
UndA (4WWJ_A)	*Pseudomonas protegens* Pf-5	SAMN05216594_4735[b]	TU74_08480[c]
AcuC (NP_390849.1)	*Bacillus subtilis* 168	SAMN05216594_2987[b]	TU74_12385[c]
Methionine gamma lyase (AAY92781.1)	*Pseudomonas protegens* Pf-5	SAMN05216594_0122[b]	TU74_09140[c]

[a] – indicates that a homologous sequence was not identified.

[b] GenBank accession number GCA_900105835.1

[c] GenBank accession number GCA_001042985.1

Metabolic analyses

Sample preparation in headspace vials

Large 1-2 kg beef samples were surface sterilized by cooking in boiling water for 10 min. Cooked surface meat was removed aseptically and the raw, sterile inner was cut into small pieces in a sterile manner. Cut meat pieces were mixed with 0.9% sterile NaCl solution (ratio of 1 g:0.67 mL) and homogenized for 60 sec with a Sorvall Omni-Mixer to produce a smooth meat paste. 3 g of sterile meat paste were aliquoted with a 100 mL sterile syringe into sterilized 20 mL glass headspace vials (Grace Davidson Discovery Sciences, Rowville, VIC, Australia).

Bacterial inocula were prepared by inoculating 3 mL of sterile TSB with single colonies and incubating the cultures in a shaking water bath at 25 °C for 18 h. Per experiment, three cultures were prepared for each isolate (n=3) on one day. To inoculate meat paste samples, cultures were diluted 1:50 with sterile 0.9% NaCl solution and 50 µL of the diluted cultures, containing approximately 10^6 cells, were distributed on the meat paste surface. For VOC analysis, each inoculum was used to inoculate two meat paste samples (one sample for headspace analysis and one sample for analysis of atmospheric gases and bacterial enumeration). For the determination of bacterial growth and consumption/production of atmospheric gases of low (F1794, F1815 and L1788) and high (F1801 and L1814) VOC-producers over a 4 d incubation period, each inoculum was used to inoculate four samples (one sample per day). Control meat paste samples were inoculated with 50 µL of diluted sterile TSB. Three controls were frozen immediately at -20 °C to act as time point 0 controls (C0) and three controls (Cd4) were incubated together with the inoculated vials for 4 d at 8 °C.

In anticipation of considerable biological variability, the VOC analysis was repeated two times constituting a total of three independent experiments, each experiment conducted with three biological replicates of the strains.

Determination of atmospheric gases and bacterial numbers in headspace vials

To analyse the levels of atmospheric gases (CO_2, O_2 and N_2) in the 20 mL headspace vials containing the inoculated samples and non-inoculated controls, a Shimadzu gas chromatograph (GC model GC8A, Shimadzu Scientific Instruments (Oceania) Pty Ltd, Rydalmere NSW, Australia) fitted with a CTR-1 column (180 cm long, 0.64 cm and 0.32 cm diameter of outer and inner columns, respectively; Alltech Deerfield, IL. USA) was used. An

aliquot of 1 mL of headspace gas was withdrawn from the vials and injected using an on-column injector (50 °C). Column and thermal conductivity detector were maintained at 30 °C and 50 °C, respectively. Helium, at a constant pressure of 3 kg/cm^2, was used as the carrier gas. The Shimadzu GC was interfaced with the computer, running Delta 5.5 chromatography Data System, using an analog-to-digital converter (Data Centre 4000). Peak areas obtained for CO_2, O_2 and N_2 were used to calculate relative amounts (%) of the composition gases and the means and standard deviations of the three independent experiments were calculated.

The number of bacteria on the inoculated meat paste samples and non-inoculated controls was determined by adding 10 mL of sterile MRD to the 20 mL headspace vials. Vial contents were transferred to sterile stomacher bags and then crushed for 1 min at 230 rpm in a Seward Stomacher 400. Samples of homogenate were decimally diluted, 100 µL of appropriate dilutions were spread on TSA and incubated for 24 h to 48 h at 25 °C. Levels of bacteria were enumerated for each sample, converted to $\log_{10}$ cfu/g, and the means and standard deviations of the three independent experiments calculated.

Headspace analysis by solid phase micro-extraction-gas chromatography mass spectrometry (SPME-GCMS)

Headspace analysis was used to analyse the VOCs in the inoculated meat paste samples and non-inoculated controls. An Agilent Technologies gas chromatograph-mass spectrometer system (GC model 6890N, MS model 5975 series B; Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a Combi PAL robotic auto sampler (CTC Analytics AG, Zwingen, Switzerland) and a programmed temperature vaporization injector (PTV inlet set in splitless mode, temperature 260 °C, splitless time 1 min; Gerstel, Mülheim, Germany) was used. The SPME fiber, coated with carboxen/polydimethylsiloxane (CAR/PDMS StableFlex fibre, phase

thickness 85 µm, length 1 cm; Supelco, Bellefonte, Pa, USA), was preconditioned prior to analysis at 270 °C for 1.5 h. 1 mL of internal standard solution (2-methyl-3-heptanone, 3.5 mg, and 2-ethyl butanoic acid, 7.1 mg, per 10 mL of methanol) was added to the samples. The autosampler was operated under the following conditions: pre-incubation time of 5 min, pre-incubation and absorption temperature of 45 °C, absorption time of 30 min, desorption time of 8 min (purge after 1 min), and desorption temperature of 260 °C. The volatile compounds of the meat paste samples were chromatographed on a VF-WAXms column (30 m length, 0.32 mm i.d., 1.0 mm film thickness, Agilent Technologies Australia, Mulgrave, VIC, Australia) using a temperature gradient: 35 °C for 5 min, raised to 225 °C at a rate of 10 °C/min and finally held at 225 °C for 10 min. Helium was used as carrier gas at a constant flow rate of 1.6 mL/min. The MSD conditions were as follows: capillary direct interface temperature of 280 °C, ionization energy of 70 eV, mass range of 35-300 amu; EM voltage of autotune +200 V, with a scan rate of 5.27 scans/s.

VOCs were identified by comparison of mass spectra with the spectra in the NIST11 database (National Institute of Standards Technology; United States of America), and linear retention indices determined using a set of saturated alkanes C7–C22 and spectra of authentic standards. VOC profiles of the isolates were established by calculating the relative amounts of VOCs as percentages based on the total peak area. To assess VOCs that played key roles in the metabolism of the isolates on the beef paste, those present in trace amounts (i.e. constituted <1% of the total volatile profile of the isolates) were not considered further. The relative amounts of VOCs shown were calculated using the means of the three independent experiments. Quantitative analysis was performed using an internal standard methodology.

RESULTS

Phylogeny

In this study, the genomes of 12 *P. fragi* and seven *P. lundensis* isolates of red meat and raw milk origin were sequenced and draft genome assemblies deposited in GenBank to provide an analysis data set (Table 4.1). Metrics of the draft genomes of the isolates correlated well with genome metrics of the respective type strains. Size estimation of the assemblies varied between 4.97 Mb and 5.30 Mb for the *P. fragi* and 4.80 Mb and 5.04 Mb for the *P. lundensis* isolates. The GC-content of the *P. fragi* and *P. lundensis* draft genomes ranged from 58% to 59.6% and 57.7% to 58.7%, respectively. Between 4,441 and 4,797 protein coding genes were predicted for the *P. fragi* genomes, while 4,336 to 4,487 were identified in the *P. lundensis* genomes. Genomic similarity of the isolates was investigated by pairwise calculation of average nucleotide identity (ANI) (Figure 4.1) and included the publically available genome sequences of the type strains and the environmental isolate *P. fragi* P121. A high degree of variation was observed among the *P. fragi*, with P121 clearly distinct from all other *P. fragi* with ANI values of 84.7% to 85.1% (Figure 4.1A). Three distinct *P. fragi* groups were otherwise observed, including 1) a group of eight closely related isolates (F1791, F1792, F1793, F1794, F1813, F1815, F1816 and F1821) as indicated by shared ANI values above 98.9%, 2) a group that consisted of two isolates (F1786, F1818) and the type strain with ANI values between 96.9% to 98.7%, and 3) a group consisting of two isolates (F1801 and F1820) that shared 95.8% ANI. Seven of the eight *P. lundensis* were closely related with ANI values between 97.7% and 99.9%, while low ANI values were determined for isolate L1802 (90.3%-90.5%) (Figure 4.1B).

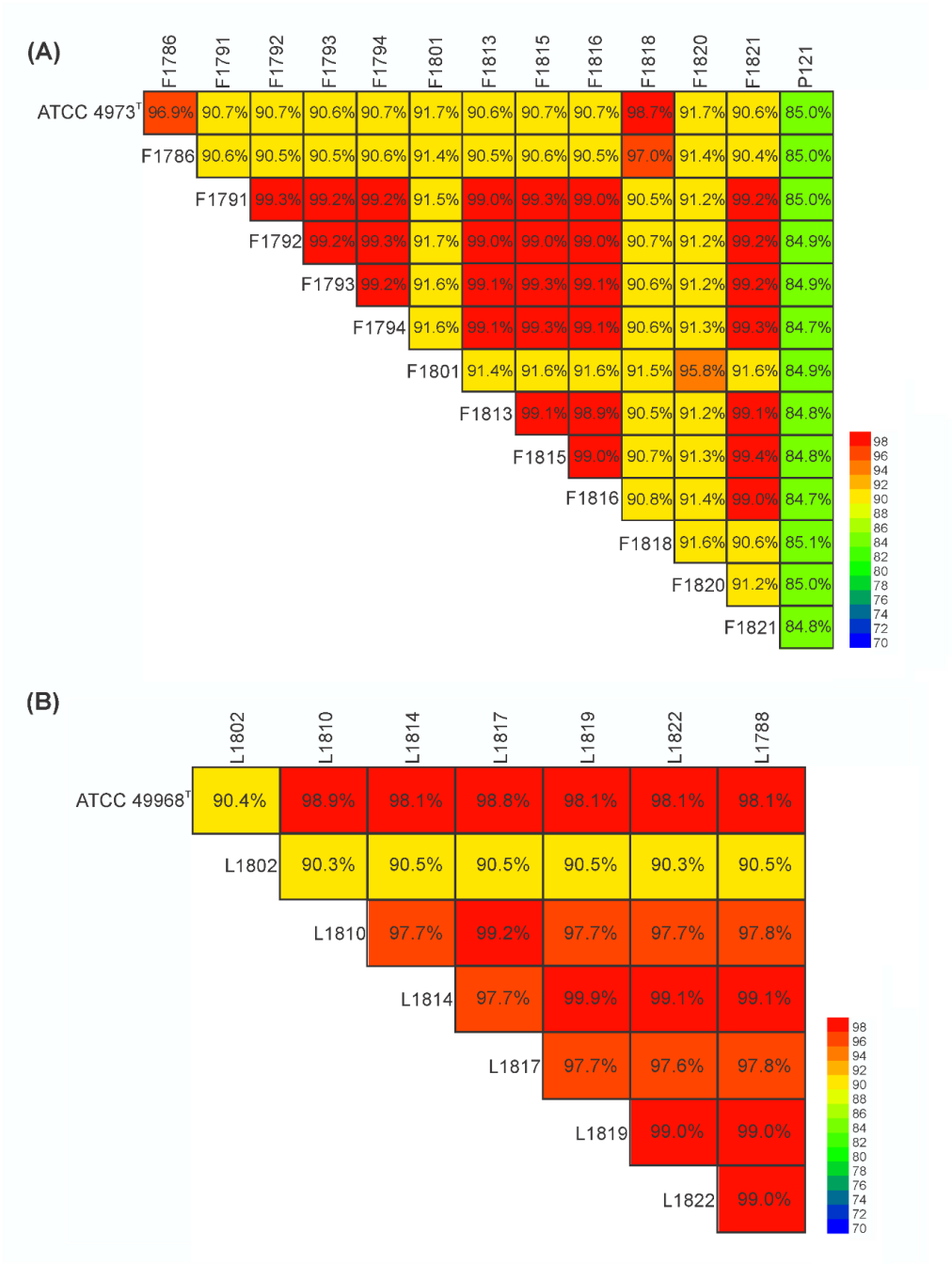

Figure 4.1: Pairwise comparison of the average nucleotide identity (ANI) of the genomes. Heat maps show the pairwise ANI as determined for the *P. fragi* **(A)** and *P. lundensis* **(B)** genomes. The colour scale is shown to the right of the heat maps.

To better understand the intra-species relationships, core SNPs were used to generate SNP matrices for phylogenetic reconstruction (Figure 4.2). The *P. fragi* SNP matrix consisted of 11,192 SNPs, whereby 5,098 of these were unique to *P. fragi* P121. Poor branch support values were obtained for many branches of the *P. fragi* SNP tree (Figure B1A: Appendix B),

indicating that a bifurcating tree was unlikely to accurately represent the ancestry. Therefore, the SNP matrix was examined with a NeighborNet network with uncorrected nucleotide distances (Figure 4.2A). The resultant network was not tree-like displaying a high level of reticulation (δ-score = 0.1915, Q-residual score = 0.00035). This network revealed three main groups of taxa with isolate P121 clearly distant from the others. These groups were consistent with the groups identified by ANI calculations. The low numbers of unique core SNPs and short network branches of the eight meat-derived Group 1 isolates underlined their close phylogenetic relationship. The exact relationship between the three Group 2 organisms and the two isolates from Group 3 could not be resolved. Interestingly, despite their geographic and temporal diversity and their different isolation sources, the soil-derived type strain and meat isolate F1818 were phylogenetically close. Moreover, the third Group 2 isolate (F1786) was derived from milk, demonstrating the Group 2 diversity.

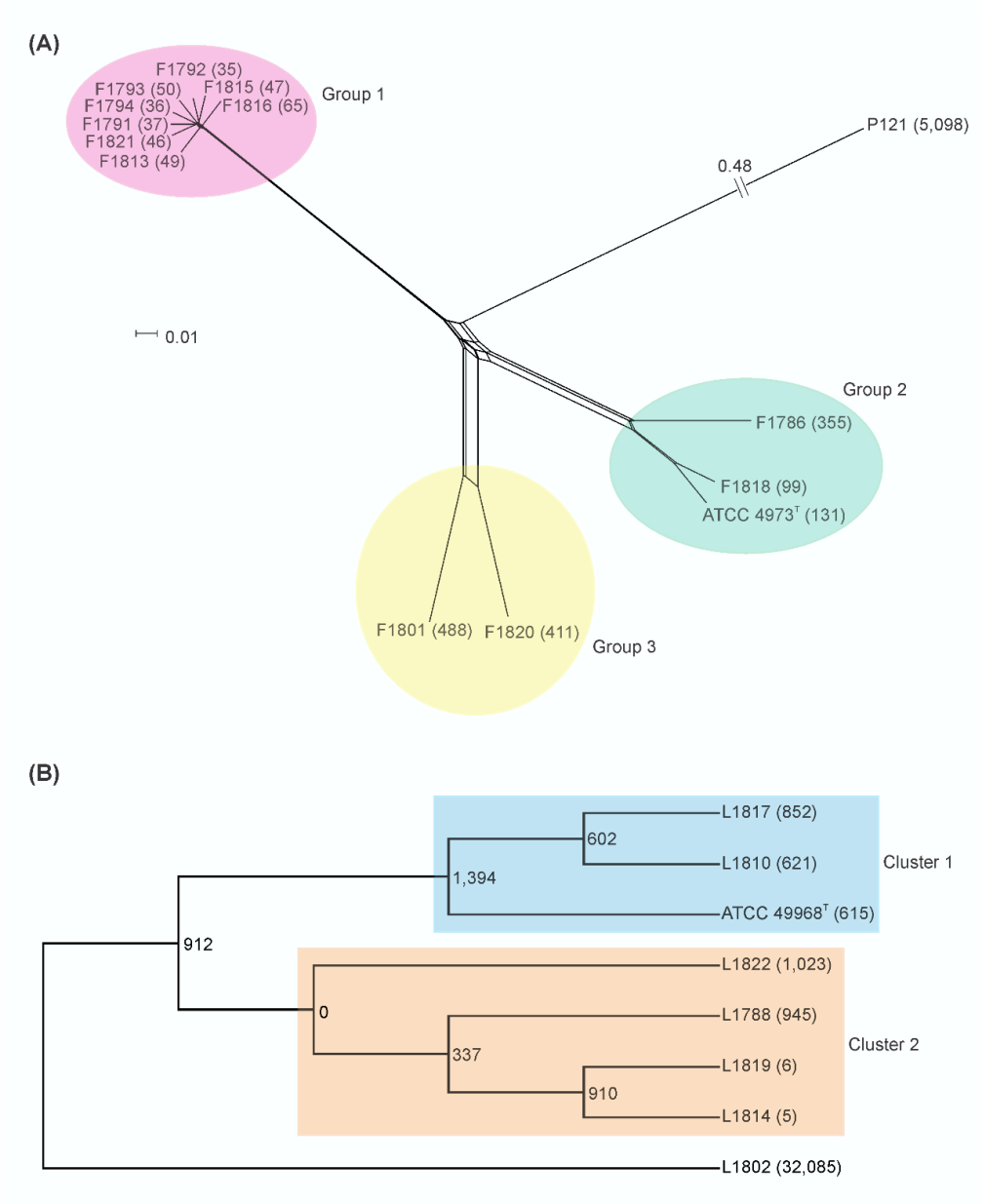

Figure 4.2: *P. fragi* and *P. lundensis* core SNP phylogenies. Coloured shading was used to highlight the main groups of taxa. Parentheses at the branch tips indicate the number of core SNPs exclusive to the isolates and type strains. **(A)** NeighborNet network analysis of *P. fragi* core SNP matrix was plotted using uncorrected P-distances. The distance scale indicates the number of nucleotide substitutions per number of SNPs. Truncated network branch is marked with // to aid visualisation and the original branch length is indicated above. **(B)** Mid-point rooted consensus parsimony cladogram of *P. lundensis* core SNPs. Support values of all nodes were 1 as determined by Shimodiara-Hasegawa resampling test. Numbers at the internal nodes represent the number of core SNPs that are shared exclusively by the descendants of that node.

Of the 42,696 core SNPs identified for the *P. lundensis*, 32,085 were exclusive to L1802. Resampling branch supports of the *P. lundensis* core SNP tree (Figure 4.2B) were high with support values of 1 for all branch points. The tree topology showed two main groups of taxa with isolate L1802 clearly separated from the other organisms. Cluster 1 was comprised of two isolates (L1817 and L1810) and the type strain. With a high number of unique SNPs and no additional shared alleles, the meat isolate L1822 was clearly distant from the other Cluster 2 isolates. Cluster 2 also comprised two closely related meat isolates (L1814 and L1819) with only five and six unique core SNPs, respectively, as well as one milk isolate (L1788). A NeighborNet network derived from the core SNP matrix yielded a tree-like network (δ score = 0.083, Q-residual score = 0.00054) confirming that a bifurcating tree adequately describes their phylogeny (Figure B1B: Appendix B).

Pan-genome

A total of 7,378 genes comprised the pan-genome of the *P. fragi*, with 3,286 genes identified as core genes, resulting in a core/pan-genome ratio of 45%. The *P. lundensis* pan-genome comprised 5,769 genes. Of these, 3,537 were core genes, amounting to a core/pan-genome ratio of 61%. The pan-genome matrices were used in a principal component analysis (PCA) to assess clustering of the isolates based on their gene repertoire (Figure 4.3). PCA of the *P. fragi* pan-matrix (Figure 4.3A) showed clustering correlated well with their phylogeny and P121 was clearly separated from the other *P. fragi*. PCA of the *P. lundensis* pan-genome matrix segregated some members of the same phylogenetic clusters and L1802 was clearly separated from the other isolates and the type strain (Figure 4.3B).

