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Oral and intestinal bacterial exotoxins: Potential linked to carcinogenesis

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Abstract

Growing evidence suggests that imbalances in resident microbes (dysbiosis) can promote chronic inflammation, immune-subversion, and production of carcinogenic metabolites, thus leading to neoplasia. Yet, evidence to support a direct link of individual bacteria species to human sporadic cancer is still limited. This chapter focuses on several emerging bacterial toxins that have recently been characterized for their potential oncogenic properties toward human orodigestive cancer and the presence of which in human tissue samples has been documented. These include cytolethal distending toxins produced by various members of gamma and epsilon Proteobacteria, Dentilisin from mammalian oral *Treponema, Pasteurella multocida* toxin, two Fusobacterial toxins, FadA and Fap2, *Bacteroides fragilis* toxin, colibactin, cytotoxic necrotizing factors and α-hemolysin from *Escherichia coli*, and *Salmonella enterica* AvrA. It was clear that these bacterial toxins have biological activities to induce several hallmarks of cancer. Some toxins directly interact with DNA or chromosomes leading to their breakdowns, causing mutations and genome instability, and others modulate cell proliferation, replication and death and facilitate immune evasion and tumor invasion, prying specific oncogene and tumor suppressor pathways, such as p53 and β-catenin/Wnt. In addition, most bacterial toxins control tumor-promoting inflammation in complex and diverse mechanisms. Despite growing laboratory evidence to support oncogenic potential of selected bacterial toxins, we need more direct evidence from human studies and mechanistic data from physiologically relevant experimental animal models, which can reflect chronic infection in vivo, as well as take bacterial-bacterial interactions among microbiome into consideration.

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1. Introduction

1.1 Overview of association between infectious agents, microbiome and cancer

Infections have been recognized as a major preventable cause of human cancer.[1] Plummer et al.[2] estimate that 15.4% of the worldwide cancer cases are attributable to infection, amounting to approximately 2.2 million cases per year. A variety of infectious agents have been linked to pathogenesis of human cancers, including parasites, such as *Schistosoma haematobium* (urinary bladder cancer) and *Opisthorchis viverrini* (cholangiocarcinoma), bacteria, such as, *Helicobacter* (*H*) *pylori* (stomach cancer and gastric lymphoma) and viruses, such as hepatitis virus types B and C (liver cancer), human papillomavirus (HPV) (anogenital and oropharyngeal cancer), Epstein-Barr (EB) virus (Burkitt lymphoma and nasopharyngeal cancer), human herpes virus type 8 (Kaposi's sarcoma and non-Hodgkin's lymphoma) and human T cell leukemia/lymphoma virus type 1.[2,3] Etiological roles of these oncoviruses have been appreciated more unequivocally, as viral genomes can be integrated to the host genome and transcribed, producing own oncoproteins and manipulating host signaling pathways critical in oncogenesis. On the other hand, precise mechanisms of bacteria-induced carcinogenesis have been less clear, except a well characterized oncoprotein from *H. pylori*, CagA,[4–9] despite the fact that bacteria are the most ubiquitous microorganisms that collectively make up to 100 trillion cells in our bodies, 10-fold the number of human cells.[10] Bacteria are primarily extracellular organisms and thus their oncogenic effects may be rather indirect. Growing evidence also suggests that imbalances in resident microbes (dysbiosis) can promote chronic inflammation, immune-subversion and production of carcinogenic metabolites, thus leading to neoplasia.[1,11–13] Yet, evidence to support a direct link of individual bacteria species to human sporadic cancer is still limited, except the aforementioned *H. pylori* producing a cytotoxin, CagA. There are other bacterial toxins that have recently been characterized for their potential oncogenic properties and the presence in human tissue samples. This book chapter focuses on those emerging bacterial toxins in order to expand the repertoire of bacteria to be interrogated further to establish a causal association.

1.2 Overview of bacterial toxins

Toxins are a wide range of substances biologically produced within living cells or organisms that are harmful to other organisms while benefiting own survival. They are inanimate and not capable of reproducing themselves. Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors. Bacterial toxins are broadly grouped into two main types, lipopolysaccharides (LPS), which are associated with the cell wall of Gram-negative bacteria, and proteins, which are released from bacterial cells and may act at tissue sites removed from the site of bacterial growth. The cell-associated toxins are referred to endotoxins and the extracellular diffusible toxins are referred to as exotoxins, or protein toxins.[14] This book chapter focuses on exotoxins produced by distinctive groups of bacteria, as LPS is not bacteria specific, produced by a virtually all Gram-negative bacteria and has been known for a long time causing sepsis (endotoxemia) and a myriad of innate inflammatory responses in infected hosts.[15–17] While exotoxins can be named after organisms generating the toxin or after susceptible tissue/organ types, protein

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toxins can be further divided according to modes of actions; (1) targeting host membrane receptors; (2) targeting host membrane lipid bilayer, and (3) targeting host cytosolic proteins, either through uptake by host endocytosis of paired bacterial proteins called AB toxins, or by direct delivery through molecular syringe of a bacterial injection apparatus.[18] Protein toxins are also characterized based in their secretion systems.[19] To exert virulence, bacterial toxins produced in cytosol must be transported across the bacterial membranes through co-translational and post-translational mechanisms to reach their targets. These transports are mediated through various secretion systems, which now include nine different mechanisms, types I–IX.[20,21] Gram-positive bacteria utilize only types I and VII,[19] Gram-negative bacteria exploit all but type VII,[20] while one bacterial species can possess multiple systems. Finally, toxins are also classified based on virulence mechanisms, i.e., targeting cytoskeleton components, targeting ubiquitin/proteasomal system, targeting cell translational machinery, targeting neurotransmission system, targeting cAMP production and MAP kinase signaling, and targeting DNA and inducing endoplasmic reticulum stress responses,[18] some of which may be highly relevant to carcinogenesis. Subsequent sections provide more details about individual bacterial toxins, with which potential links to human cancers have been increasingly recognized.

2. Cytolethal distending toxins (CDTs)

2.1 Biochemical properties of CDTs

CDTs belong to the AB toxin family produced by several groups of Gram-negative bacteria and is composed of an active subunit (CdtB) and two binding subunits (CdtA and CdtC) (AB_2 toxin).[22] The crystal structure solved by Nesic et al. indicates that this holotoxin is a tripartite complex,[23] and thus it is distinguished from binary CDT toxin secreted by *Clostridium difficile* in its structure and functions. CDT consists of an enzyme of the DNase-I family (CdtB) bound to two ricin-like lectin domains (CdtA and CdtC), which are required for host cell attachment and translocation of CdtB into host cells.[22] It forms a ternary complex with three interdependent molecular interfaces, characterized by globular, as well as non-globular extensions from CdtA and CdtC that facilitate interactions among the three subunits. CdtA and CdtB have also been reported to contain disordered amino acid sequences pointing to dynamic properties of this molecule.[23,24] An overall surface area of interaction is estimated to be over 10,700 $Å^2$ for the entire holotoxin, with relative molecular mass ~70,000 (M_r 70 K).[23]

The CdtA and CdtC subunits are lectin-type molecules, sharing structural homology with the B-chain repeats of the plant toxin ricin and form a deeply grooved, highly aromatic surface that is critical for toxicity.[23] The CdtB subunit is functionally and structurally homologous to mammalian DNase I, is translocated into the nucleus, and acts as a genotoxin leading to DNA damage.[25] After cell surface binding via CdtA and CdtC, CdtB has to cross the plasma membrane through endocytosis to reach the nucleus, traveling through the trans-Golgi, where sulfation occurs, and then it is retrograde translocated via the endoplasmic reticulum (ER), where it is glycosylated.[26] Further confocal microscopic analysis indicates that most likely the active subunit of CDT is directly translocated from the ER to the nucleus.[27] CdtB is a highly potent DNase, inducing DNA at an extremely low concentration (50 pg/mL),

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leading to both single-strand and double-strand breaks.[25] CdtB also possesses lipid phosphatase activity, converting PI3,4,5,P3 to PI,4,5P2.[22,28,29] This leads to the blockade of PI-3K signaling, which is required for an extremely diverse array of cellular functions, most notably cellular proliferation and survival and frequently dysregulated in many cancers.[22,30] These biochemical characteristics of this toxin are indicative of its potential involvement in human cancers.

2.2 Microbiological characteristics of CDT-producing bacteria

CDTs are produced by a small but diverse group of bacterial pathogens that primarily belong to gamma and epsilon Proteobacteria.[28] To date, CDT have been documented in *Aggregatibacter actinomycetemcomitans* (*Aa*), *Campylobacter coli/fetus/hyointestinalis/jejuni* (*Cj*)/*lari/upsaliensis*, *Escherichia albertii/coli* (*Ea/Ec*), *Haemophilus ducreyi* (*Hd*), *Helicobacter canis/cinaedi/fennelliae/hepaticus/pullorum/winghamensis*, *Salmonella enterica* (*Se*) and *Shigella dysenteriae/boydii*,[5,25,28,31] which were isolated from human samples. However, it is important to note that this toxin is not necessarily produced by all subspecies/strains that belong to these specific bacterial groups. The higher carriage rates (>80%) have been reported for *Aa*, *Hd*, *Se* and enterohepatic *Campylobacter* and *Helicobacter* species, while it is generally low in *Ec* and *Shigella*.[28,31–33]