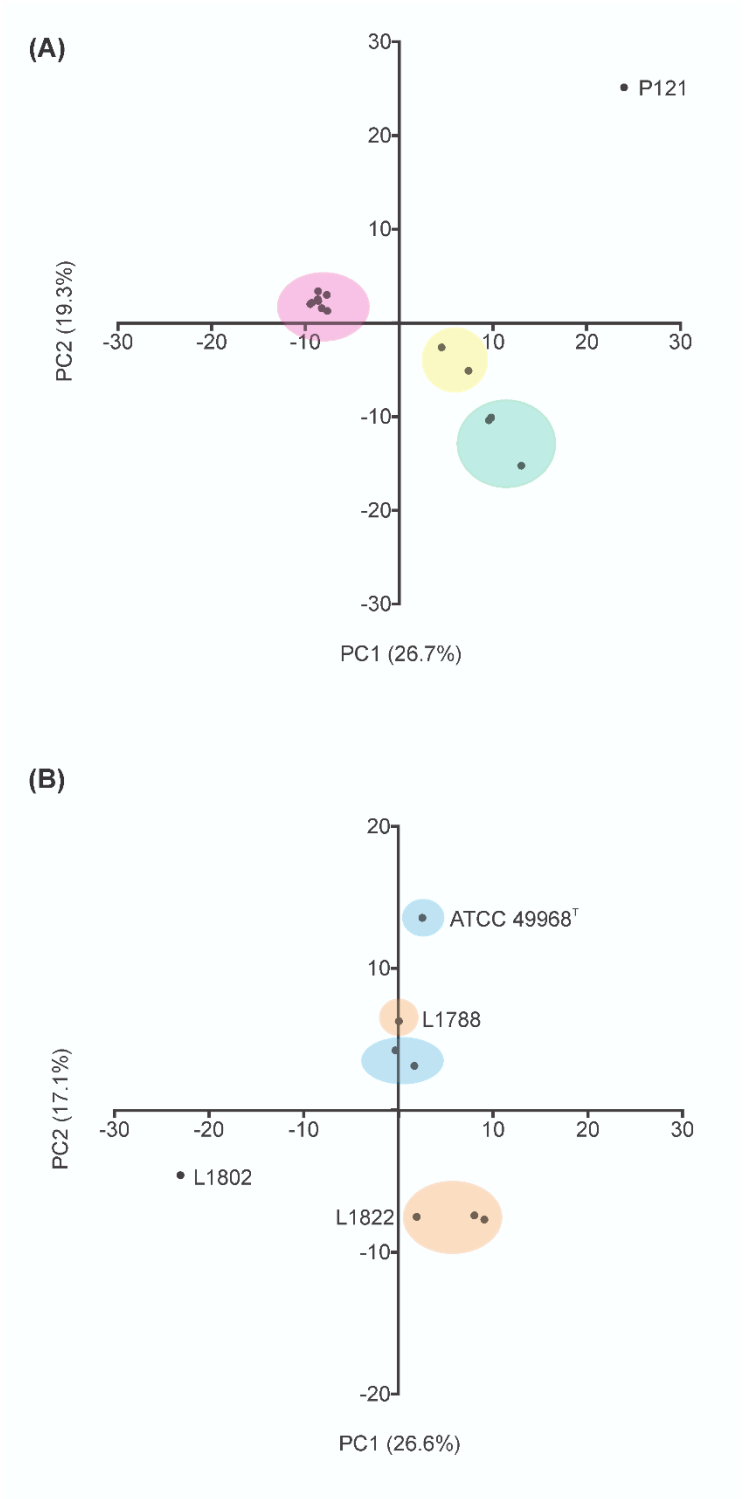

Figure 4.3: Principal component analysis of *P. fragi* (A) *and P. lundensis* (B) pan-matrices. Each dot marks a genome in the first two principal components of the pan-matrix space. Percentages in the axis labels show the amount of total pan-matrix variation seen along each principal component. Coloured ellipses and circles highlight the phylogenetic groupings of the organisms as defined in Figure 4.2.

The accessory genome was analysed to understand functional groups of genes responsible for intra-species differences. A large percentage of genes of both accessory genomes were assigned to the categories general function prediction and function unknown. Genes involved in transcription and signal transduction mechanisms represented large proportions of both accessory genomes (Figure 4.4). Other well represented functional groups (i.e. more than 5% of the accessory pool) of the *P. fragi* accessory genome included carbohydrate and amino acid transport, replication, recombination and repair, cell wall/membrane/envelope biogenesis, inorganic ion transport and the mobilome (Figure 4.4A). A notably large proportion of the *P. lundensis* accessory genes were assigned to the mobilome, and replication, recombination and repair groups (Figure 4.4B). Additional well represented categories were cell wall/membrane/envelope biogenesis and inorganic ion transport.

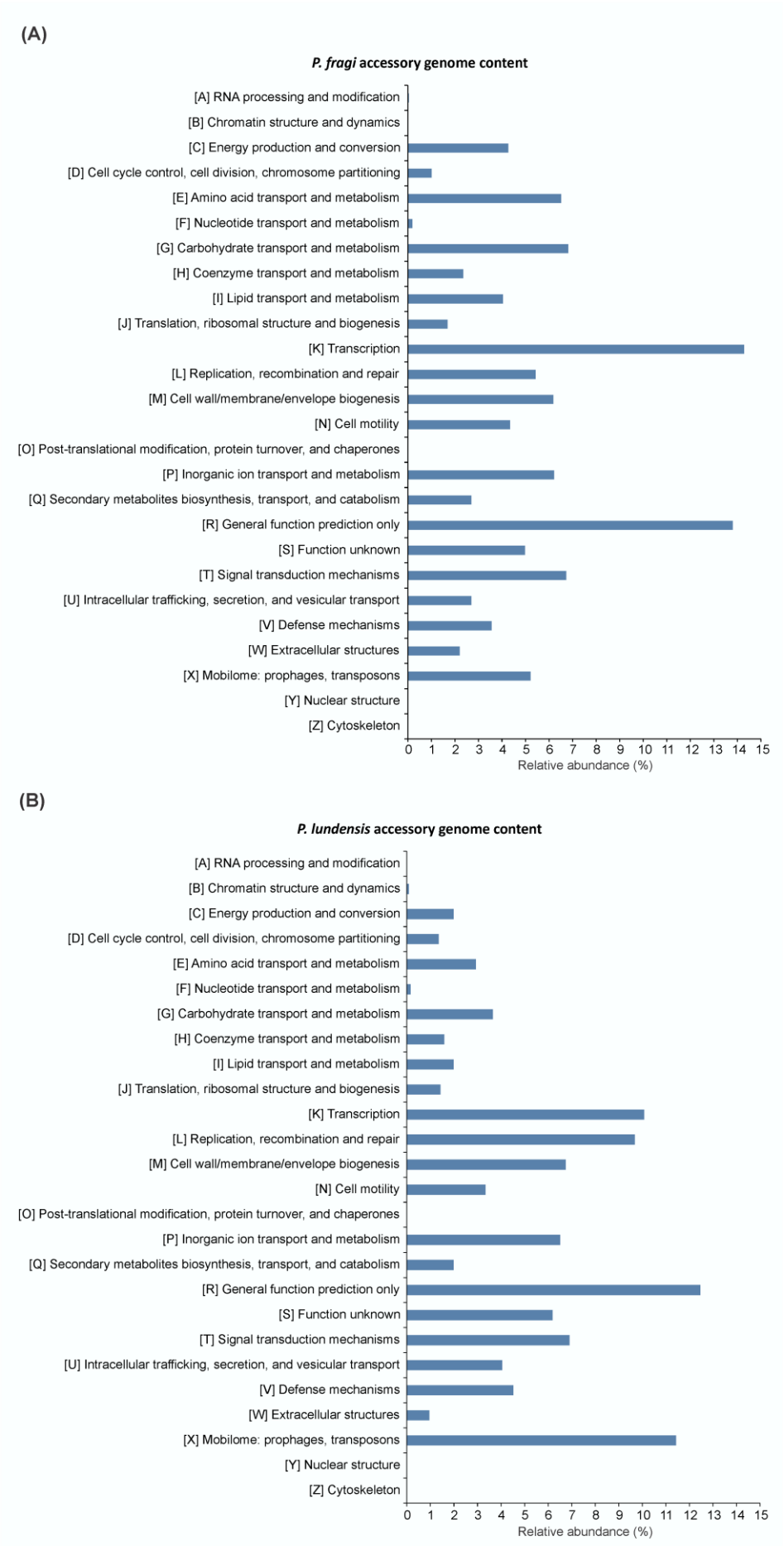
(A)
P. fragi accessory genome content
[A] RNA processing and modification
[B] Chromatin structure and dynamics
[C] Energy production and conversion
[D] Cell cycle control, cell division, chromosome partitioning
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[O] Post-translational modification, protein turnover, and chaperones
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport, and catabolism
[R] General function prediction only
[S] Function unknown
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion, and vesicular transport
[V] Defense mechanisms
[W] Extracellular structures
[X] Mobilome: prophages, transposons
[Y] Nuclear structure
[Z] Cytoskeleton
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Relative abundance (%)
(B)
P. lundensis accessory genome content
[A] RNA processing and modification
[B] Chromatin structure and dynamics
[C] Energy production and conversion
[D] Cell cycle control, cell division, chromosome partitioning
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[O] Post-translational modification, protein turnover, and chaperones
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport, and catabolism
[R] General function prediction only
[S] Function unknown
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion, and vesicular transport
[V] Defense mechanisms
[W] Extracellular structures
[X] Mobilome: prophages, transposons
[Y] Nuclear structure
[Z] Cytoskeleton
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Relative abundance (%)

Figure 4.4: COG functional categories of accessory genomes. The relative abundances of COG functional categories for the *P. fragi* **(A)** and *P. lundensis* **(B)** accessory genomes are shown. On the vertical axis, COG functional categories are depicted with their letter association, while the horizontal axis indicates their relative abundance.

Volatile analysis

To assess the main intra- and inter-species metabolic differences, the major VOCs produced or metabolised by the isolates were investigated using a meat model system, enabling controlled, reproducible conditions and sufficient replicates. Where possible, metabolic activity was linked to gene repertoire. Isolates were inoculated onto precise amounts of sterile beef paste in headspace vials and poor refrigeration conditions were simulated by incubating the vials at 8 °C. Preliminary tests showed the transition to stationary phase growth occurred around day 4 of incubation and oxygen levels on this day were reduced to approximately 2% to 3%. Pseudomonads grow well in oxygen concentrations down to 2%, but are progressively inhibited at lower concentrations (Clark and Burki, 1972). Therefore, VOCs were determined on day 4 before oxygen levels were low enough to impact on the growth of the isolates.

Samples were inoculated with log 6 cfu/g; after the 4-day incubation period numbers increased to log 9.4-9.6 cfu/g, while oxygen in the headspaces of the inoculated samples constituted between 1.8%-5.5% of the volume and carbon dioxide and nitrogen between 7.6%-10.1% and 86.2%-88.9%, respectively (Table B1: Appendix B).

A total of 16 VOCs (Figure 4.5) were found to be the major VOCs produced or metabolised by the isolates. Among these were short and medium-length fatty acid esters, ketones,

hydrocarbons as well as a fatty acid, alcohol and a sulphur compound. Strong differences were observed in the total amounts of detected VOCs between the isolates.

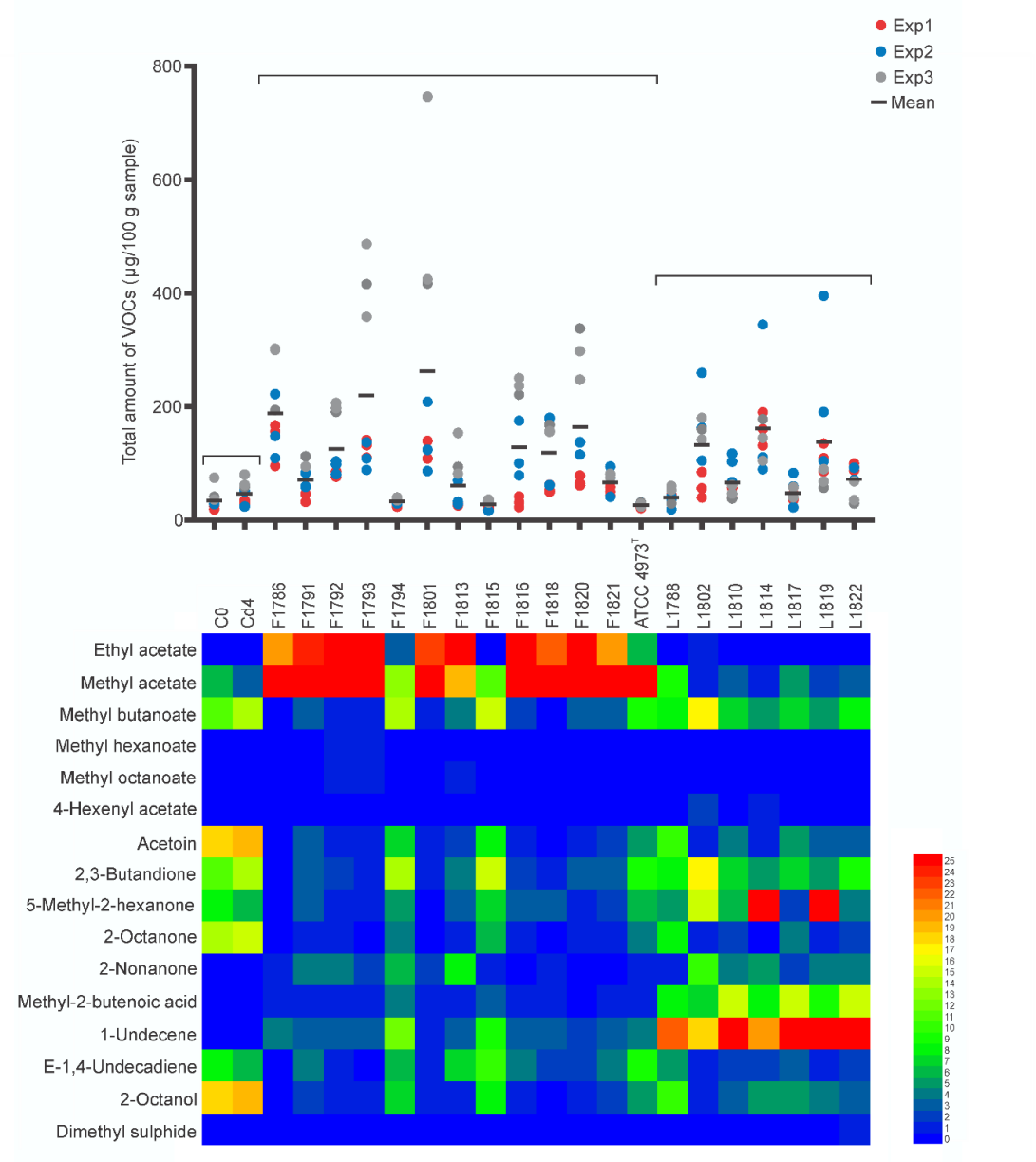

Figure 4.5: VOC profiles of inoculated meat paste samples and non-inoculated meat paste controls. Scatter plot shows total amounts of the 16 VOCs detected in the inoculated samples and non-inoculated controls prior to (C0) and after (Cd4) incubation at 8 °C for 4 days. Replicates from experiment 1 (Exp1), 2 (Exp2) and 3 (Exp3) are coloured in red, blue and grey, respectively, while mean values are shown with black horizontal bars. Each sample

is represented by nine data points with overlapping potentially obscuring some points. Lines above the data points demarcate between control samples and samples of the two species. The heat map was generated with the means of the area ratios (%) of the 16 VOCs; the colour scale shown to the right of the heat map indicates area ratio differences of 1% between 0% and 25%.

Esters

Six different esters of short and medium chain fatty acids with equal carbon numbers were identified. These included one ethyl ester (ethyl acetate), four linear methyl esters (methyl acetate, methyl butanoate, methyl hexanoate and methyl octanoate) and 4-hexenyl acetate. Methyl acetate was the only ester detected in all samples. Ethyl acetate was found exclusively in samples inoculated with *P. fragi*, with the exception of L1802, which was the only *P. lundensis* isolate for which this compound was found. Methyl butanoate was detected in most samples with the exception of three *P. fragi* isolates (F1786, F1801 and F1818). Methyl hexanoate and methyl octanoate were only identified in samples inoculated with *P. fragi* F1792, F1793 and F1813, while 4-hexenyl-acetate only constituted more than 1% of the volatile profiles of *P. lundensis* L1802 and L1814 and the detected amounts of these three esters were low (data not shown). Notably, the majority of the *P. fragi* demonstrated extensive production of ethyl and methyl acetate (Figure 4.6). In marked contrast, *P. fragi* isolates F1815 and F1794 as well as the *P. fragi* type strain produced extremely low levels of these esters. For isolate F1815, ethyl acetate levels did not constitute more than 1% of its volatile profile. Although the *P. fragi* and *P. lundensis* genomes harboured a multitude of predicted lipase and esterase genes, no obvious genetic variations were identified to explain the difference in levels of esters produced.

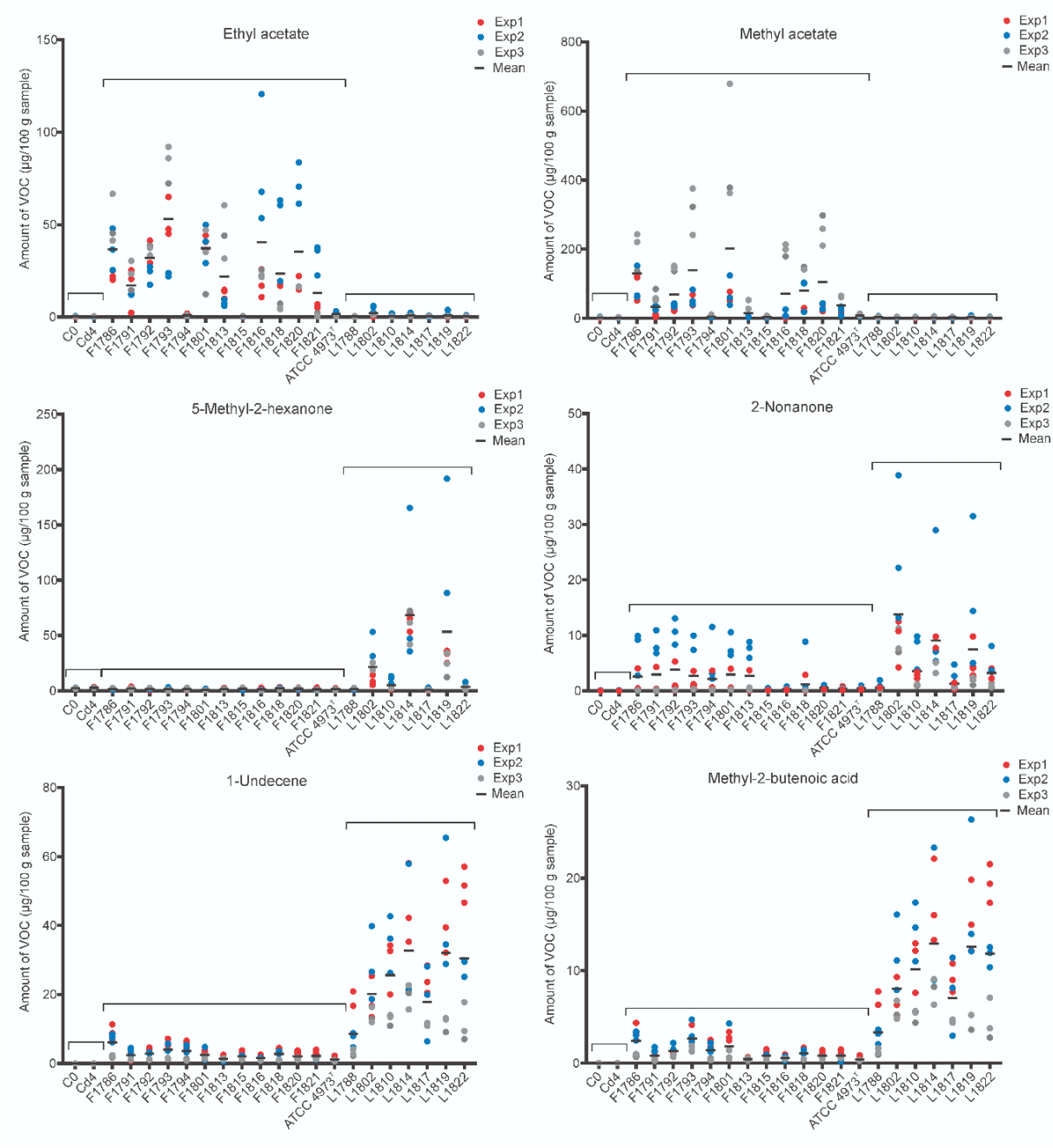

Figure 4.6: VOCs produced by *P. fragi* and *P. lundensis* isolates. Absolute amounts of key VOCs of the inoculated samples compared to their detected amounts in non-inoculated controls prior to (C0) and after (Cd4) incubation at 8 °C for 4 days. Replicates from experiment 1 (Exp1), 2 (Exp2) and 3 (Exp3) are coloured in red, blue and grey respectively, while the mean values are shown with black horizontal bars. Each sample is represented by nine data points with overlapping potentially obscuring some points. Lines above the data points demarcate between control samples and samples of the two species.

Ketones

Five different ketones constituted a significant proportion of the volatile profiles of samples, including 5-methyl-2-hexanone, 2-nonanone, acetoin (3-hydroxy-2-butanone), 2-octanone and 2,3-butandione. *P. lundensis* isolates L1802, L1814 and L1819 produced the largest amounts of 5-methyl-2-hexanone and 2-nonanone (Figure 4.6), with particularly high levels of 2-nonanone detected in Experiment 2, as observed for most of the organisms. In contrast, comparatively low amounts of 2-nonanone were detected for *P. lundensis* L1788 and five *P. fragi* (F1815, F1816, F1820, F1821 and ATCC 4973^{T}).

Mean values of the ketones 2-octanone and acetoin, which were present in non-inoculated controls, notably decreased in all inoculated samples (Figure 4.7) and genome inspections showed all genomes encoded a gene with homology to the acetoin utilisation protein (AcuC) of *Bacillus subtilis* strain 168 (Table 4.2).

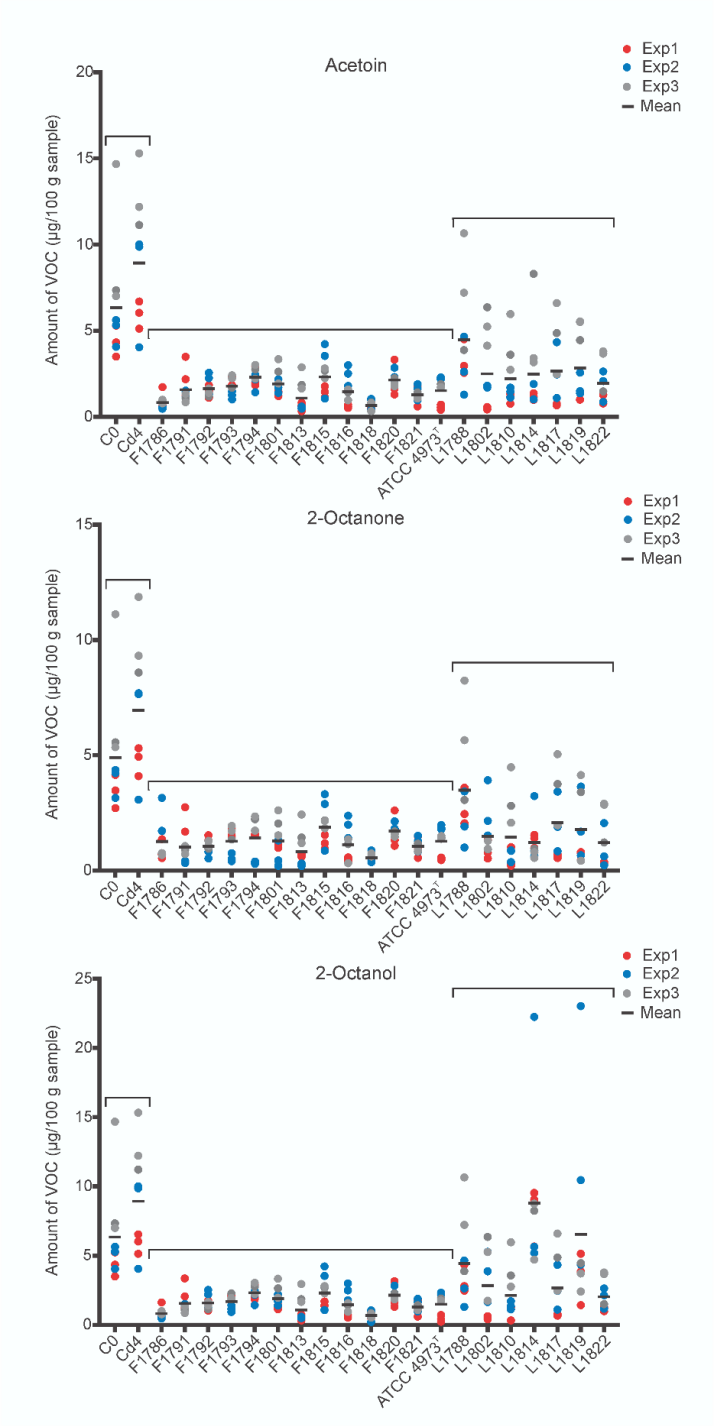

Figure 4.7: VOCs metabolised by *P. fragi* and *P. lundensis* isolates. Absolute amounts of VOCs detected in reduced amounts in inoculated samples compared to non-inoculated controls prior to (C0) and after (Cd4) incubation at 8 °C for 4 days. Replicates from experiment 1 (Exp1), 2 (Exp2) and 3 (Exp3) are coloured in red, blue and grey respectively, while the mean values are shown with black horizontal bars. Each sample is represented by nine data points with overlapping potentially obscuring some points. Lines above the data points demarcate between control samples and samples of the two species.

Other volatile compounds

The hydrocarbon 1-undecene and volatile fatty acid methyl-2-butenoic acid were identified in all inoculated samples and amounts were notably higher in samples inoculated with *P. lundensis* isolates (Figure 4.6). In line with the ubiquitous production of 1-undecene, an *undA* homolog was identified in all genomes. The sequences of the *P. lundensis* and *P. fragi* UndA proteins shared a high degree of homology with 73%-74% and 68%-72% identity with the *Pseudomonas protegens* Pf-5 sequence, respectively (Table 4.2). The VOCs E-1,4-undecadiene and 2-octanol were identified in non-inoculated controls and inoculated samples, with a marked reduction in mean 2-octanol levels detected for all samples inoculated with *P. fragi* and some samples inoculated with *P. lundensis* isolates (Figure 4.7). Closely related isolates L1814 and L1819 were exceptions with little to no reduction in their mean 2-octanol values, and an increase in levels of this compound were observed for one replicate of each isolate from Experiment 2. Dimethyl sulphide was only found in notable quantities in samples inoculated with *P. lundensis* L1822, although, a methionine gamma lyase gene (EC 4.4.1.11) was identified in all of the genomes.

To better understand differences in levels of VOCs, in particular the striking absence of ester production of *P. fragi* F1815, F1794 and ATCC 4973^{T}, growth of three isolates with overall low levels of VOC production (F1815, F1794, L1788) and growth of two isolates with high levels of VOC production (F1801 and L1814) were monitored over a 4-day incubation period. Percentages of atmospheric gases in the headspace vials were also measured prior to sampling. Results in Figure B2 (Appendix B) show notable interspecies differences in oxygen consumption, however intra-species differences were not observed. Moreover, major intra-

species differences, which might explain differences in levels of produced VOCs, were not seen in growth rates of the isolates.

DISCUSSION

Genomic analyses

Unresolved taxonomy of the *Pseudomonas* genus complicates our understanding of the ecology of aerobic meat spoilage communities. The results of our study, which included the genomes of isolates identified using a multiplex PCR targeting the carbamoyl phosphate synthase (*carA*) gene (Ercolini et al., 2007), as well as a number of publically available genome sequences from NCBI, highlight this. The ANI for 11 of the *P. fragi* and one of the *P. lundensis* isolates with their respective type strains was well below the suggested threshold of 95%-96% for species demarcation (Richter and Rossello, 2009). More notably, based on these recommendations for species delineation, the four distinct *P. fragi* groups determined with pairwise calculation of ANI and SNP phylogeny, indicate that a larger diversity of *Pseudomonas* microbiota may exist with possible spoilage potential. As taxonomy is essential for identification and classification of bacterial species, it is not surprising that an unambiguous, molecular-based identification does not yet exist for this group of bacteria. To tackle this problem effectively, comprehensive comparative genomic and systematic phylogenetic studies of spoilage-associated *Pseudomonas* species are required.

Although, the pan-genomes must be considered as rough estimates due to the nature of draft genomes, estimates of the core to pan-genome ratios provided insight into the isolate diversity. A relatively high degree of diversity was observed for the 14 *P. fragi* included in this analysis (core/pan-genome ratio of 45%). Recent genome investigations of the meat

spoilage bacterium *Brochothrix thermosphacta* revealed a substantially higher core/pan-genome ratio of 66% for 12 draft genomes of this organism (Stanborough et al., 2017a), which is likely influenced by the smaller genome size of this bacterium. A similar degree of diversity was observed for the eight analysed *P. lundensis* (core/pan-genome ratio of 61%).

Clustering of the pan-genome matrices did not reveal a correlation between the sources of the isolates and type strains, and their gene inventory. The separation of *P. fragi* P121 and *P. lundensis* L1802 from the other isolates indicates notable differences in their gene repertoire, and the clear segregation of these two isolates from the respective type strains in all genomic analyses suggests that taxonomic reclassification may be required.

Components of the accessory genome are significant contributors to genome evolution, but they also confer phenotypes that are advantageous under specific conditions (Kung et al., 2010). The accessory genomes of both species contained a large proportion of regulatory genes (assigned to transcription and signal transduction groups), which suggests genetic flexibility and diversity are traits of these organisms. While the large proportion of phage and transposon-related genes (assigned to mobilome and replication, recombination and repair groups) in the *P. lundensis* accessory genome indicates that bacteriophages and mobile genetic elements are differentiating genetic features of this group, metabolic diverseness appears to contribute to the overall diversity of the *P. fragi*, as carbohydrate and amino acid transport and metabolism, and inorganic ion transport were well represented functional groups.

VOC analysis

The utilisation of a meat paste model system in this study has its limitations, as these conditions provide higher accessibility of nutrients, therefore promoting spoilage. Although

higher in abundance, the types of compounds available for microbial utilisation remain the same. Therefore volatile metabolic end-products of the isolates observed in this work are likely to be similar to those encountered during their growth and metabolism on beef cuts. The volatile analysis showed that a number of characteristic species- and isolate-specific VOCs were produced or metabolised during growth of the isolates on the meat paste, many of which are associated with the undesirable characteristics of meat spoilage. A correlation was not observed between the isolation sources of the bacteria and the types and amounts of VOCs detected. In agreement with previous studies (Morales et al., 2005b, Edwards et al., 1987), short chain fatty acid esters responsible for the fruity flavour defect dominated the volatile profiles of most of the *P. fragi*. The results of a number of studies have led to the suggestion that ethanol is the preferred substrate for ester synthesis (Casaburi et al., 2015, Edwards et al., 1987). However, our results showed that methyl acetate was produced in greater amounts than ethyl acetate and while small amounts of methyl butanoate, hexanoate and octanoate were detected, ethyl esters other than ethyl acetate did not constitute more than 1% of the total volatile profile of the strains. Thus, the enzyme(s) involved in ester production may not be as substrate-specific as previously thought. Instead the production of either ethyl or methyl esters may be a result of the availability of the substrates in the matrix. Interestingly, two *P. fragi* isolates and the *P. fragi* type strain consistently produced very low levels of methyl and ethyl acetate. This observation was all the more intriguing considering ester production is a distinguishing taxonomic feature of the type strain of this species (Breed et al., 1957). Their lack of ester production was not accompanied by the production of alternative compounds, rather, these organisms showed an overall low production of VOCs. These differences in metabolic activity could not be easily explained by notably slower growth rates or discrepancies in gene content. As ester

production requires the availability and/or production of alcohols and fatty acids and the activity of esterases, absence of these components, for example due to single enzyme defects or deficiencies, could result in the absence of ester production (Ercolini et al., 2010). We cannot exclude the possibility that these three organisms produced alternative odour-active compounds, which were not detected with our methods, but these results raise the question of whether strains of presumed spoilage species have similar meat-spoilage potential. They also highlight the importance of strain-specific studies in the meat environment to better understand the heterogeneity of strains of the same species as well as the heterogeneous behaviour of strains in general.

Currently, very limited information is available about the production of odour-active VOCs produced by *P. lundensis* strains. Therefore little is known about their contribution to meat spoilage. Growth and metabolism of the *P. lundensis* isolates from this study was associated in particular with the production of 1-undecene as well as the odour-active compounds 2-nonanone and methyl-2-butenoic acid. Production of 1-undecene is considered to be ubiquitous for members of the *Pseudomonas* genus (Rui et al., 2014) and its potential as a marker compound for acceptability/degree of spoilage has been discussed (Dainty et al., 1984). In this work, 1-undecene production was ubiquitously observed, although in notably greater amounts for *P. lundensis* isolates. This may be attributed to activity of the identified homologous UndA protein, which was shown to convert lauric acid to 1-undecene in *P. protegens* Pf-5 (Rui et al., 2014). The ketone 2-nonanone, which has an earthy, dirty, dairy odour, is commonly associated with the presence of pseudomonads including the species *P. fragi* and *P. lundensis* (Morales et al., 2005b). Our study showed that 2-nonanone was a key VOC of most of the *P. fragi* and all *P. lundensis* isolates. For three *P. lundensis* isolates, the fruity odour VOC 5-methyl-2-hexanone, was produced in large amounts, suggesting that

strains of this species may also contribute substantially to fruity off-odours. Furthermore, methyl-2-butenoic acid with its rancid, pungent odour was a characteristic volatile metabolite of all *P. lundensis* isolates and to a lesser extent the *P. fragi*. A similar compound, 2-methyl butanoic acid, was previously found associated with aerobically stored beef and *Pseudomonas* populations (Ercolini et al., 2011).

Pseudomonads metabolise acetoin (Edwards et al., 1987), and accordingly, a strong reduction in mean acetoin levels compared to the control samples was observed, which may be the result of the identified AcuC homolog. Comparisons with the control samples also showed marked reductions in 2-octanone and 2-octanol levels for all *P. fragi* and some *P. lundensis* strains, likely due to dehydrogenase activity of the bacteria. Interestingly, the closely related isolates L1814 and L1819 showed little to no reduction in mean 2-octanol levels and an increase was observed in replicates from Experiment 2, which may indicate an ability of these isolates to produce this compound with its oily, earthy odour under favouring conditions. The production of 2-octanol could result from specific methyl ketone reductase activity of the isolates, as other *Pseudomonas* species were shown to be capable of catalysing the reduction of 2-octanone to 2-octanol (Du et al., 2014). Although sulphurous compounds are commonly associated with *Pseudomonas* at late stages of spoilage, they did not represent major VOC of the strains' volatile profiles. However, the presence of a methionine gamma lyase gene in all genomes is indicative of an ability to degrade L-methionine to methanethiol, which can in turn give rise to dimethyl sulphide via auto-oxidation. Thus, under different experimental conditions dimethyl sulphide production of the isolates may have been detected. It is possible that the homogenisation of the meat in our study increased the availability of sugar compounds, and at the sampling time point substantial degradation of sulphurous amino acids had not yet occurred.

Conclusions

The isolates included in this work may belong to more than two *Pseudomonas* species with possible spoilage potential. Among the *P. fragi*, a high degree of genetic diversity was observed, and the large proportion of regulatory genes in the accessory genomes, suggests genetic flexibility and diversity are traits of both species. Most of the organisms produced significant levels of odour-active compounds, however three *P. fragi* strains that produced extremely low levels of VOCs, led us to question whether all strains of presumed spoilage species have the same spoilage potential.

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Chapter 5

Vibrioferrin production by the food spoilage bacterium *Pseudomonas fragi*

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ABSTRACT

Pseudomonas fragi is a meat and milk spoilage bacterium with high iron requirements; however, mechanisms of iron acquisition remain largely unknown. The aim of this work was to investigate siderophore production as an iron acquisition system for *P. fragi*. A vibrioferrin siderophore gene cluster was identified in 13 *P. fragi* and experiments were conducted with a representative strain of this group (F1801). Chromeazurol S assays showed that *P. fragi* F1801 produced siderophores under iron starvation at optimum growth and refrigeration temperature. Conversely, supplementation of low iron media with 50 µM $FeCl_3$ repressed transcription of the vibrioferrin genes and siderophore production. Disruption of the siderophore receptor (*pvuA*) caused polar effects on downstream vibrioferrin genes, resulting in impaired siderophore production of the Δ*pvuA* mutant. Growth of this mutant was compared to growth of a control strain (Δ*lip*) with wild-type vibrioferrin genes in low iron media supplemented with iron chelators 2,2'-bipyridyl or apo-transferrin. While 25 µM 2,2'-bipyridyl caused impaired growth of Δ*pvuA*, growth of the mutant was completely

inhibited by 2.5 µM apo-transferrin, but could be restored by $FeCl_3$ addition. In summary, this work identifies a vibrioferrin-mediated iron acquisition system of *P. fragi*, which is required for growth of this bacterium under iron-starvation.

INTRODUCTION

Iron is required for fundamental cellular processes such as respiration and the synthesis of amino acids and DNA, and is therefore an essential element for the majority of microorganisms (Ilbert and Bonnefoy, 2013). However, iron is in short supply in many habitats. For example, in aerobic environments at neutral pH, Fe^{2+} is rapidly oxidised to Fe^{3+}, leading to the formation of insoluble hydroxide salts and rendering iron scarce in the environment (Colombo et al., 2014). Similarly, in the human host and foods such as milk and meat, iron is sequestered by iron-binding proteins including transferrin family proteins, transferrin and lactoferrin, haemproteins myoglobin and haemoglobin, and ferritin (Skaar, 2010).

Microorganisms have evolved a variety of mechanisms to obtain their iron. In iron-rich environments, iron acquisition occurs through energy-independent, low-affinity iron uptake systems (Jones and Niederweis, 2010). In iron-limited environments, a common strategy involves the excretion of small molecular weight iron-chelating compounds termed siderophores (Noinaj et al., 2010). Fe^{3+}-siderophore complexes that form outside the cell are taken up in Gram-negative bacteria by specific outer membrane receptors, which are supplied with energy for transport from the cytoplasmic membrane by the Ton system (Faraldo-Gomez and Sansom, 2003).

Excess iron causes toxic cellular effects, necessitating the tight regulation of iron uptake in microorganisms. The ferric uptake regulator protein (Fur) regulates iron homoeostasis at the transcription level in many Gram-negative and some Gram-positive organisms (Carpenter et al., 2009). When intracellular Fe^{2+}-concentrations exceed a certain threshold level, Fur binds to specific regulatory DNA sequences, termed Fur-boxes, preventing RNA polymerase binding to promotors (Troxell and Hassan, 2013). In contrast, scarcity of intracellular iron causes Fur to lose its ability to bind to Fur-boxes, resulting in gene transcription. Thus, the transcription of iron uptake genes is ultimately regulated by the concentration of intracellular iron.

Pseudomonas have some of the best studied siderophore-mediated iron uptake systems such as the pyoverdine siderophores of the fluorescent pseudomonads (Meyer and Abdallah, 1978, Cox and Adams, 1985, Cezard et al., 2015, Trapet et al., 2016). In contrast, *Pseudomonas fragi* has been considered a non-siderophore producing member of this genus and is described as not producing siderophores in detectable amounts in Bergey's Manual (Garrity et al., 2005, Champomier-Vergès et al., 1996). *P. fragi* is a problematic meat and milk spoilage bacterium with high iron requirements, but beyond its ability to scavenge foreign Fe^{3+}-siderophore complexes, very little is known about the strategies this bacterium employs to obtain iron (Casaburi et al., 2015, De Jonghe et al., 2011, Champomier-Vergès et al., 1996). Interestingly, in a recent study on plant growth-promoting bacteria, a putative *P. fragi* isolate derived from the rhizosphere was shown to produce siderophores (Farh et al., 2017), suggesting previous conclusions that this bacterium is a non-siderophore producer may not have been accurate.

In this work, siderophore production was investigated as an iron acquisition system for *P. fragi*. The genomes of 13 *P. fragi* were examined for the presence of siderophore biosynthetic gene clusters, which led to the identification of vibrioferrin biosynthesis and vibrioferrin-mediated iron acquisition genes. This work describes experiments that determine siderophore production for *P. fragi* F1801 and show a role for the siderophore in iron metabolism of this bacterium under low iron conditions.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

Strains, plasmids, and genome and gene sequences used in this study are included in Table 5.1.

Table 5.1: Strains, plasmids, and genome and gene sequences used in this study

Strains, plasmids, and genome and gene sequences	Relevant characteristics	Locus tags of vibrioferrin genes	Accession numbers[a]
***P. fragi* genome sequences**			
F1786[b]		CJU73_02415–CJU73_02445	GCA_002269585.1
F1791[b]		CJU79_02430–CJU79_02400	GCA_002269515.1
F1792[b]		CJU72_13900–CJU72_13930	GCA_002269595.1
F1793[b]		CJU75_07595–CJU75_07565	GCA_002269565.1
F1794[b]		CJU80_05030–CJU80_05000	GCA_002269445.1
F1813[b]		CJU77_09910–CJU77_09940	GCA_002269465.1
F1815[b]		CJU76_02305–CJU76_02275	GCA_002269545.1
F1816[b]		CJU74_06845–CJU76_06815	GCA_002269485.1
F1818[b]		CJU78_20565–CJU78_20595	GCA_002269625.1
F1820[b]		CJF43_16240–CJF43_16270	GCA_002269055.1
F1821[b]		CJF37_06830–CJF37_06800	GCA_002269155.1
ATCC 4973[Tb]		SAMN05216594_0902–SAMN05216594_0896	GCA_900105835.1
F1801[b]		CJU81_14170–CJU81_14200	GCA_002269505.1
***P. fragi* strains**			
F1801[c]	Wild-type		SRX3235903
F1801Δ*lip*[c]	Disruption of CJU81_12870[b] at 167[th] amino acid encoding codon, Km^r		SRX3235904
F1801Δ*pvuA*[c]	Disruption of CJU81_14195[b] at 419[th] amino acid encoding codon, Km^r		SRX3235905
***Escherichia coli* strains**			
Escherichia coli S17-1 *λpir*	*thi*, *pro*, *hsd* (r^- m^+) *recA*::RP4-2-Tc^r::Mu Km^r::Tn7 Tp^r Sm^r *λpir*		
Genome/gene sequences of other bacteria			
Azotobacter vinelandii CA			GCA_000380335.1

Strains, plasmids, and genome and gene sequences	Relevant characteristics	Locus tags of vibrioferrin genes	Accession numbers[a]
Xanthomonas campestris 8004			GCA_000012105.1
Vibrio parahaemolyticus WP1			AB048250.2 and AB082123.1
Plasmids			
pJP5603	Suicide plasmid, Km^r		
pJP5603_lip323-783	pJP5603::disruption construct for gene CJU81_12870[c], Km^r		
pJP5603_pvuA886-1523	pJP5603::disruption construct for gene CJU81_14195[c], Km^r		

[a] Relevant GenBank and Sequence Read Archive accession numbers provided.