In nearly all bacteria, CDTs are encoded by the *cdtABC* gene cluster, a constitutively expressed operon on the chromosome, which yields molecular masses of 23–30, 28–29 and 19–21 kDa of CdtA, CdtB and CdtC subunits, respectively, according to bacterial species.[28] Among these three genes, *cdtB* is the most conserved with homology across species of 50% or higher, while that of *cdtA* and *cdtC* is generally less than 30%.[22,31,34] Nonsynonymous mutation in any conserved residue that is critical for the catalytic activity or the Mg^{2+} binding abolishes the ability of CdtB to cleave DNA.[22,23,35] Single nucleotide polymorphisms at amino acid 281 (H/R) in *Aa cdtB* and at 95 (P/S) in *Cj cdtB* have been shown to change its biological activity by several log orders.[36,37] Strains carrying the high activity allele were reported to be dominant in clinical isolates.[36,37] A notable exception for the structure of this gene cluster is *Se*, which is an intracellular pathogen and expresses CDT only after bacterial internalization into host cells.[22,38] *Se* does not carry any homologous genes to *cdtA* and *cdtC*, but possesses two other genes called, *pltA* and *pltb* (persussis-like toxin A and B), located upstream of *cdtB* in the same pathogenicity island, the products of which form with CdtB a tripartite.[39,40] These two subunits have been projected to facilitate the transport of CdtB from its production site within infected host cells to extracellular medium, from where CdtB can intoxicate other non-infected cells.[22,39]

Each of the translated CdtA, CdtB and CdtC subunits is independently translocated across the cytoplasmic membrane, through a standard mechanism for the movement of secreted proteins across the cytoplasmic membrane of bacteria, where precursor proteins are exported by a common sec-dependent pathway that involves both signal peptidase I and signal peptidase II.[41,42] The CdtB and CdtC proteins processed by signal peptidase I and the CdtA processed by signal peptidase II accumulate in the periplasmic space and all three proteins self-assemble at the outer membrane. Catalyzed by an unidentified processing enzyme to remove the hydrophobic glycerolipid of CdtA, the holotoxin is translocated across the outer

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membrane.[41,42] The hydrophilic holotoxin accumulates in the aqueous environment outside the bacterium until it recognizes a specific receptor on the target cell surface.

An alternative mode of bacterial toxin secretion is the release of holotoxins into enclosed outer membrane vesicles (OMVs). Bacterial OMVs are nano-sized compartments consisting of a lipid bilayer that encapsulates periplasm-derived, luminal content. OMVs produced by of Gram-negative bacteria are now recognized as a generalized secretion pathway that provides a means to transfer cargo to the host cells.[43] In the context of intestinal colonization, packaging and release of CDT into outer membrane vesicles may provide the toxin with extra protection against enzymatic digestion, thus increasing the likelihood of the uptake of intact toxin by host cells. Although a mechanism of delivering the toxin from the vesicles to the target cell has not been well understood, several potential routes, including clathrin or caveolin mediated or non-mediated endocytosis and membrane fusion, have been demonstrated.[43] OMVs-like vesicles have been also found to be synthesized by internalized Se, transported and released to the extracellular medium through host microtubule and actin tracks.[44]

2.3 In vitro cellular responses to CDTs

Biological consequences from CDT intoxication in mammalian cells have been evaluated in vitro using various normal and tumor cell lines that are exposed to bacterial lysates or purified toxins or are transfected with *cdtB* or CdtB, of different bacterial origins. DNA damage caused by CDT has been shown to induce the activation of DNA damage response (DDR), leading to an ATM (Ataxia telangiectasia mutated)-dependent cell cycle arrest at G2/M and/or G1/S transition, and initiation of multiple DNA repair pathways. However, the DDR system sometimes fails to properly repair DNA damage, leading to cell death by apoptosis or to a long-term cell cycle arrest (senescence).[25] This cellular senescence may alternatively lead to a senescence-associated secretory phenotype (SASP), which is characterized by secretion of a large number of growth factors and pro-inflammatory cytokines and can promote survival and proliferation of transformed cells.[45]

Cell fate following CDT-induced DNA damage depends on types of cells.[46,47] Hematopoietic lineages, particularly lymphocyte, are known to be greatly more sensitive to CDT than most other cells, inducing rapid move toward apoptosis after a brief cell cycle arrest.[29,46] Accordingly, CDT is considered an immunotoxin. It is capable of inhibiting phagocytosis in murine macrophages[48] as well of inhibiting functions of both T- and B-cells.[29,46] Furthermore, CDT has other immunomodulatory properties as it has been documented to induce the expression of pro-inflammatory cytokines/chemokines (such as IL-1β, IL-6, IL-8) in human macrophages and peripheral mononuclear cells.[25,49,50]