[b] Previously sequenced *P. fragi* genomes that were examined for the presence of siderophore biosynthetic gene clusters.

[c] Genomes sequenced in this study.

[d] Locus tags of respective coding sequences.

Conditions of iron starvation were achieved in modified M9 media (MM9) comprising 10% v/v of MM9 salts (5 g/L NaCl, 10 g/L NH_4Cl, 0.59 g/L $Na_2HPO_4.H_2O$ and 0.45 g/L KH_2PO_4), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% w/v glucose, 0.3% casamino acids w/v, 0.2% w/v succinate and 0.1 M PIPES (pH 6.8, NaOH). *P. fragi* cultures were grown under agitation unless otherwise specified. Kanamycin was added to culture media of gene disruption mutants at a concentration of 50 μg/mL.

Identification of siderophore gene cluster

Examination of the 13 previously sequenced *P. fragi* genomes (Table 5.1) for siderophore biosynthetic gene clusters was performed with antiSMASH version 4 (Blin et al., 2017).

Generation of disruption mutants, genomic DNA isolation, and genome sequencing and analysis

A gene disruption mutant was generated of the TonB-dependent outer membrane siderophore receptor (Δ*pvuA*) by homologous recombination with a pJP5603 suicide vector construct. This system results in a single cross-over event, whereby the pJP5603 vector (Riedel et al., 2013) encoding a kanamycin resistance gene remains integrated in the genome (Penfold and Pemberton, 1992). Thus, a control strain with wild-type vibrioferrin genes and a disrupted lipase gene (Δ*lip*) was generated for subsequent experimental comparisons. The *lip* gene was chosen because the disruption of this gene would not affect iron metabolism of the strain, nor would it be required for growth of the strain in the culture media used in this work. Furthermore, because it is present as a single gene rather than in an operon cluster, polar effects on downstream genes were highly unlikely.

DNA manipulations were performed using standard protocols (Sambrook and Russell, 2001). Plasmid constructs were generated by ligating BamHI-digested pJP5603 and BamHI-restriction fragments of the *P. fragi* F1801 *lip* and *pvuA* genes (Table 5.1), which were amplified using primers included in Table C1 (Appendix C).

pJP5603 gene disruption constructs were transferred from *Escherichia coli* S17-1 *λpir* (Simon et al., 1983) to *P. fragi* F1801 by biparental filter matings as previously described (Windgassen et al., 2000) with some adjustments; recipient cells were grown at 30 °C and the ratio of donor to recipient cells was 1:2 ($5x10^8$ cfu to 10^9 cfu), respectively. After mating, cells were spread on *Pseudomonas* agar containing cetrimide-fucidin-cephaloridine selective supplement (Oxoid) for *Pseudomonas* and 50 µg/mL kanamycin sulphate to select for transconjugants. Plates were incubated at 25 °C for 30 h.

Strains (wild-type F1801, Δ*lip* and Δ*pvuA*) were grown for 18 h in tryptone soya broth (TSB, Oxoid) at 25 °C. Genomic DNA was isolated with the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's protocol for Gram-negative bacteria. Library preparation and genome sequencing were carried out at Queensland Health (Health Support Queensland) using MiniSeq High Output kits with 150 cycles and the Illumina MiniSeq System (Illumina). Genome assembly was performed as previously described (Stanborough et al., 2017a). Confirmation of correct insertion of the vector was achieved by manual inspection of the genomes with Geneious version 9.0.5 (Kearse et al., 2012). Snippy (https://github.com/tseemann/snippy) was used to confirm an absence of additional genetic variants in the Δ*lip* and Δ*pvuA* mutants' genomes by aligning sequence reads of wild-type F1801, Δ*pvuA* and Δ*lip* strains to the reference genome of F1801.

Sequence reads of wild-type F1801, Δ*lip* and Δ*pvuA* strains were deposited in the Sequence Read Archive and the respective accession numbers are provided in Table 5.1.

Phylogenetic tree of vibrioferrin sequences

MAFFT multiple sequence alignments (Katoh et al., 2002) of the vibrioferrin PvuA and PvsA-PvsE protein sequences were performed using the default settings. The six multiple alignments were concatenated and Gblocks version 0.91b (Castresana, 2000, Talavera and Castresana, 2007) was run with the default settings to remove poorly aligned positions and divergent regions of the alignments. Bayesian inference (BI), performed with MrBayes version 3.2.6 (Ronquist and Huelsenbeck, 2003), was conducted with a run of 1000,000 generations and sampling every 1000. A mixed amino acid model analysis was set, enabling the MCMC sampler to test all of the fixed rate models. MrBayes determined that the Wheeler and Goldman (WAG) model of amino acid replacement (Whelan and Goldman,

2001) had the best likelihood score and was chosen for the analysis. Convergence parameters were assessed using Tracer version 1.6 (Rambaut et al., 2014) and the majority rule consensus tree was rendered with Figtree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). BIs were run three times to ensure reproducibility of the resulting trees.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was isolated from 1 mL of mid-log phase iron-starved cultures ($1.5x10^8$ cfu). Stabilisation of RNA was achieved with RNAprotect Bacteria Reagent (QIAGEN) following the manufacturer's recommendations and total RNA was extracted with the RNeasy Mini Kit (QIAGEN) according to the supplier's instructions and stored in H_2O at -80 °C. cDNA was synthesised from 1 µg of total RNA using iScript gDNA Clear cDNA Synthesis Kit (Biorad) as suggested by the manufacturer, however DNase treatment was increased from the recommended 5 to 30 min. qRT-PCR was performed on an AriaMx Real-time PCR System (Agilent) using the iTaq Universal SYBR Green Supermix (Biorad) as a fluorescence source. PCR reaction mixes included 10 µL iTaq Universal SYBR Green Supermix (Biorad), 500 nM forward and reverse primers and 1 µL template cDNA (diluted 1:10 in H_2O) in a total volume of 20 µL. Primer sequences (Table C1: Appendix C) were designed with Primer3 version 2.3.4 (Untergasser et al., 2012) . Acceptable primer efficiency was confirmed for each target gene with standard curves, and melt-curve analysis of amplicons of each target gene was performed to ensure amplification of single gene products. PCR conditions were as follows: 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 30 sec at 60 °C. Melt-curve analysis involved an incremental increase of 0.5 °C every 2 s from 65 to 95 °C. Each assay included a non-template control, and each sample a control without reverse transcriptase. Raw data

were analysed with Agilent AriaMx version 1.0 software and exported to excel for further analysis. Ct-values were normalised to the isocitrate dehydrogenase gene (gene locus tag CJU81_09775 in F1801, GenBank accession no. GCA_002269505.1) and relative expression levels were calculated according to the $2^{\Delta\Delta Ct}$ method.

Chromeazurol S (CAS) supernatant assays

CAS assay solution was prepared as previously described (Alexander and Zuberer, 1991). Siderophore detection from culture supernatants was performed using the method of Schwyn and Neilands (1987). Briefly, bacterial cultures were spun down at 2,500 x g for 5 min and the supernatants were filtered through a 0.20 µm cellulose acetate membrane filter (Sartorius). Siderophores were detected in the supernatants by mixing equal volumes of filtered supernatant and CAS solution. After allowing the solutions to equilibrate for 1.5 h, a visible change in the colour of the mixture from blue to orange was considered a positive reaction. To detect siderophore production at 25 °C, strains were grown in 4 mL MM9 for 18 h prior to supernatant collection. To monitor siderophore production at 4 °C, F1801 was grown in 50 mL MM9 for 4 d and every 24 h 3 mL of culture were removed to measure growth (A_{600}), and detect siderophores by CAS supernatant assays. To monitor the increase in siderophore levels over the 4 d, absorbance measurements of the CAS reaction mixtures were performed at 630 nm.

Growth experiments with 2,2'-bipyridyl and bovine apo-transferrin

Single colonies were used to inoculate 3 mL MM9. Cultures were grown at 25 °C for 18 h. 20 µL of these cultures were used to inoculate 180 µL fresh MM9, supplemented as indicated in the text with 2,2'-bipyridyl (Sigma-Aldrich), bovine apo-transferrin (Sigma-Aldrich) and/or

sodium bicarbonate and $FeCl_3$, in Greiner 96-well flat bottom microtitre plates (Sigma-Aldrich). Growth was monitored (A_{600}) using an EON microplate reader (BioTek).

RESULTS AND DISCUSSION

In this work, 13 previously sequenced *P. fragi* genomes were examined for the presence of siderophore biosynthetic gene clusters. This search resulted in the identification of a conserved gene cluster, homologs of which were shown to be responsible for vibrioferrin biosynthesis and vibrioferrin-mediated iron uptake in the terrestrial bacteria *Azotobacter vinelandii* and *Xanthomonas* spp., and in the marine bacterium *Vibrio parahaemolyticus* (Pandey and Sonti, 2010, Yamamoto et al., 1994, Baars et al., 2016, Pandey et al., 2017) (Figure 5.1). Similar to the vibrioferrin gene clusters in *A. vinelandii* and *Xanthomonas campestris*, this ~10 kb region in *P. fragi* comprised seven open reading frames (Figure 5.1A). Previous work in *Xanthomonas* spp. and *V. parahaemolyticus* showed that five of these genes (*mhpE*, *pvsA*, *pvsB*, *pvsD* and *pvsE*) are involved in the biosynthesis of vibrioferrin, while *pvuA* is a TonB-dependent outer-membrane receptor and *pvsC* an inner-membrane exporter for vibrioferrin (Tanabe et al., 2006, Funahashi et al., 2002, Fujita et al., 2011, Pandey and Sonti, 2010). Locus tags of these genes in the 13 strains are shown in Table 5.1. Analysis of the upstream region of this gene cluster revealed the presence of a 19 bp sequence, matching 14 of the 19 nucleotides of the *X. campestris* consensus Fur-box (Blanvillain et al., 2007) and 11 of the 19 nucleotides of the *Escherichia coli* and *Pseudomonas aeruginosa* consensus Fur-box. This sequence was located 129 bases upstream of the *mhpE* start codon. Fur homologs were also identified in the *P. fragi* genomes with the locus tag CJU81_16820 in F1801.

To investigate the relatedness of the vibrioferrin genes between the 13 strains and *A. vinelandii*, *X. campestris* and *V. parahaemolyticus*, a phylogenetic tree of the PvuA and PvsA-PvsE sequences was determined by Bayesian inference (BI) (Figure 5.1B). Topology of the BI consensus tree showed three clades of *P. fragi* vibrioferrin sequences. The largest of the three clades, comprising closely related sequences of eight of the 13 strains, was clearly separated from the other two clades, which share a more recent common ancestral sequence. The sequences of the *P. fragi* type strain were found in one of these two clades with those of two additional strains. Sequences of F1801, the *P. fragi* strain used for subsequent experiments, were grouped together in the second of these clades with vibrioferrin sequences of one other strain. In accordance with their phylogeny, sequences of the *P. fragi* were closest to those of *A. vinelandii*, while those of *X. campestris* and *V. parahaemolyticus* were substantially more distant.

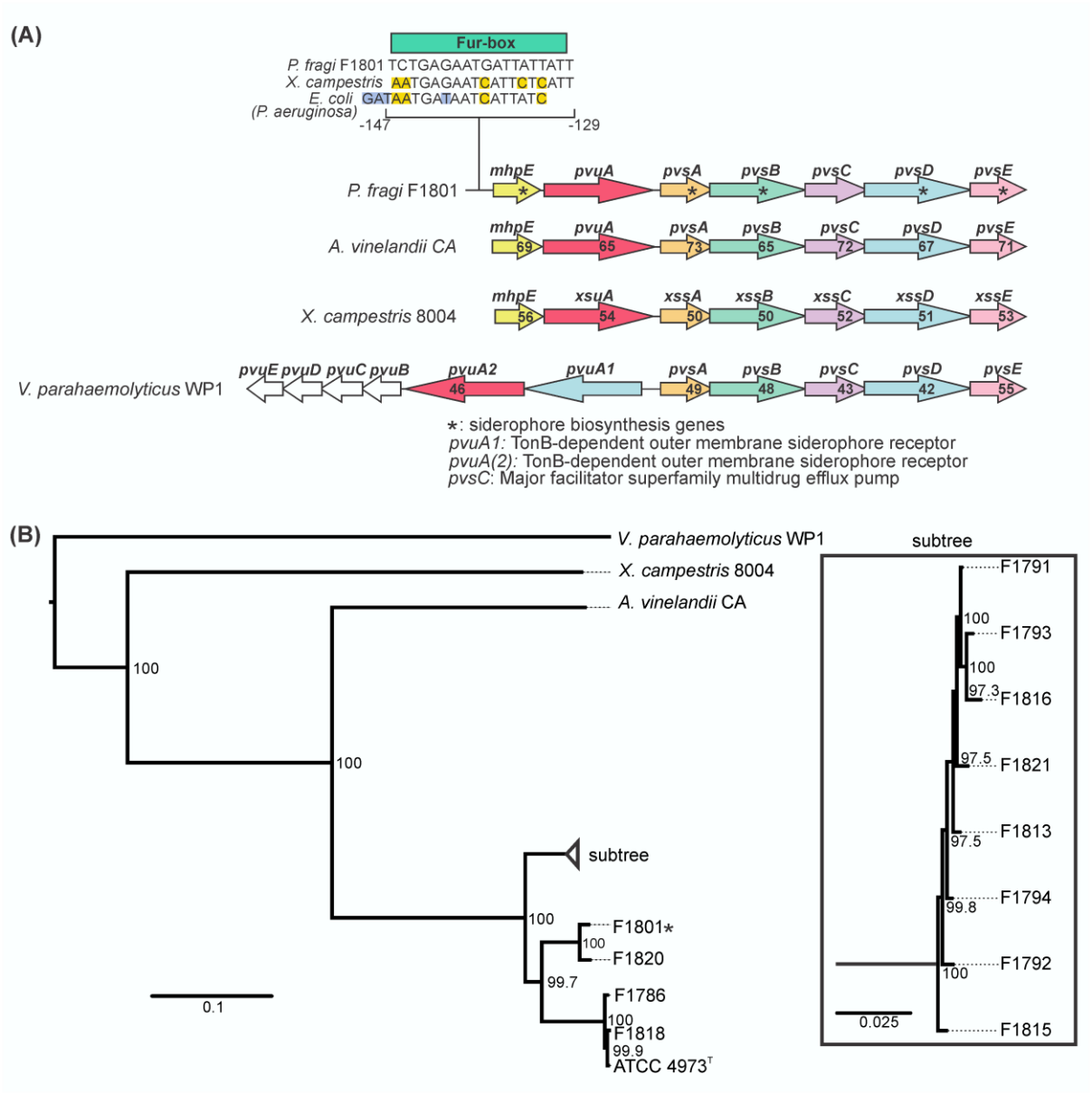

Figure 5.1: Vibrioferrin gene cluster identified in 13 *P. fragi*. (A) Schematic diagram of the vibrioferrin gene cluster identified in *P. fragi* and conserved synteny of these genes in known vibrioferrin producing bacteria *A. vinelandii*, *X. campestris* and *V. parahaemolyticus*. Predicted biosynthesis genes are marked with an asterisk in the *P. fragi* genes. Numbers shown in genes indicate percent sequence identity of proteins to respective homologs in *P. fragi* F1801, as determined by MAFFT protein alignment. The putative Fur-box identified upstream of the first gene in the *P. fragi* cluster is shown and consensus Fur-box sequences of *X. campestris* and *E. coli* (identical to the *P. aeruginosa* consensus Fur-box) are included for comparison. **(B)** Mid-point rooted phylogram of the consensus BI tree of the PvuA and PvsA-PvsE sequences of the 13 *P. fragi*, *A. vinelandii*, *X. campestris* and *V. parahaemolyticus*. *P. fragi* F1801 that was used in subsequent experiments is marked with an asterisk. The

subtree shown in the box represents BI branching on a different scale to the tree. Posterior probability values are shown as percentages at the nodes and scale bars denote the number of substitutions per site.

Previous work suggested that *P. fragi* does not synthesise siderophores (Champomier-Vergès et al., 1996), and utilising the succinate media (non-deferrated and deferrated forms) presented in the work, *P. fragi* F1801 displayed little to no growth and siderophore production of this strain was not observed (data not shown). Therefore in this study, a non-deferrated, modified M9 medium (MM9) was used containing glucose that was not present in the media used by Champomier-Verges *et al.* (1996). MM9 enabled both satisfactory growth, as demonstrated by growth of F1801 at 25 °C, while still ensuring conditions of iron-starvation, which can be seen by the pronounced increase in growth of F1801 in MM9 supplemented with 50 µM $FeCl_3$ (Figure 5.2A).

The sensitive CAS supernatant assay was used to detect the presence of siderophores in culture supernatants of F1801 grown at optimal growth temperature (25 °C) in MM9. The CAS assay is based on the removal of iron from the chromogenic dye CAS by siderophores, whereby a colour change is observed from blue to orange (Schwyn and Neilands, 1987). Figure 5.2B shows that siderophores were clearly detected in cell-free supernatants of iron-starved cultures of F1801. Reaction times for the exchange of iron from CAS were within 1.5 h, a time-frame consistent with carboxylate-type siderophores (Schwyn and Neilands, 1987). In contrast, when F1801 was grown in MM9 supplemented with 50 mM $FeCl_3$, siderophores were largely absent from culture supernatants (Figure 5.2B). Furthermore, expression of the vibrioferrin genes was heavily down-regulated (between 12- (*pvsE*) and 120-fold (*pvsD*) reduction) relative to their expression in MM9 without iron-supplementation (Figure 5.2C),

indicating repression of siderophore production under iron-rich conditions. These results are in agreement with other studies, which showed that vibrioferrin production in *A. vinelandii*, *Xanthomonas* and *V. parahaemolyticus* increased under iron-limitation, while under iron-replete conditions vibrioferrin production ceased (Pandey and Sonti, 2010, Funahashi et al., 2000, Pandey et al., 2017, McRose et al., 2017). Thus, together with the presence of a putative Fur-box upstream of the vibrioferrin gene cluster, these results suggest Fur-mediated regulation of the *P. fragi* vibrioferrin genes.

As a key meat and milk spoilage bacterium, *P. fragi* F1801 was tested for siderophore production at refrigeration temperature by monitoring growth and the accumulation of siderophores in supernatants at 4 °C over 4 days. Blue-coloured CAS solution has a maximum absorption at 630 nm. Orange-coloured CAS reaction mixtures, in which siderophores have removed iron from CAS, have essentially no absorbance at this wavelength and there is a largely linear dependence of A_{630}-values of CAS reaction mixtures versus concentration of the chelator (Schwyn and Neilands, 1987). Figure 5.2D shows a steady decrease in A_{630} values of CAS reaction mixtures over the 4 days, indicating an accumulation of siderophores in culture supernatants of F1801. This accumulation correlated well with cell growth of the strain, demonstrating siderophore production of *P. fragi* at a temperature relevant to the storage of meat and milk.

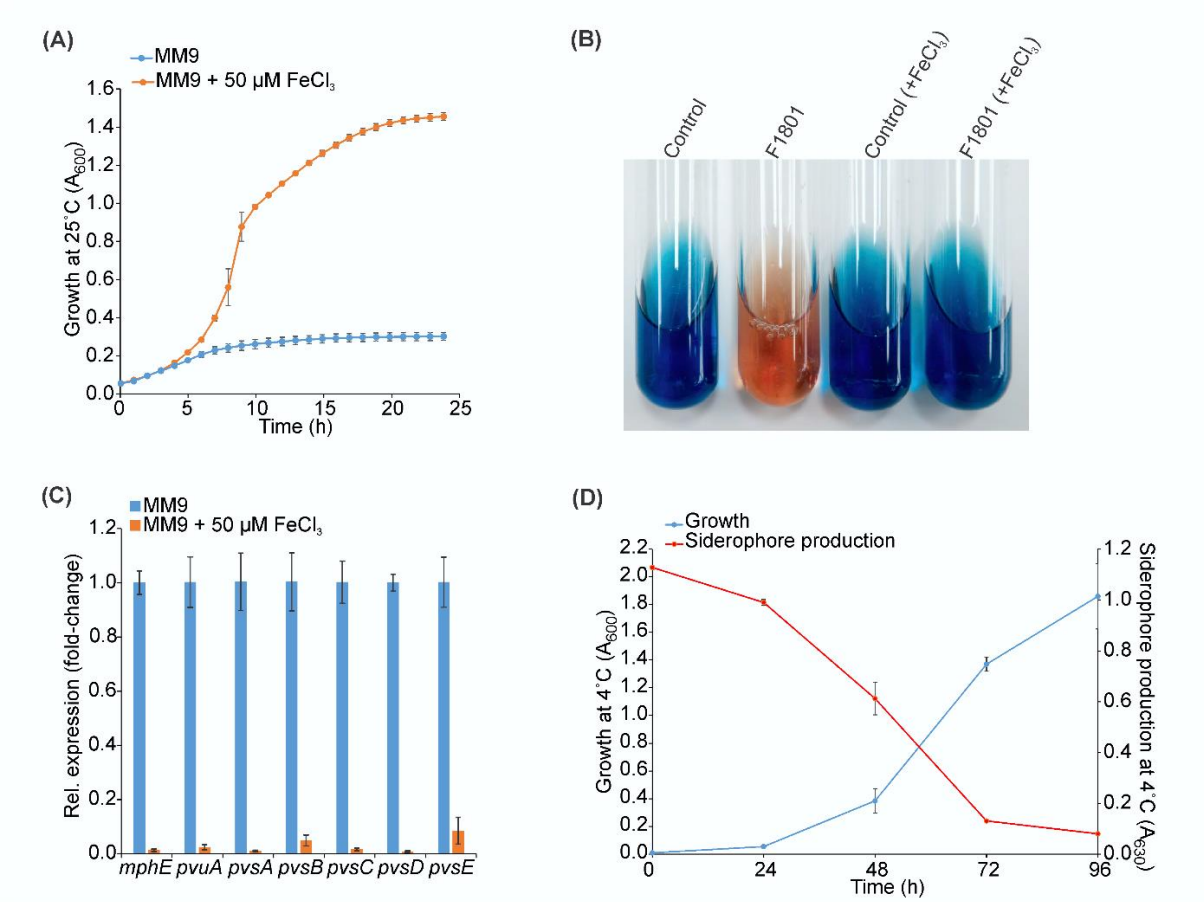

Figure 5.2: Siderophore production by *P. fragi* F1801 at 25 °C and 4 °C. (A) Growth of F1801 in MM9 or in MM9 that was supplemented with 50 µM $FeCl_3$. Optical density of the cultures was measured at 600 nm every hour in an EON microplate reader (BioTek). Data shown are the means and error bars the standard deviations of three biological replicates. **(B)** CAS assay results of F1801. Shown from left to right is CAS solution that was added to; sterile MM9 (control), filtered culture supernatant of F1801 grown in MM9 at 25 °C (F1801), sterile MM9 supplemented with 50 µM $FeCl_3$ (control (+$FeCl_3$)), and filtered culture supernatant of F1801 grown at 25 °C in MM9 supplemented with 50 µM $FeCl_3$ (F1801 (+$FeCl_3$)). **(C)** Relative quantification by qRT-PCR of the vibrioferrin genes in F1801 under iron-replete conditions (MM9 supplemented with 50 µM $FeCl_3$) compared to conditions of iron-starvation (MM9). Data shown are the means and error bars the standard deviations of three biological replicates. **(D)** Growth of and siderophore production by F1801 in MM9 at 4 °C. Optical density of the cultures and CAS assay absorbance measurements were carried out with a Novaspec Plus visible spectrophotometer (VWR). Data shown are the means and error bars the standard deviations of three biological replicates.

To confirm a role for the vibrioferrin gene cluster in siderophore-mediated iron acquisition, a gene disruption mutant of the TonB-dependent outer membrane siderophore receptor (Δ*pvuA*) was generated, as well as a control strain with wild-type vibrioferrin genes and a disrupted lipase gene (Δ*lip*) for subsequent experimental comparisons. qRT-PCR analysis revealed that expression of genes downstream of *pvuA* in the Δ*pvuA* mutant was markedly reduced (between 7- (*pvsE*) and 128-fold (*pvsA*) reduction) relative to the Δ*lip* control strain (Figure 5.3A), indicating polar effects caused by integration of the suicide vector in the *pvuA* gene. As our aim was to confirm a role for this gene cluster in siderophore-mediated iron uptake, rather than to study the function of the vibrioferrin genes, which has been addressed in other studies (Pandey and Sonti, 2010, Tanabe et al., 2003, Funahashi et al., 2002, Fujita et al., 2011, Tanabe et al., 2006, Pandey et al., 2017), the Δ*pvuA* mutant was used for further investigations.

A marked reduction in siderophore levels was detected in culture supernatants of Δ*pvuA* compared to the Δ*lip* control strain (Figure 5.3B). Gene deletion of *pvuA*, which encodes an outer membrane vibrioferrin receptor, should result in an accumulation of siderophores in culture supernatants. Therefore the observed reduction was likely due to the polar effects on the downstream biosynthesis genes. Genetic variants were not found in genes or in non-coding regions associated with the vibrioferrin genes or other iron-related genes of the Δ*pvuA* mutant's genome (Table C2: Appendix C), demonstrating that phenotypes of the Δ*pvuA* mutant were solely due to the disruption of the target gene and associated polar effects.

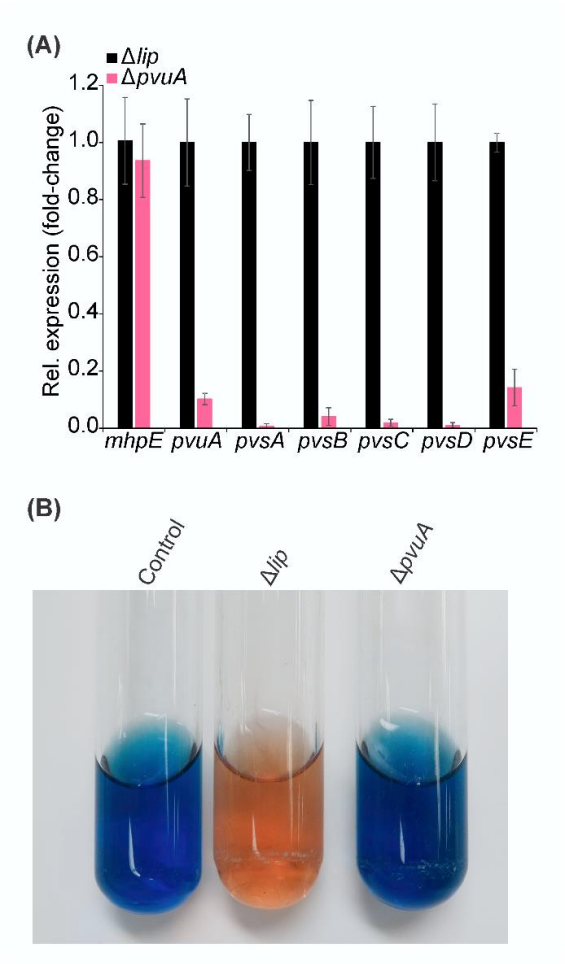

Figure 5.3: Δ*pvuA* shows impaired siderophore production. (A) Relative quantification by qRT-PCR of expression of the vibrioferrin genes in Δ*pvuA* compared with the Δ*lip* control strain. Data shown are the means and error bars the standard deviations of three biological replicates. **(B)** CAS assay results of the Δ*pvuA* mutant and Δ*lip* control strain. Shown from left to right is CAS solution that was added to; sterile MM9 (control), filtered culture supernatant of Δ*lip* grown in MM9 at 25 °C, and filtered culture supernatant of Δ*pvuA* grown in MM9 at 25 °C.

A clear growth defect of the Δ*pvuA* mutant was not observed in MM9 when compared to the Δ*lip* control strain (Figure 5.4A). Therefore, to understand the role of the vibrioferrin siderophore under low iron conditions, growth of the Δ*pvuA* and Δ*lip* strains was tested under more severe iron-depleted conditions by adding 25 µM of the iron chelator 2,2'-

bipyridyl (BP) to MM9. While growth of the Δ*lip* control strain was unaffected at 25 µM BP, addition of the ferrous iron chelator at these concentrations markedly impaired growth of the Δ*pvuA* mutant, demonstrating that the siderophore is involved in iron acquisition under low iron conditions.

To investigate whether the vibrioferrin siderophore could play a role in iron metabolism of *P. fragi* in foods such as meat and milk, where iron is sequestered by high-affinity iron-binding proteins, growth of Δ*pvuA* and Δ*lip* strains was compared in MM9, to which bovine apo-transferrin (apo-Tsf), as a model transferrin protein, was added. In all transferrin proteins, the carbonate (or bicarbonate) of the binding cleft is the synergistic anion, without which the protein loses its affinity for iron (Pakdaman and El Hage Chahine, 1997). Thus, to ensure the specific binding of Fe^{3+} by apo-Tsf, the growth media was also supplemented with 20 mM bicarbonate (BC) (Chung, 1984). BC at a concentration of 20 mM had no effect on the strains' growth when compared to their growth in MM9 without BC addition (Figure 5.4B). When the growth medium was supplemented with apo-Tsf at a concentration of 2.5 µM in the presence of 20 mM BC, growth, although slowed, was observed for the Δ*lip* control strain (Figure 5.4C). In contrast, growth of Δ*pvuA* was completely inhibited in the presence of apo-Tsf and BC, but could be rescued by supplementation of this media with 100 µM $FeCl_3$ (Figure 5.4D). These data suggest that the vibrioferrin siderophore may be capable of removing ferric iron from transferrin proteins.

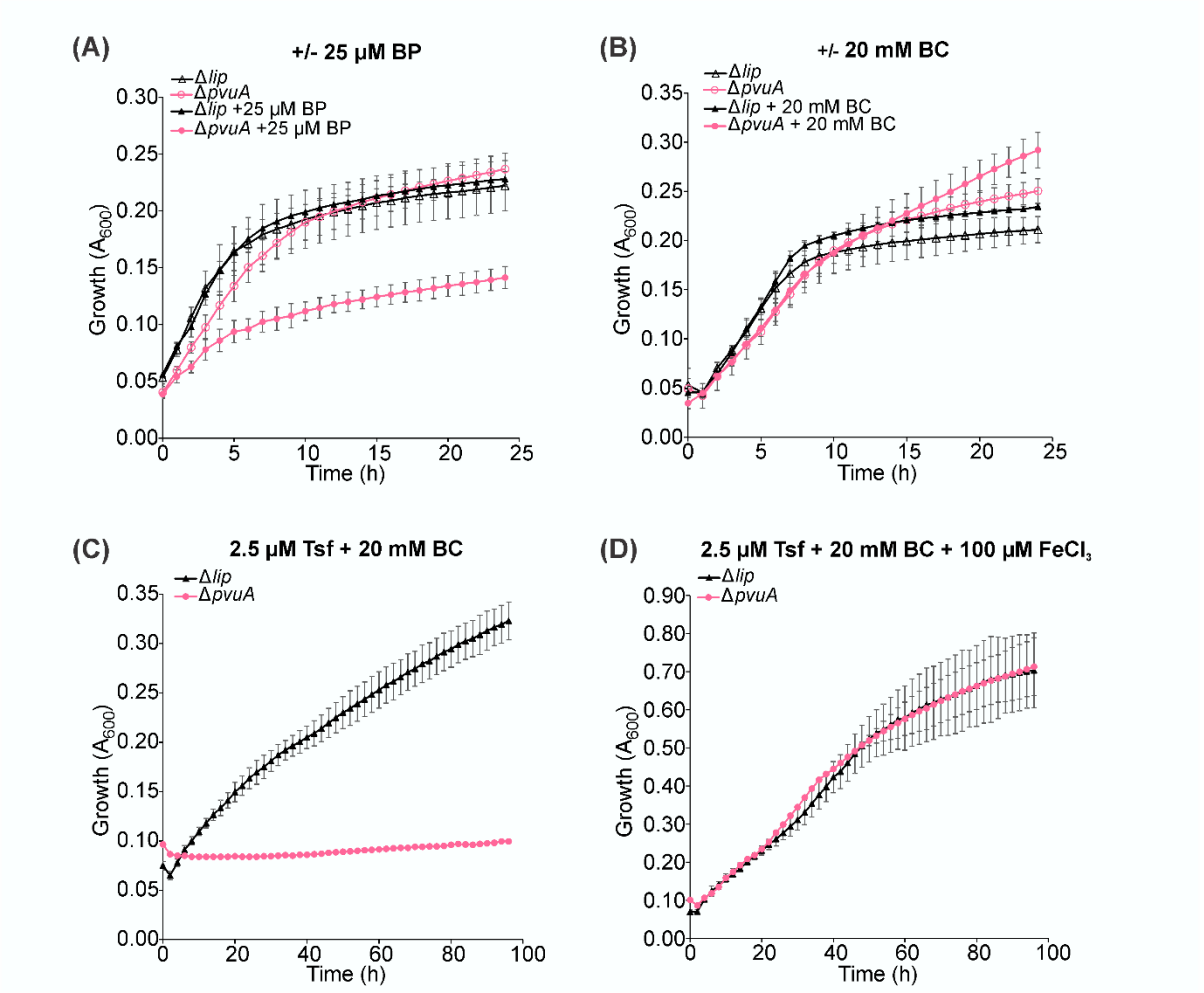

Figure 5.4: Vibrioferrin siderophore plays a role under iron-starvation and scavenges iron from transferrin. Growth of Δ*pvuA* and Δ*lip* control strain in MM9, and in MM9 supplemented with 25 µM 2,2'-bipyridyl (BP) **(A)**, in MM9, and in MM9 supplemented with 20 mM bicarbonate (BC) **(B)**, in MM9 supplemented with 2.5 µM bovine apo-transferrin (apo-Tsf) and 20 mM BC **(C)** and in MM9 supplemented with 2.5 µM apo-Tsf, 20 mM BC and 100 µM $FeCl_3$ **(D)**. Data shown for growth experiments in **(A–D)** are the means and error bars the standard deviations of three biological replicates. All experiments were repeated a minimum of three times with similar results observed.

Remarkably, despite considerable divergence between the six vibrioferrin protein sequences shared by *A. vinelandii* and *V. parahaemolyticus*, the absence of an *mhpE* gene in *V. parahaemolyticus*, and the absence of the vibrioferrin-uptake genes *pvuA(1)-pvuE* in *A.*

vinelandii, both bacteria were shown to produce identical vibrioferrin compounds (Baars et al., 2016, Yamamoto et al., 1994). Based on percent identities and the evolutionary relationship of the *P. fragi* vibrioferrin sequences with *A. vinelandii* and *V. parahaemolyticus* counterparts, it is likely that the *P. fragi* siderophore is an α-hydroxycarboxylate type siderophore, similar to or identical to vibrioferrin.

Conclusion

A vibrioferrin gene cluster was identified in 13 strains of the food spoilage bacterium *P. fragi* and siderophore production of *P. fragi* F1801 was observed. Based on the homology of the identified genes, *P. fragi* likely excretes an α-hydroxycarboxylate type siderophore, similar to or identical to vibrioferrin, which plays a role in iron-metabolism of this bacterium under low iron conditions. More experiments are required to determine whether the siderophore contributes to the successful growth and colonisation of milk and meat by *P fragi*; however, these data suggest that the vibrioferrin siderophore is capable of removing ferric iron from transferrin proteins.

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Chapter 6

The effect of a low field strength pulsed electric field on the aerobic microbiota of beef

ABSTRACT

This study aimed to investigate the effect of a low field strength pulsed electric field (PEF) (0.25 kV/cm, 3000 pulses), with potential for beef tenderisation, on the aerobic microbiota of beef stored at 4 °C. The microbial load of naturally contaminated PEF-treated and -untreated samples was compared along with assessing potential damage to muscle cells and enhanced proteolysis using reversed phase-high performance liquid chromatography (RP-HPLC). For time points up to and including 3 days post-treatment, little difference was detected in the mean log cfu/g of treated and untreated samples. Later time points indicated that PEF may promote microbial growth, as differences in the sample means were observed. On day-eight and -nine post-processing, these differences were statisically significant (P<0.05). RP-HPLC showed no differences in the profiles of phosphate buffered saline-soluble compounds derived from the meat surface of treated and untreated samples 2 hours post-treatment. In summary, the tested PEF-settings slightly enhanced growth of the aerobic microbiota, but evidence for muscle cell leakage and enhanced proteolysis was not obtained with the applied method.

INTRODUCTION

Meat tenderness is recognised as the most important meat eating quality attribute and therefore it has a significant impact on the value of meat cuts and the re-purchase intent of consumers (Miller et al., 2001, Troy and Kerry, 2010). This drives a demand for processing technologies that can improve meat tenderness of low value cuts and ensure the consistency of high value muscles.

The emerging food processing technology, pulsed electric field (PEF), refers to the use of very short pulses (µs to ms range) of low to high electric field intensity (0.1-50 kV/cm) to foods (Buckow et al., 2013, Toepfl et al., 2014). PEF-treatment induces external electric fields, which cause an increase in the electric potential across cell membranes (Zimmermann et al., 1976). If the electric field intensity exceeds a critical limit then electroporation of cells, disruption of cell organelles and further structural changes to foods can occur (Zimmermann et al., 1976, Toepfl et al., 2014, Gudmundsson and Hafsteinsson, 2001). While high field strength PEF (20-50 kV/cm) is effective for microbial inactivation in fruit juices owing to the disruption of microbial cell membranes (Buckow et al., 2013), it is not suitable as a decontamination method for muscle foods. The absence of a protective cell wall and larger size renders muscle cells highly susceptible to PEF-induced damage (Hülsheger et al., 1981, Grahl and Märkl, 1996). Low field strength PEF is however considered to have potential to improve meat tenderness due to limited muscle cell disruption, potential accelerated release of Ca^{2+} and proteases post-mortem and stimulation of the glycolysis process, all of which could contribute to enhanced post-mortem proteolysis (Suwandy et al., 2015, Bekhit and Hopkins, 2014). A real advantage of this technology over other available methods is the ability to modify the processing parameters to treat different

meat cuts, which may be translated into quality upgrading of low value cuts (Bekhit et al., 2014b).

Due to muscle cell damage and enhanced proteolysis, PEF-treatment could promote the development of meat spoilage owing to an increased availability of nutrients and precursor metabolites of microbial spoilage. Currently, the impact of low field strength PEF on the microorganisms that grow on beef during storage is unknown. Microorganisms that play a major role in the spoilage of aerobically stored, refrigerated meat are psychrotrophic *Pseudomonas*, whose growth is promoted by oxygen and chilled temperatures (Gill and Newton, 1978, Hernández-Macedo et al., 2011b). Mesophiles, although initially present on carcasses, do not grow at chilled temperatures and are quickly outgrown by psychrotrophic members of the microbiota (Gill and Newton, 1978). In this context, the aim of this work was to investigate the effects of a low field strength PEF-treatment (0.25 kV/cm, 3000 pulses) with potential for beef tenderisation on the aerobic microbiota developing on beef stored aerobically at 4 °C. The microbial load of naturally contaminated PEF-treated and -untreated samples was compared over the storage period of 9 days and reversed phase-high performance liquid chromatography (RP-HPLC) was used to determine potential damage to muscle cells and connective tissue of samples following PEF-treatment.

MATERIALS AND METHODS

Meat sampling and preparation

Beef topside, muscle *M. semimembranosus*, was sourced from a local butcher. The muscle was trimmed of excess fat and connective tissue and 20 samples of an average size of 65 x 30 x 25 mm were cut and weighed. Of the 20 portions, 10 of these pieces were randomly

chosen for PEF-treatment (i.e. 10 treated replicates) and 10 were randomly chosen as untreated control samples (i.e. 10 control replicates).

PEF equipment and treatment of meat

A Diversified Technologies Power ModTM 25 kW Pulsed Electric Field System (Diversified Technologies, Inc., Bedford, MA, USA) was used, consisting of an adjustable treatment chamber and a modulator cabinet designed and manufactured by CSIRO for the application of pulsed electric fields to solid foods. The height of the inside of the treatment chamber was 40 mm, the gap between the parallel plate electrodes was set to 65 mm and Teflon blocks were inserted in the sides of the chamber to define a width of 30 mm. To eliminate temperature effects, the PEF-treatment chamber and the 20 beef portions were placed in separate sterile Stomacher bags and cooled to approximately 1-2 °C in an ice slurry prior to treatment. The PEF-treatment chamber was then dried of excess water and the samples were placed into the treatment chamber ensuring there were no air pockets at the surface of the electrodes.

Only one set of parameters were tested. These settings represented the harshest of a range of previously trialled PEF-conditions with meat tenderisation potential that did not result in temperature effects (Bolumar, T. G., personal communication): Square wave pulses of 10 µs width at a peak voltage of 1,500 V were applied, resulting in an electrical field strength of 0.25 kV/cm. The pulse repetition rate was set to 100 Hz and PEF was applied to the meat for a treatment time of 30 ms.

Following PEF-treatment, samples were removed from the chamber, placed in separate sterile Stomacher bags and kept at 4 °C. Handling of the control samples was identical to treated samples with the exception of the PEF-treatment.

Microbiological analysis

Microbiological analysis of the samples was carried out over a period of 9 d. Sampling took place every 24 h, beginning at 2 h post PEF-treatment: 47.5 mL sterile Dulbecco A phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK) were added to the Stomacher bags containing the samples. Samples were washed by vigorously shaking the bags for 30 s in a horizontal direction. Washing buffer was then aspirated from the bags and ten-fold dilutions of the buffer were prepared. 1 mL of the dilutions was spread in duplicate onto aerobic plate count Petrifilm (3M, Maplewood, USA). To ensure good recovery and growth of psychrotropic spoilage bacteria, petrifilms were incubated at 25 °C for 72 h.

To compare the aerobic populations of microorganisms between treated and untreated samples with a limited number of samples, following aspiration of the washing buffer, washed samples were not discarded. Instead samples were aseptically placed into fresh, sterile Stomacher bags, returned to storage at 4 °C and sampled again as described at each time point.

For each sample at each time point, cfu were enumerated and converted to $\log_{10}$ cfu/g of the meat sample. Mean $\log_{10}$ cfu/g values were calculated for treated and untreated samples for each time point and compared using a two-sample T-test (assuming unequal variances). Statistical significance was assigned to comparisons with a *P* value below 0.05. The Baranyi model (Baranyi and Roberts, 1994) was fitted to the growth curves of the means of the treated and non-treated samples using DMFit (v3.5 [ComBase; http://www.combase.cc/index.php/en/tools]) to calculate the kinetic growth parameters: maximum growth rate ($\log_{10}$ cfu/h), maximum population density ($\log_{10}$ cfu/g) and lag time

(h). These parameters were compared using a two sample z-test with a significance level of $\alpha<0.05$ (critical z-score values: -1.96 and +1.96).

Reversed phase-high performance liquid chromatography (RP-HPLC)

PBS-soluble compounds derived from the washing buffer of the 10 treated and 10 untreated samples 2 h post-treatment were analysed using RP-HPLC. Extracts were filtered using 0.2 µm cellulose acetate filters (Advantec MFS, Dublin, USA) before analysis. The filtered extracts were chromatographed by an automated Shimadzu system (consisting of two LC-10AD VP pumps, FCV-10AL VP mixer, DGU-14A degasser and SIL-10 VP auto injector; Shimadzu Corp., Kyoto, Japan) fitted with a Vydac, Everest C18 column (250x4.6 mm 300A 5um; The Separations Group Inc, CA, USA). The samples were chromatographed using a multi-step solvent gradient of solvent B (0.1% trifluoroacetic acid in 90% acetonitrile in water) in solvent A (0.1% trifluoroacetic acid in deionised water). Samples were eluted using a gradient elution method: the concentration of solvent B was increased from 27% to 32% in the first 2 min, to 39% in the next 15 min, to 42.4% in the following 13 min, to 50% in the subsequent 15 min, and finally increased to 60% in the next 3 min and maintained for 5 min. The column temperature and solvent flow rate was maintained at 40 °C and 0.7 mL/min, respectively. Eluate was monitored at 214 nm using a SPD-10A vp UV-VIS detector (Shimadzu Corp., Kyoto, Japan). The chromatography system was interfaced with computer Class VP 7.3 software (Shimadzu Corp., Kyoto, Japan).

RESULTS AND DISCUSSION

The sample means of the log cfu/g for PEF-treated and -untreated beef samples are plotted in Figure 6.1 (n=10 for both treated and untreated beef samples). The initial microbial load

of the samples was determined two hours post-treatment, demonstrating that the observed mean starting population density was 1.84 ± 0.28 log cfu/g and 1.88 ± 0.14 log cfu/g for the treated and untreated samples, respectively (Table 6.1).

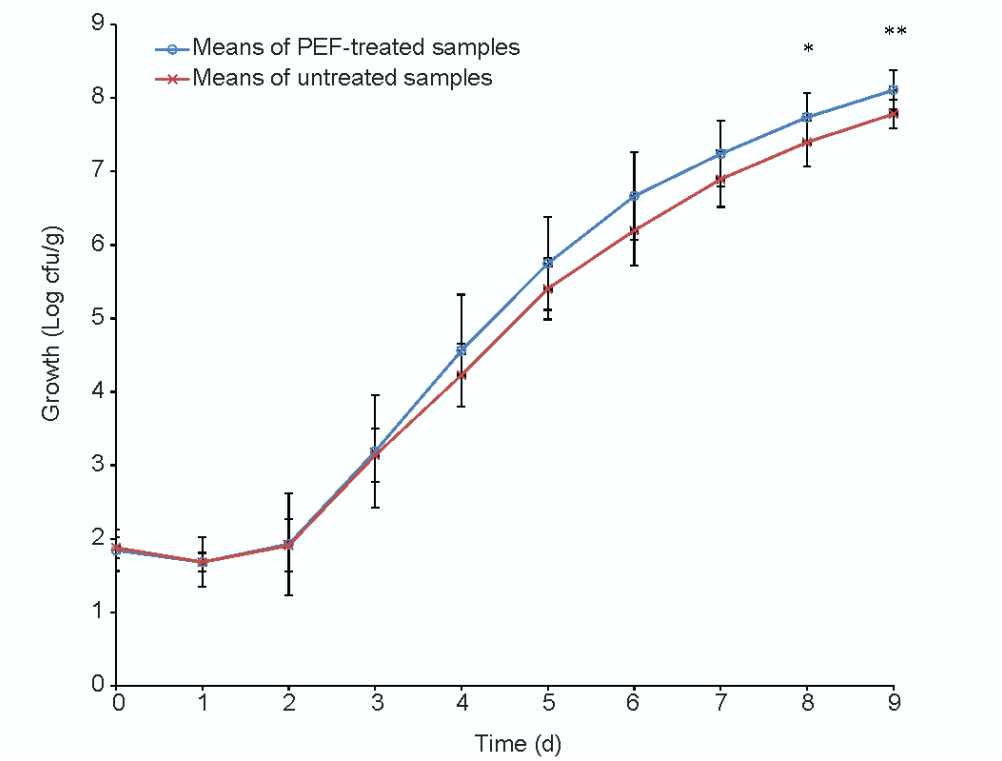

Figure 6.1: Growth promotion of aerobic microorganisms at late storage time points of PEF-treated samples. Shown are the mean curves of aerobic microbial counts of aerobically stored PEF-treated and -untreated beef samples (n=10 for both treated and untreated samples) determined over the storage period of 9 d at 4 °C. The blue curve shows the means of PEF-treated samples, while the red curve represents the means of untreated samples. On day-eight and -nine post-treatment, the difference in the means of the log cfu/g of the PEF-treated and -untreated beef samples were statistically significant. *: *P* value of 0.03, **: *P* value of 0.01.

Table 6.1: Aerobic microbial counts and statistical comparison.

	Log cfu/g[a]		T-test assuming unequal variances
Time points (d)	**Treated samples**	**Untreated samples**	***P* (T<=t) two-tail**
0	1.84 ± 0.28	1.88 ± 0.14	0.74
1	1.68 ± 0.34	1.68 ± 0.12	0.10
2	1.93 ± 0.69	1.91 ± 0.36	0.95
3	3.19 ± 0.77	3.13 ± 0.36	0.84
4	4.56 ± 0.76	4.23 ± 0.43	0.25
5	5.75 ± 0.63	5.41 ± 0.42	0.17
6	6.67 ± 0.60	6.20 ± 0.47	0.07
7	7.24 ± 0.45	6.90 ± 0.38	0.08
8	7.74 ± 0.33	7.40 ± 0.33	0.03
9	8.12 ± 0.27	7.78 ± 0.19	0.01

[a] Values are means ± standard deviation of 10 replicates.

The sample means of the log cfu/g remained similar for times up to and including 3 days post-processing, after which, the means of the treated samples were consistently higher than those of the controls. After day-five, the means of the treated samples were more than two-fold greater than those of the control samples at all time points, and on day-eight and -nine post-treatment, statistically significant differences (P <0.05) in the sample means were observed, indicating that the PEF-treatment may enhance microbial growth. Interestingly, despite the potential stress conditions exerted by the PEF-treatment, no noticeable difference in the lag phase of the growth curves was observed.

Using the Baranyi model, the growth curves were fitted to obtain estimates of the kinetic growth parameters of the curves. For the fitted curves of the treated and untreated

samples, a lag parameter of 38.7 and 41.6 h, a maximum specific growth rate of 0.04 and 0.05 log cfu/h, and a maximum population density of 7.68 and 7.88 log cfu/g were observed, respectively (Table 6.2). As demonstrated by z-scores within the critical z-score values (-1.96 and +1.96), these minor differences in the three growth parameters between the treated and untreated samples were not statistically significant (Table 6.2).

Table 6.2: Kinetic growth parameters determined by DMFit and statistical comparison.

Kinetic parameters	Treated samples	Untreated samples	z-score[a]
Lag parameter	41.6	38.7	0.21
Maximum growth rate	0.05	0.04	0.93
Maximum population density	7.88	7.68	0.69

[a] z-score: as calculated by a two-sample z-test.

Mild PEF-processing of muscle meat may result in differences in the types and amounts of compounds that are present on the meat surface, such as myocyte constituents due to cell disruption and peptides resulting from enhanced proteolytic activity (Gudmundsson and Hafsteinsson, 2001, Suwandy et al., 2015). To investigate whether the microbial growth promotion that was observed for the treated samples could be attributed to an increase in available nutrients, PBS-soluble compounds derived from the beef sample surfaces two hours post-treatment were analysed by RP-HPLC. Representative chromatograms of a treated and untreated sample are shown in Figure 6.2. Comparison of the chromatogram profiles showed that all peaks associated with the treated samples were also present in the profiles of the untreated samples. Furthermore, significant differences in the mean peak heights and peak areas of each peak were not found. As these results did not indicate

changes to the types and amounts of PBS-soluble compounds that were present on the surfaces of the treated samples, no further analysis of the compounds of these peaks was undertaken. It is possible that longer incubation of the samples post-treatment may be required to detect PEF-induced cell leakage and enhanced proteolysis.

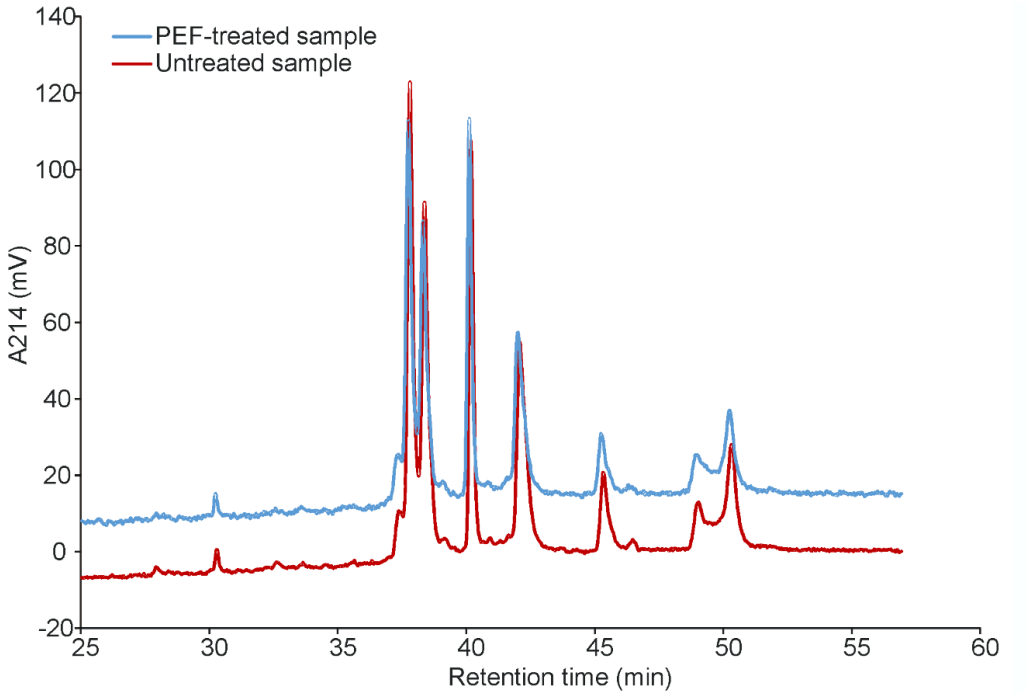

Figure 6.2: Representative RP-HPLC chromatograms of PBS-soluble compounds from the surface of a PEF-treated and untreated beef sample show no notable profile changes. PBS-soluble compounds derived from the surface of PEF-treated and -untreated beef samples 2 h post-treatment were analysed by RP-HPLC using a UV detector (214 nm). Representative chromatograms of a treated sample (blue chromatogram) and an untreated sample (red chromatogram) show that profiles were highly similar, indicating no detectable changes to the types and amounts of PBS-soluble compounds that were present on the surfaces of the treated samples.

In summary, the mild PEF-settings tested here had only a minor impact on the development of the aerobic microbiota of the beef samples post-treatment. A slight growth enhancement

was observed for processed samples, however significant differences were only seen at late time points, where the microbial counts were at levels high enough to characterise the meat as spoiled (Nychas and Drosinos, 2014). Furthermore, no differences in the profiles of PBS-soluble compounds derived from the meat surface of treated and untreated samples were detected. Thus, cell leakage and enhanced proteolysis, as a result of PEF-processing, were not observed with the PEF-conditions and methods used in this study. Due to the emerging interest in this technology for its potential to improve quality attributes of meat, the minor growth enhancement of aerobic microorganisms on treated samples observed in this work, and the limited scope of this study, further work addressing the impact of low field strength PEF on the microbiota of meat is warranted. In particular, a larger sample size enabling the use of conventional microbiology testing, a greater range of tested PEF-settings, and testing of different microbial subpopulations and longer incubation times following treatment should be trialled.

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Chapter 7

General discussion

Fresh meat is a highly nutritious commodity, conducive to the rapid growth of microorganisms and spoilage. Despite the negative economic and social consequences associated with meat spoilage, the physiology of many of the microorganisms involved in meat spoilage remains poorly studied. The objective of this thesis was to improve current knowledge of the biology of the important meat spoilage bacteria *B. thermosphacta*, *P. fragi* and *P. lundensis*, which is critical to ensure improvements in the control of meat contamination and spoilage, and to facilitate shelf-life extension.

Advances in sequencing technologies and the field of genomics has enabled rapid and affordable whole genome sequencing and analysis of bacterial genomes. Not only do genome sequences facilitate reliable identification of bacteria, but access to the genome can provide crucial insight into the evolution of bacteria, their metabolic potential, environmental adaptability, resistance mechanisms and more. Analysis of genome sequences of the meat spoilage bacterium *B. thermosphacta* were lacking in the literature. Therefore chapter 2 (Stanborough et al., 2017a) focused on analysing draft genomes of 12 strains of *B. thermosphacta* and compared these to genomes of *B. campestris* and *L. monocytogenes.* Data from these analyses revealed a high degree of genomic similarity among the 12 strains, with bacteriophage genes representing a large proportion of their accessory genome. Particularly striking was the genomic similarity between the type strain of this species and the other strains, considering both the temporal and geographic

differences between them. In the future, sequencing of more strains of this organism will shed light on the overall intraspecies diversity.

Links between previously published phenotypic data on *B. thermosphacta*, as well as data provided during this study, and the gene inventory of this bacterium were explored. For example *B. thermosphacta* is commonly associated with cheesy, buttery, sweaty odours, and the production of the metabolites acetate, acetoin, butanediol and branched-chain fatty acids isobutyric, isovaleric and 2-methylbutyric acid (Dainty and Hibbard, 1983, Dainty and Hofman, 1983, Grau, 1983, Casaburi et al., 2014). Strong evidence existed for the production of these branched-chain fatty acids from amino acids rather than lipolysis (Dainty and Hibbard, 1983). The results of this study confirmed that genes for the conversion of the branched-chain amino acids leucine, valine, and isoleucine to their respective branched-chain fatty acid moieties were present in both *Brochothrix* species. Genes for the production of acetoin, butanediol and additional metabolic end-products from pyruvate such as ethanol, lactic acid and acetate were also identified in both species. As a previous study showed that *B. campestris* can cause spoilage characterised by off-odours and green drip (Gribble and Brightwell, 2013), and genes for the production of acetoin and butanediol were also identified in the *B. campestris* genome in this work, further emphasis should be placed on determining whether *B. campestris* also plays a role in meat spoilage.

Contrary to available data, amino acid decarboxylase genes were not found in the *Brochothrix* genomes and BA production by the *B. thermosphacta* strains was not observed. As the production of BA by *B. thermosphacta* strains has been observed in previous studies (Casaburi et al., 2014, Nowak and Czyzowska, 2011, Paleologos et al., 2004), it is likely that

the production of these compounds by this bacterium is strain-dependent. Considering the spoilage impact of BA like cadaverine and putrescine and the health implications associated with the secretion of BA into food environments, further studies on BA production of *B. thermosphacta* strains and the responsible gene repertoire are warranted. An interesting starting point would be to examine the genomes of known BA-producing strains for the presence of amino acid decarboxylase genes.

Analysis of antibiotic susceptibility of the 12 strains showed that the bacteria were sensitive to the four tested antibiotics, except for one tetracycline-resistant strain with plasmid-mediated tetracycline resistance genes. The strains were also tested for their tolerance to copper, cobalt and cadmium, however, tolerance levels were not high enough to categorise the strains as resistant. Literature searches identified only a limited number of studies on the prevalence of resistance genes in non-pathogenic, meat-associated bacteria. Monitoring of resistance genes in these bacteria should be addressed in the future for the following reasons: 1) increasing concern about bacterial cross-resistance to antibiotics, disinfectants and/or heavy metals, 2) the relative ease with which resistance genes can be transferred between organisms, 3) the use of antibiotics in animal husbandry and, 4) *B. thermosphacta* and other meat-associated bacteria share their environmental niche (i.e. food, food processing environment, abattoir, farm) with human and livestock pathogens.

A search for *L. monocytogenes* virulence gene orthologues in the *Brochothrix* genomes showed key virulence genes were absent, consistent with the lack of evidence to suggest that *Brochothrix* have pathogenic potential. However, orthologues of other *L. monocytogenes* virulence genes such as factors involved in surface protein anchoring (*srtA*, *srtB*) and intracellular survival, as well as stress-response regulatory genes (σ^B and σ^B-related

genes) were found, which could be involved in spoilage-relevant pathways and play a role in the persistence of this organism in food processing environments. Knowledge of these genes led to the work comprising chapter 3 and could also be applied for future studies, especially considering genes such as σ^B, whose activation during food processing steps or abiotic surface cleaning, could have consequences for increased stress resistance and persistence of the bacteria in food and food processing environments.

The discovery of an orthologue of the *L. monocytogenes srtA* (involved in surface protein anchoring) in the *B. thermosphacta* genomes formed the basis of the work presented in chapter 3, in which the putative BtSrtA was characterised biochemically and potential substrate proteins were determined. Successful expression and purification of a recombinant, truncated version of this enzyme was achieved. Investigations of its catalytic activity showed the BtSrtA was capable of attaching pseudo-substrates containing LPXTG sorting motifs to a cell wall mimic, demonstrating *in vitro* SrtA bioconjugation activity. In addition, genome examination of *B. thermosphacta* identified 11 potential BtSrtA substrates, two of which contained protein domains associated with adherence of pathogens to host extracellular matrix proteins and cells. Clearly the attachment of these identified substrates by BtSrtA needs to be confirmed in future work, but this study provides a robust starting point for investigating surface protein anchoring in *B. thermosphacta*. As surface proteins mediate interactions of bacteria with their environment, and two of the substrate proteins identified in this work contained conserved domains that are involved in adhesion of pathogens to host cells and ECM, further work in this direction could provide crucial insight into molecular mechanisms involved in the colonisation of meat by *B. thermosphacta*.

The most important meat spoilage bacteria under aerobic conditions are psychrotrophic *Pseudomonas* species. In particular, the species *P. fragi* and *P. lundensis* constitute a significant proportion of the aerobic spoilage microbiota, but remain surprisingly poorly studied and genome analyses of these bacteria were missing. Therefore chapter 4 (Stanborough et al., 2018) aimed to provide insight into genomes of *P. lundensis* and *P. fragi*. The genome analyses showed that the isolates included in this work may belong to more than two *Pseudomonas* species with possible spoilage potential. These results highlight the existing difficulty of species identification of this group and suggest that the exact composition of aerobic meat spoilage communities remains to be elucidated. To improve our understanding of psychrotrophic *Pseudomonas* species associated with meat, future work will need to focus on increasing the availability of genome sequences of these bacteria and systematic studies of the phylogeny of this group will be required.

As very limited information was available on the odour-active compounds produced by *P. lundensis*, and a thorough understanding of the metabolic potential of *P. fragi* at the strain level was missing, the second aim of this chapter was to compare the metabolic profiles of the *P. lundensis* and *P. fragi* isolates during their growth on a beef matrix. Most of the organisms produced significant levels of odour-active compounds, however three *P. fragi* strains produced extremely low levels of VOCs, which suggested that not all strains of presumed spoilage species have the same spoilage potential. These results demonstrate that strain-specific studies in the meat environment are required to better understand the heterogeneity of strains of the same species as well as the heterogeneous behaviour of strains in general. The *P. fragi* isolates were associated in particular with the production of ethyl and methyl esters, which is in line with previous studies on this bacterium (Casaburi et al., 2015, Ercolini et al., 2010, Morales et al., 2005a). The *P. lundensis* isolates produced

substantial amounts of 2-nonanone and methyl-2-butenoic acid, confirming that isolates of this species can be significant producers of odour-active ketones (Morales et al., 2005b) and demonstrating how isolates of this species may contribute to the spoilage of meat. 1-undecene production was ubiquitous among isolates of both species, further confirming that production of this compound is a feature of *Pseudomonas* species, and as such, 1-undecene may have potential to be a marker compound for acceptability/degree of spoilage (Rui et al., 2014, Dainty et al., 1984).

The work presented in chapter 5 (Stanborough et al., 2017b) was initiated following the discovery of putative siderophore biosynthetic genes in the *P. fragi* genomes from chapter 4. *P. fragi* was thought not to produce siderophores (Champomier-Vergès et al., 1996) and is described as not producing siderophores in detectable amounts in Bergey's Manual (Garrity et al., 2005). Not only is iron acquisition a fundamental aspect of the biology of microorganisms, but efficient iron acquisition may play a role in the dominance of this bacterial species in spoilage communities. Results of this chapter showed *P. fragi* likely secretes an α-hydroxycarboxylate type siderophore, similar to or identical to vibrioferrin, which plays a role in iron-metabolism of this bacterium under low iron conditions. Remarkably, both *A. vinelandii* and *V. parahaemolyticus* were shown to produce vibrioferrin siderophores with identical structures, despite considerable divergence between their vibrioferrin protein sequences and the absence of the vibrioferrin-uptake genes in *A. vinelandii* (Baars et al., 2016, Yamamoto et al., 1994). Next steps following on from this research could be to purify and determine the chemical structure of the *P. fragi* siderophore, which is required to understand its mechanism of iron coordination. To investigate whether the siderophore contributes to the successful growth of *P fragi* on meat, expression of the vibrioferrin genes should be tested during its growth on meat.

Furthermore, mutants deficient in siderophore production could be used to determine whether the vibrioferrin siderophore contributes to providing *P. fragi* with a competitive advantage over other members of the meat microbiota. For example, growth of mutant and wild type strains could be compared in single or multiorganism biofilms on meat.

Meat tenderness is considered the most important quality attribute contributing to consumer choice and thus considerable interest exists for the development of novel technologies, which can improve the tenderness of meat. As PEF is a processing technology with potential for meat tenderisation, but also with a potential to enhance meat spoilage, a small study was conducted on the effects of a low field strength PEF on the aerobic microbiota of beef. The mild PEF-settings tested in this work had only a minor impact on the total aerobic microbial counts of treated samples. A slight growth enhancement was observed for PEF-processed samples, however significant differences were only seen at time points, where the microbial counts were at levels high enough to characterise the meat as spoiled. Furthermore, cell leakage and enhanced proteolysis as a result of PEF-processing was not observed with the PEF-conditions and methods used in this work. Due to the emerging interest in this technology for its potential to improve quality attributes of meat, the minor growth enhancement of aerobic microorganisms on treated samples observed in this work, and the overall limited scope of this study, further work addressing the impact of low field strength PEF on the microbiota of meat is warranted. Larger sample sizes would be required to enable a conventional approach to microbiology testing, and to account for variations in the application of PEF and the heterogeneity of meat between samples. The effects of low field strength PEF should also be tested on different subpopulations of the meat microbiota and systematic testing of various PEF settings would be needed. Additionally, a longer incubation time following treatment may reveal increased proteolysis.

In conclusion, the genomic and physiological studies of key meat spoilage bacteria that form this thesis enhance knowledge of these poorly studied bacteria by: 1) providing insight into genomic and metabolic diversity of strains of these bacteria, 2) demonstrating how strains of these bacteria may or may not contribute to spoilage by the production of various malodourous compounds, 3) providing a starting point for further studies on surface protein attachment of *B. thermosphacta* and understanding mechanisms with which this bacterium interacts with its environment and 4) by revealing a siderophore-mediated iron acquisition system of *P. fragi*.

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Appendix A

Chapter 2 - Comparative genomic study of the meat spoilage organism *Brochothrix thermosphacta*

Table A1. OrthoANI values[a].

Genome 1	Genome 2	OrthoANI (%)
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7803	99.19
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7804	99.09
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7806	99.11
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7807	99.00
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7808	99.20
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7809	98.99
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7810	99.26
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7811	99.23
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7813	99.12
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7816	99.54
B. thermosphacta DSM 20171^T	*B. thermosphacta* 7818	99.14
B. thermosphacta DSM 20171^T	*B. campestris* DSM 4712^T	75.05

[a] OrthoANI (Orthologous Average Nucleotide Identity) between *B. thermosphacta* DSM 20171^T and the draft genomes (listed in column two) is shown in column three.

Table A2. DDH estimates[a].

	Formula 2[b]			
Reference genome	**DDH (%)**	**Model C.I. (%)**	**Prob. DDH >= 70% (%)**	**G+C difference[c]**
B. thermosphacta 7803	92.4	90.4–94.0	96.54	0.12
B. thermosphacta 7804	91.9	89.9–93.6	96.41	0.13
B. thermosphacta 7806	91.8	89.7–93.5	96.36	0.12
B. thermosphacta 7807	90.7	88.5–92.6	96.04	0.00
B. thermosphacta 7808	92.6	90.6–94.2	96.59	0.07
B. thermosphacta 7809	91.1	88.9–92.9	96.16	0.04
B. thermosphacta 7810	93.1	91.2–94.6	96.73	0.02
B. thermosphacta 7811	92.5	90.4–94.1	96.55	0.03
B. thermosphacta 7813	92.4	90.4–94.0	96.53	0.16
B. thermosphacta 7816	92.6	90.6–94.2	96.59	0.13
B. thermosphacta 7818	92.5	90.5–94.1	96.56	0.09
B. campestris DSM 4712^T	20.6	18.3–23.0	0.00	3.81

[a] DDH (digital DNA:DNA hybridisation) estimates determined for the comparison of the draft genome of *B. thermosphacta* DSM 20171^T to the listed reference draft genomes in column one.

[b] DDH values, which correlate well with wet lab percentages are shown in the second column. In column three, model-based confidence intervals (Model C.I.) are listed followed by the probability that DDH values are >70% in column four.

[c] GC-differences between the query (*B. thermosphacta* DSM 20171^T) and reference genomes, which cannot differ by more than one within a single species.

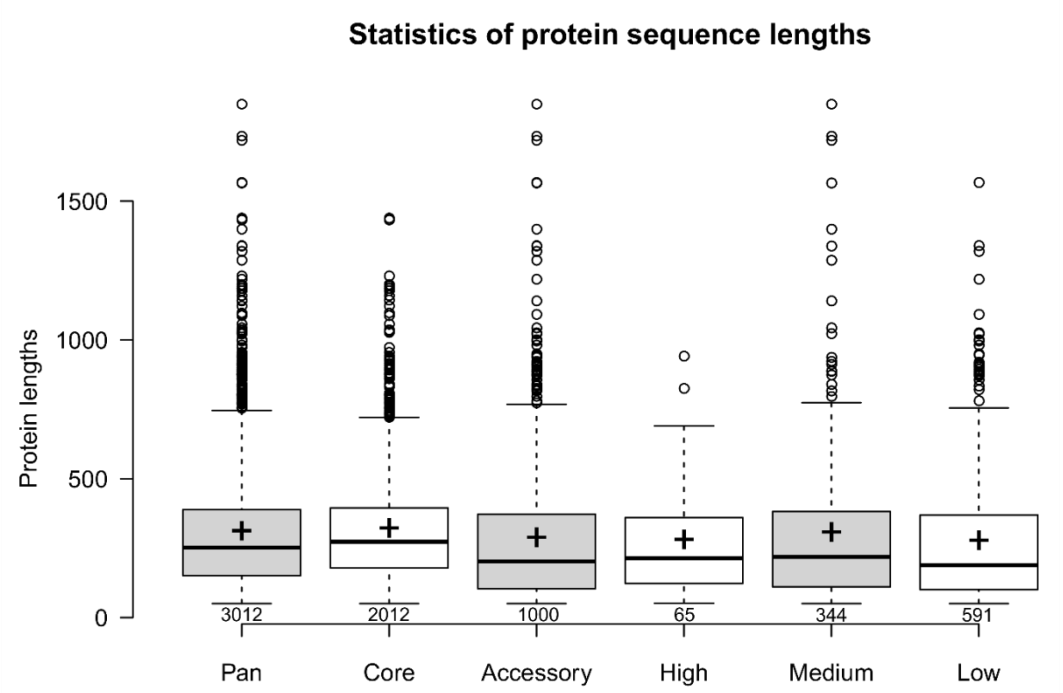

Figure A1. Statistics of protein sequence lengths. Box plots of protein lengths of all predicted coding regions in the pan-genome of the 12 *B. thermosphacta* strains are shown. Crosses represent sample means and whiskers extend 1.5 times the interquartile. Outliers are represented by circles. The number of protein sequences analysed are listed below each plot.

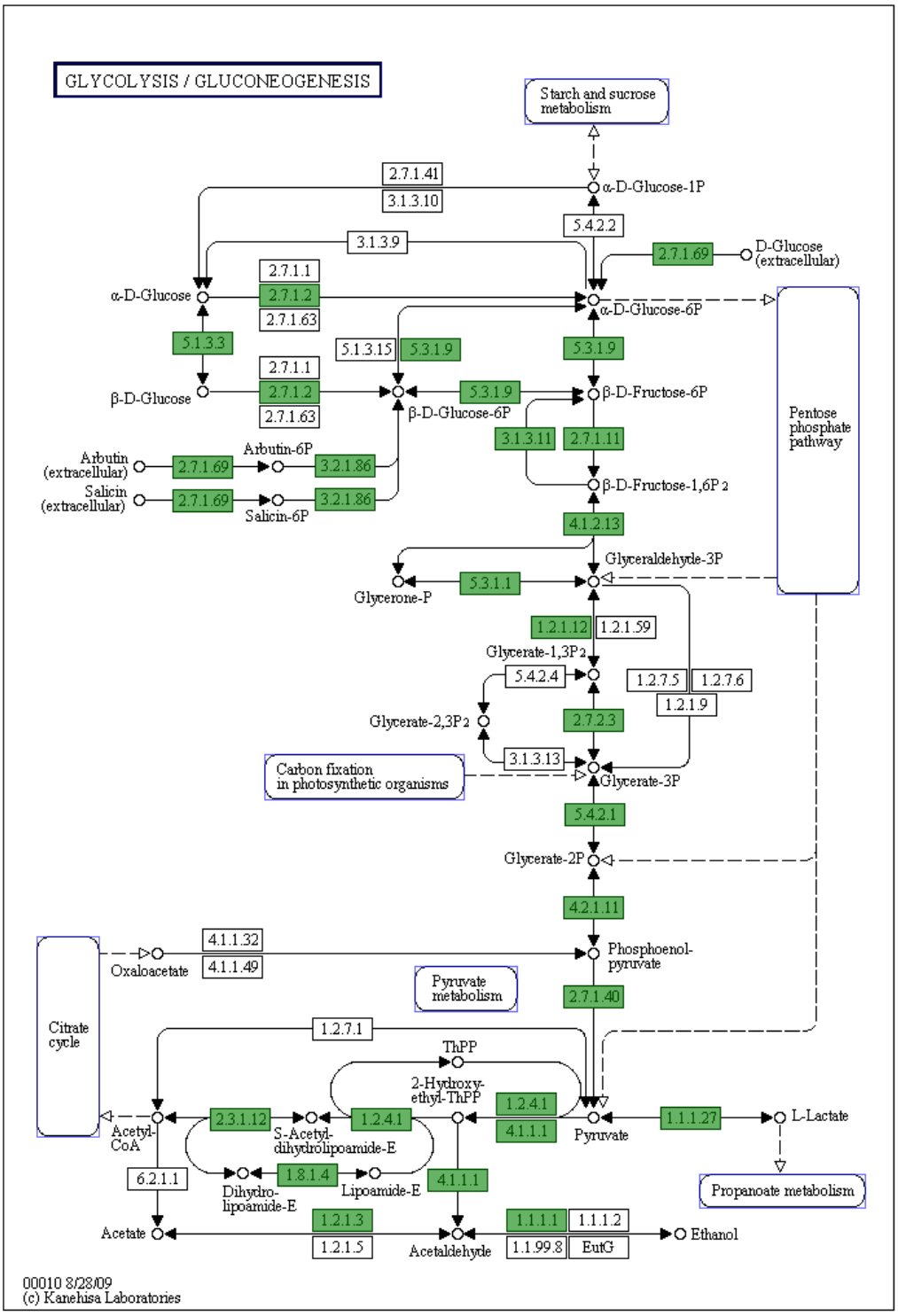

Figure A2. KEGG map of the glycolysis/gluconeogenesis pathways and subsequent pyruvate reactions in *Brochothrix*. Enzymes that are present in *Brochothrix* species are shown in green boxes, while enzymes that are absent are represented in uncoloured boxes, with the exception of a pyruvate decarboxylase (EC 4.1.1.1) gene, which was not identified in the draft genome of *B. campestris* DSM 4712^{T}.

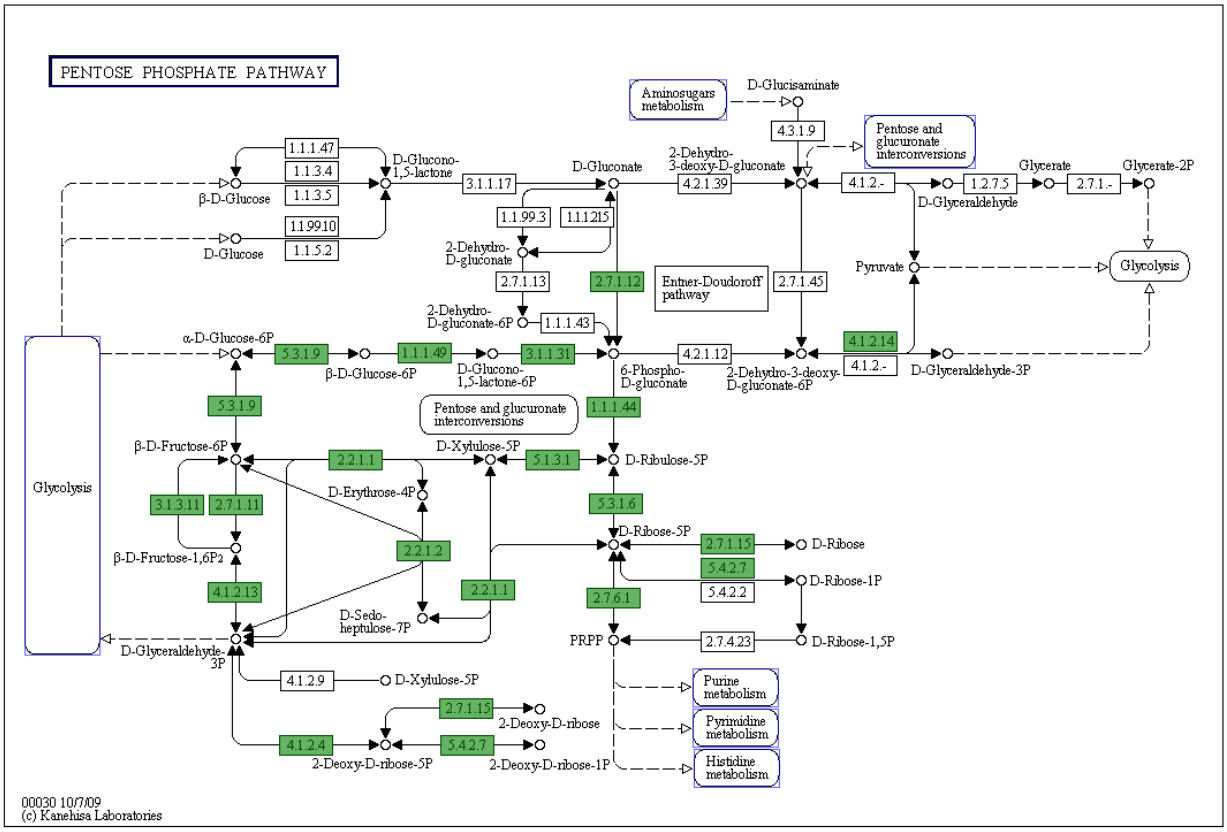

Figure A3. KEGG map of the pentose-phosphate pathway in *Brochothrix*. Enzymes that are present in *Brochothrix* species are shown in green boxes. Enzymes that are absent are represented in uncoloured boxes.

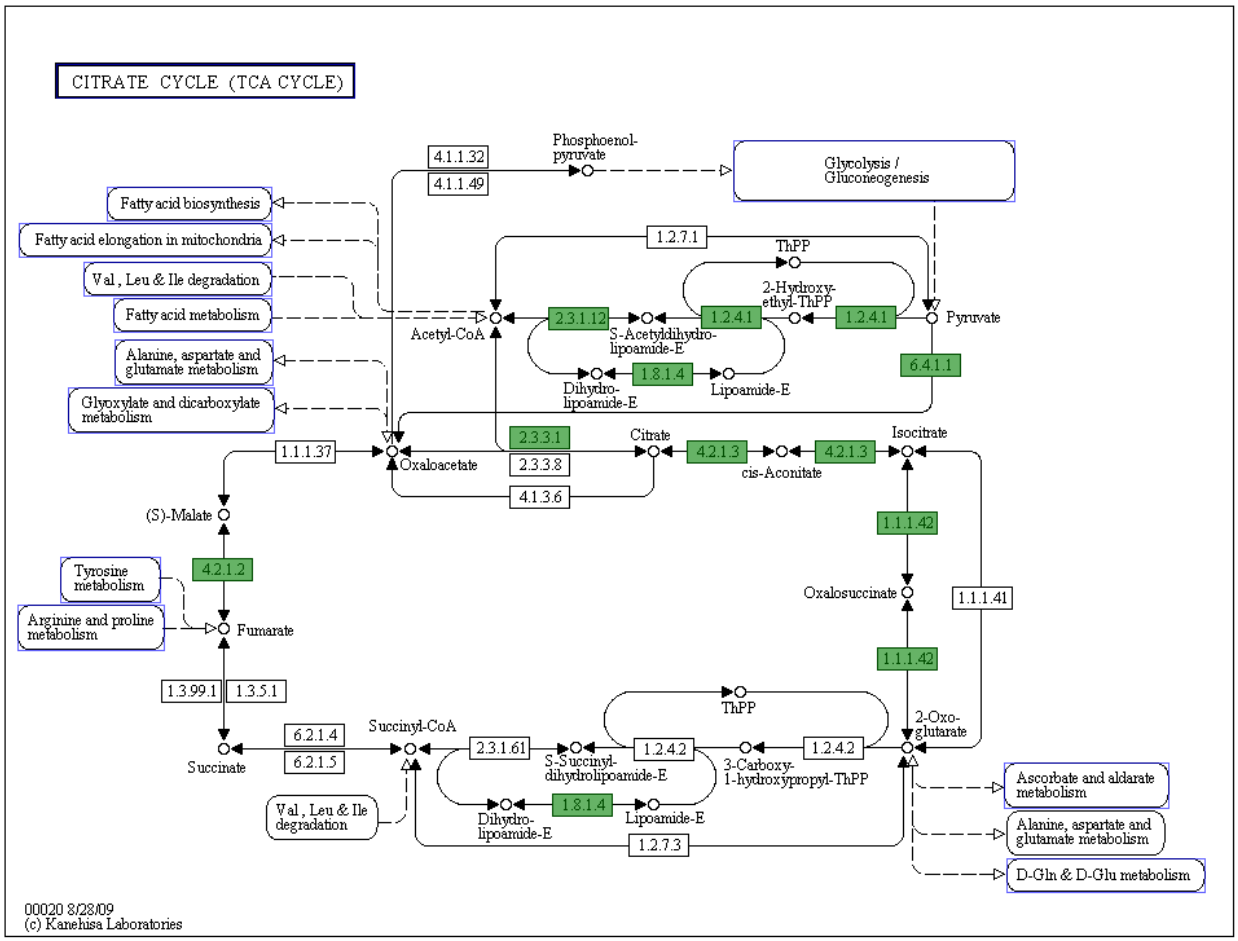

Figure A4. KEGG map of the citrate cycle shows key enzymes are missing in *Brochothrix*. Enzymes that are present in *Brochothrix* are shown in green boxes and enzymes that are absent are represented in uncoloured boxes. Although not shown here, a gene encoding an adihydrolipoamide S-succinyltransferase enzyme (EC 2.3.1.61) was also identified in the genome of *B. campestris* DSM 4712^{T}.

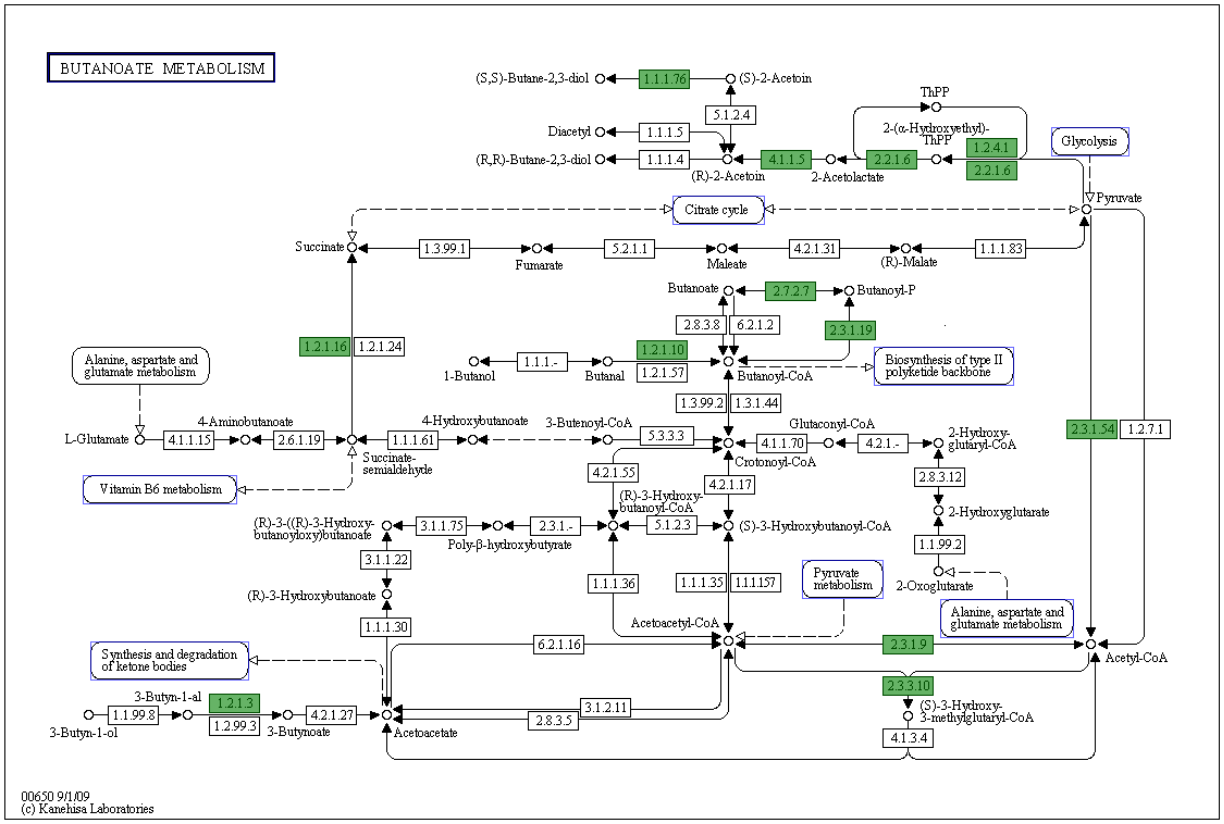

Figure A5. KEGG map of butanoate metabolism indicates that enzymes are present in *Brochothrix* for the conversion of pyruvate to acetoin and 2,3-butanediol. Enzymes that are present in *Brochothrix* species are shown in green boxes, while enzymes that are absent are represented in uncoloured boxes.

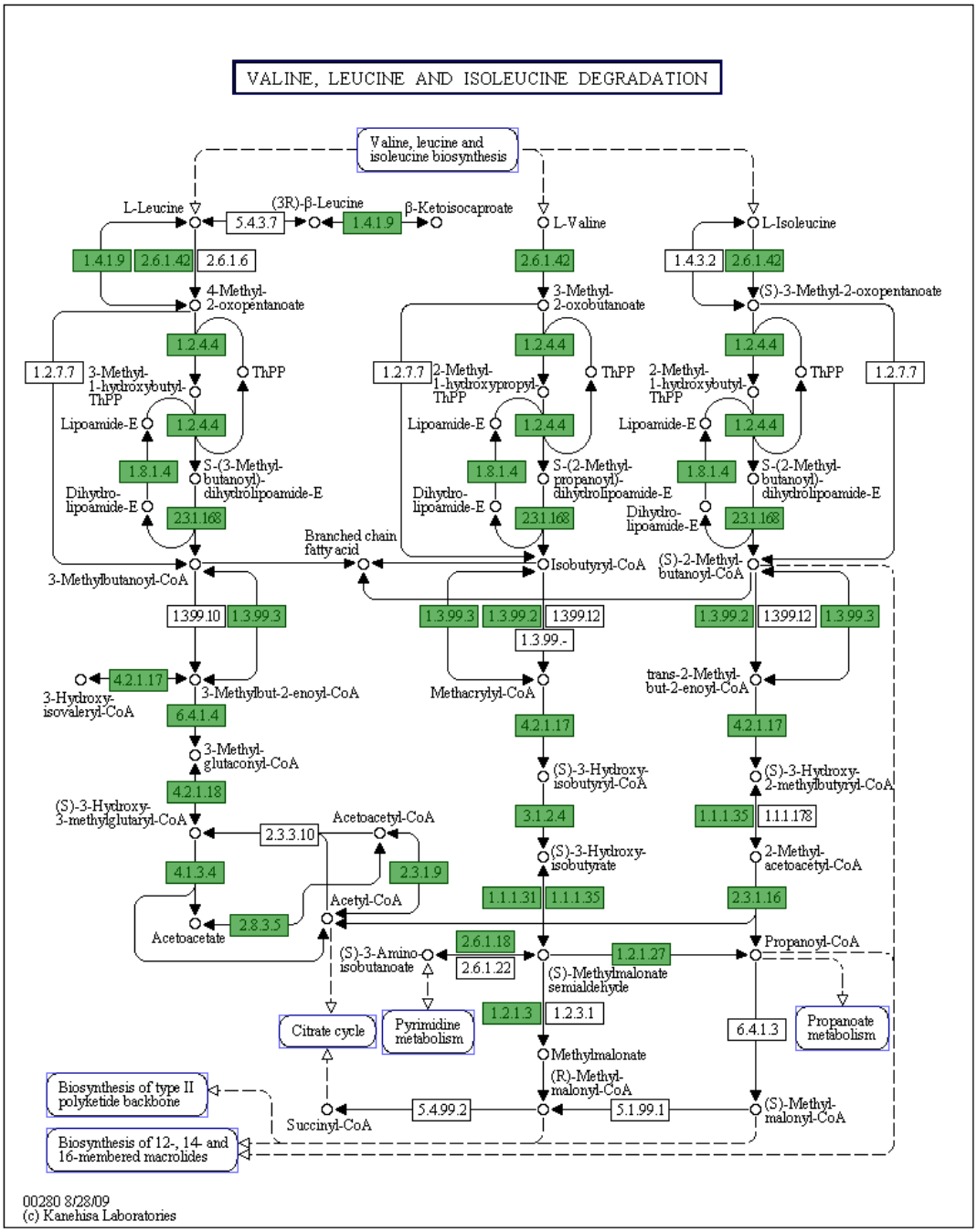

Figure A6. KEGG map of valine, leucine and isoleucine degradation pathways indicates all enzymes are present for the conversion of these amino acids to the acyl-CoA derivatives isobutyryl-CoA, isovaleryl-CoA and 2-methylbutananoyl-CoA, and propanoyl-CoA. Enzymes present in the *B. thermosphacta* strains are marked in green boxes and enzymes that are absent are in uncoloured boxes. While all enzymes required for the conversion of valine, leucine and isoleucine to their branched chain acyl-CoA derivatives are present in *B. campestris* DSM 4712^{T}, many of the enzymes downstream of these are missing including enzymes required for propanoyl-CoA production.

Appendix B

Chapter 4 - Genomic and metabolic characterisation of spoilage-associated *Pseudomonas* species

Table B1. Bacterial numbers of inoculated samples and non-inoculated controls, and percentages of atmospheric gases in headspace vials after 4 d of incubation at 8 °C.[a]

Samples	Log cfu/g sample		CO_2 levels (%)		O_2 levels (%)		N_2 levels (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C0	BLD[b]	-	0.1	0.1	21.2	0.1	78.8	0.1
Cd4	5.37	1.08	0.2	0.1	20.3	0.2	79.4	0.3
F1786	9.59	0.05	9.5	0.2	2.3	0.6	88.1	0.6
F1791	9.50	0.11	9.0	0.8	2.6	0.2	88.4	1.0
F1792	9.39	0.09	8.3	0.5	3.2	1.7	88.5	1.6
F1793	9.48	0.02	7.6	0.4	4.7	1.5	87.7	1.9
F1794	9.50	0.06	8.8	0.9	2.8	1.1	88.4	1.4
F1801	9.44	0.13	9.1	0.8	2.7	0.5	88.2	1.1
F1813	9.46	0.04	8.8	0.7	1.9	0.1	89.4	0.8
F1815	9.53	0.12	8.7	0.6	2.7	1.3	88.6	1.1
F1816	9.37	0.04	8.8	0.6	2.3	1.0	88.9	1.2
F1818	9.41	0.06	9.3	0.5	3.1	1.4	87.6	1.1
F1820	9.41	0.05	8.7	0.8	2.7	0.9	88.6	0.6
F1821	9.47	0.04	9.2	0.3	2.8	0.9	88.0	0.9
ATCC 4973^T	9.56	0.07	10.0	0.4	1.8	0.3	88.2	0.7
L1788	9.43	0.04	8.3	0.6	5.5	1.4	86.2	0.8
L1802	9.62	0.04	9.9	0.4	3.3	0.8	86.8	1.1
L1810	9.59	0.02	9.5	0.3	3.3	0.9	87.2	0.7
L1814	9.59	0.06	9.1	0.2	3.9	0.7	87.1	0.6
L1817	9.60	0.01	9.8	0.9	3.6	0.5	86.6	0.8
L1819	9.56	0.02	10.1	0.8	3.1	0.4	86.9	1.2
L1822	9.58	0.08	10.1	1.0	3.3	1.2	86.6	0.7

[a] Shown are the means and standard deviations (SD) of three independent experiments.

[b] BLD: Below the detection limit

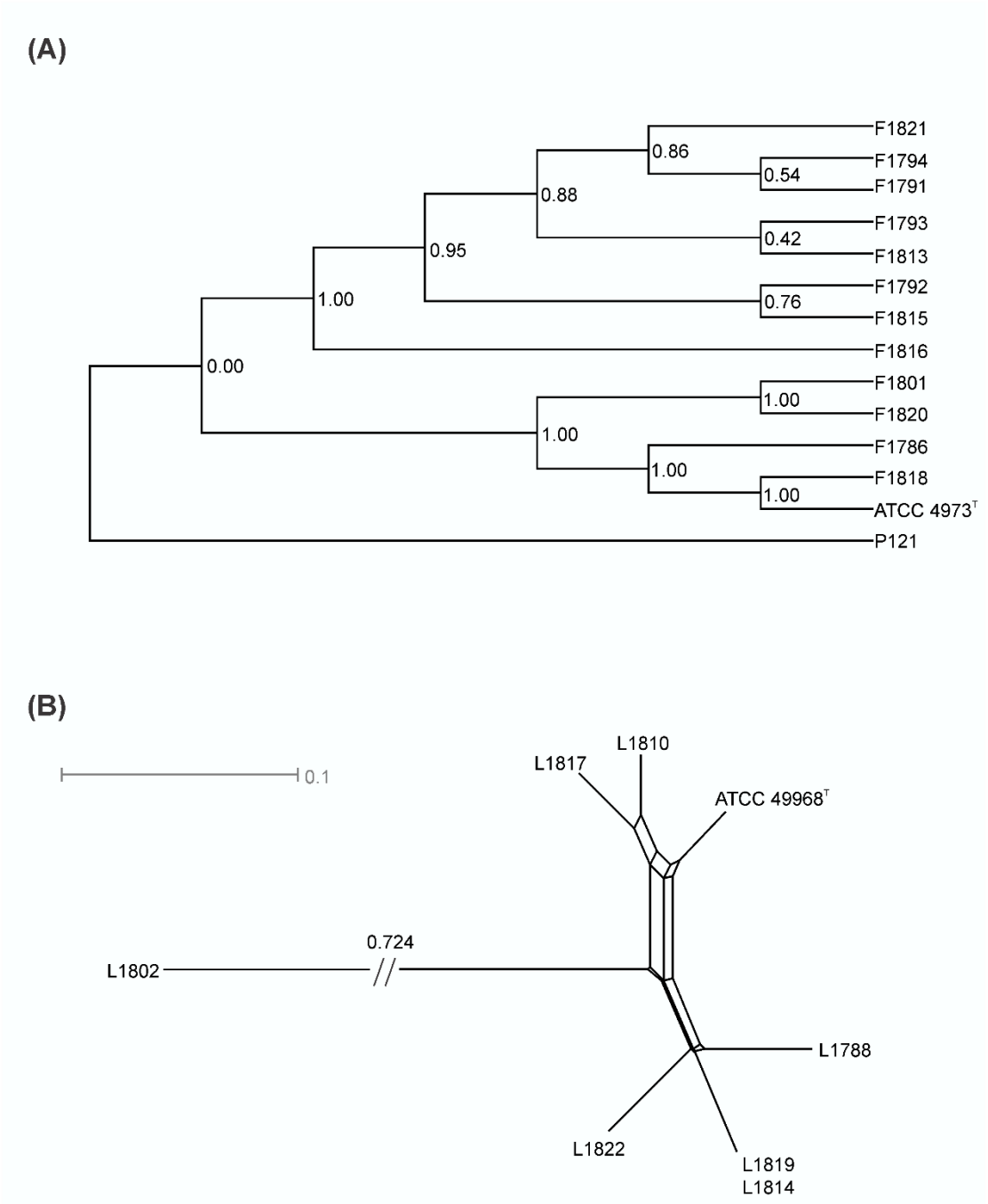

Figure B1. Core SNP phylogenies of *P. fragi* and *P. lundensis* organisms. A Mid-point rooted consensus parsimony cladogram of *P. fragi* core SNPs. Support values were determined by Shimodiara-Hasegawa resampling test. **B** NeighborNet network analysis of *P. lundensis* core SNP matrix was plotted using uncorrected P distances. The distance scale indicates the number of nucleotide substitutions per number of SNPs. Truncated network branch is marked with // to aid visualisation and the original branch length is indicated above.

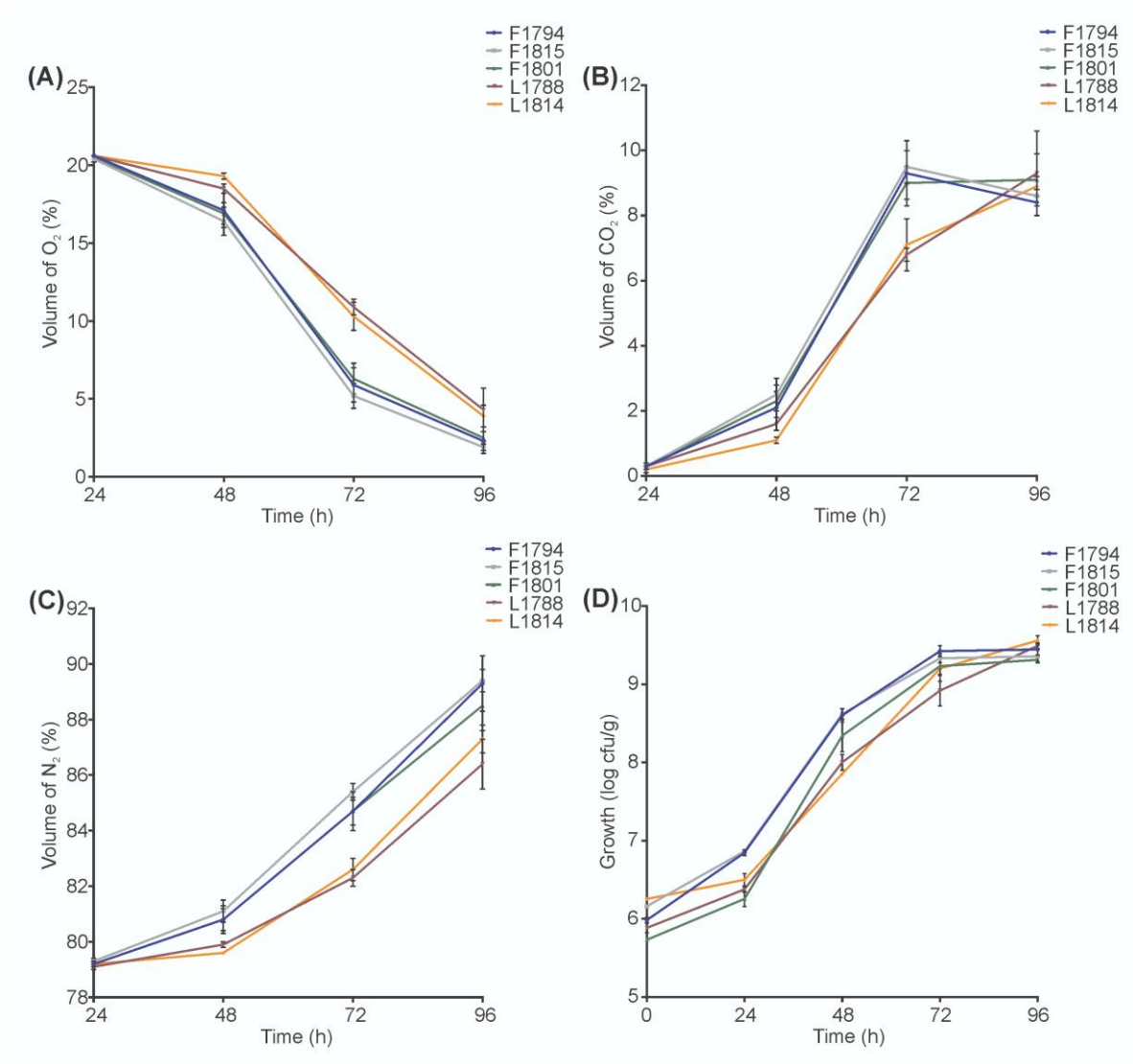

Figure B2. Growth and percentages of atmospheric gases in headspaces of vials of low (F1794, F1815 and L1788) and high (F1801 and L1814) VOC producers. Tested isolates, indicated in the legends, were inoculated onto a sterile beef paste and samples were incubated at 8 °C. The percentages of O_2 (A), CO_2 (B) and N_2 (C) in the headspaces of the vials were determined at 24 h intervals, prior to sampling of the vial contents to determine the log cfu/g (D). Each point represents the mean and error bars show the standard deviation of three experimental replicates.

Appendix C

Chapter 5 - Vibrioferrin production by the food spoilage bacterium *Pseudomonas fragi*

Table C1. Primers used in this study.

Primer name	Primer sequence[a]
Primers used for generating gene disruption constructs	
lip323fwd	AAAAAGGATCCGTCCCAATCATGGCTCCGAA
lip783rev	AAAAAGGATCCCAGGTGGTCCATTGCGTAGT
pvuA886fwd	AAAAAGGATCCAGCTACCGCAATTTCGTCGTC
pvuA1523rev	AAAAAGGATCCATCAGTGGTGGCGTTGTAGAC
qRT-PCR primers	
icd481fwd	AAAATGGGTGCCTGGGCTGC
icd623rev	GTGGTGGTGCCGTCTTGAGC
aldo559fwd	GCCGAACAGAACGCGCAATG
aldo664rev	GGAGAGAATGCAGGCGGGCT
pvuA1898fwd	CCTCGGCAGGCATCAGCAAC
pvuA2005rev	CGCCTAAGGTGAGCGAACGC
pvsA162fwd	GCTGCAGGTGATCGAGTGCG
pvsA282rev	GGCTGTCGCGGTTTGCAGAT
pvsB1190fwd	CCGGCTCCTTGCAACACTGG
pvsB1292rev	TGCTGATTGGCCTCAAGCGC
pvsC1028fwd	TGGTCAGCCAAATGTGCGGG
pvsC1127rev	CGGGAAGTGATCATTAGAGGGCG
pvsD505fwd	ACGCCGAAAAGCCGTGAAGG
pvsD657rev	CGCCAGTTGCTGCAGGGTAC
pvsE915fwd	CCATCACTTCCGCACGCCTG
pvsE1038rev	CTTGGGCGTACACAGCTGGC

[a] Underlined sequences indicate BamHI restriction enzyme sites.

Table C2. Genetic variants present in Δ*pvuA*.

Contig[a]	Position[b]	Variant type	Ref[c]	Alt[d]	Evidence[e]	FTYPE[f]
NQKQ01000013	14617	Complex	ACAGG	GCAGA	GCAGA:17 ACAGG:1	Non-coding region
NQKQ01000051	87	Single nucleotide polymorphism	A	G	G:53 A:5	Non-coding region

[a] Accession numbers of the contigs the variants were found on in the draft genome of *P. fragi* F1801 (Accession no. GCA_002269505.1).

[b] Position in the sequence, counting from 1.

[c] The nucleotide(s) in the reference.

[d] The alternate nucleotide(s) supported by the reads.

[e] Frequency counts for REF and ALT.

[f] Class of feature affected: CDS, tRNA, rRNA or non-coding region.