Epithelial and mesenchymal lineages mainly undergo cell cycle arrest accompanied by cytoplasmic elongation and distension due to accumulation of F-actin assemblies.[51] DNA double-strand breaks induced by *Hd*CDT led to the formation of actin stress fibers and the rearrangement of the actin cytoskeleton in epithelial cells and fibroblasts, via activation of the small GTPase RhoA. Activation of RhoA was part of the ATM-induced cellular responses to genotoxic stresses to support cell survival.[52] Moreover, in colorectal cancer cell lines, exposure to *Hd* CDT stimulated a marker of autophagy, a pro-survival pathway, in an

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ATM and p53-dependent manner.[53] CDT also induced remodeling of adherens junctions in gingival tissue and normal epithelial cells and compromised mucosal barrier function, which was accompanied by a pronounced increase in the expression and cytosolic distribution of E-cadherin and β-catenin.[54]

Investigators have demonstrated in both fibroblast and normal and cancer cell lines the enhanced senescence-associated β-galactosidase activity, expansion of promyelocytic leukemia nuclear compartments and induced expression of several cytokines (especially interleukins IL-6, IL-8 and IL-24), which were overall features shared by cells undergoing replicative or premature cellular senescence.[55] Furthermore, a long-term (30 weeks) of exposure of fibroblast and intestinal epithelial cells to *Hd* CDT led to increased frequency of mutations (mainly transversions), accumulation of chromosomal aberrations and enhanced anchorage-independent growth.[56] These observations suggest that CDT-induced DNA damage promotes genomic instability, favoring tumor initiation and progression.[57] Graillot et al. postulate that CDT does not initiate colorectal cancer by itself, but may have promoting effects in premalignant epithelial cells. They found that chronic exposure (3 weeks) to *Ec* CDT resulted in enhanced anchorage-independent growth and genetic instability measured by the micronucleus formation in colonic epithelial cells defective in APC and p53 in comparison to normal cells, which were considered hallmarks of malignant transformation.[58] Frisan et al. also postulate that CDT may act synergistically with an oncovirus, specifically EBV. They found that exposure of gastric cancer cell lines to *Aa* CDT reactivated latent EBV infection and promoted latent infection in non-infected cells via genomic instability.[59]

2.4 CDT toxicity in animal models

Potential carcinogenic effects of CDTs in vivo have been evaluated in a few animal models. The vast majority of the studies were based on inoculation of murine enterohepatic *Helicobacter* species, either *H. hepaticus* or *H. bilis*, while a minority used enteric *Cj*. In these studies, natural or engineered CDT-deficient strains were often used for comparison.[60,61] In both liver and colon carcinogenesis, a progressive pathological sequence from chronic active inflammation (hepatitis/colitis), dysplasia, benign tumor to cancer has been acknowledged.[60,61] *Hh* inoculation to A/J, C57BL/6 and their F1 mice induced hepatitis, some of which further progressed to hepatocellular carcinomas or dysplastic liver lesions, in a gender-specific manner, resulting in 69% incidence in F1 male mice. *Hh* was isolated from hepatic tissues of all F1 mice with liver tumors, which were developed in the presence of hepatic steatosis and active hepatitis.[62] Likewise, perinatal intraperitoneal inoculation of *Hh* to A/J female mice resulted in increased incidence of hepatitis and hepatocellular tumors in their male (not female) offspring.[63] However, these experiments did not rule out the effects of other virulence factors co-expressed in CDT-positive *Hh*. Thus, others have tried to address the independent effects of CDT. While infection of A/JCr mice with wild-type (WT) *Hh* or an isogenic mutant lacking CDT activity induced comparable chronic hepatitis, the CDT mutant-infected mice did not develop any hepatic dysplastic nodules in contrast to the WT-infected male (not female) mice that developed dysplasia. Male mice infected with WT also exhibited significantly enhanced hepatic transcription of pro-inflammatory TNF-α, IFN-γ and Cox-2, growth mediators IL-6 and TGF-α, anti-apoptotic Bcl-2 and Bcl-XL, and

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increased hepatocyte proliferation compared with the CDT mutant-infected male mice, confirming that CDT plays a key role in promoting the dysplastic changes in the *Hh*-infected mouse livers.[64] Fox et al. further investigated the potential interactions of *Hh* with chemical or virial liver carcinogens. In both models, *Hh* intestinal colonization was sufficient to promote the development of hepatocellular carcinoma (HCC), via transactivation of the nuclear factor kappa N (NF-κB) network in the liver. Thus bacterial translocation to the liver was not a requisite and, in the presence of a chemical carcinogen, aflatoxin, both male female mice equally developed HCC.[65] Tumor promoting effects of *Hh* on hepatitis C virus transgenic mice shown in this study also suggest potential bacterial involvement in viral activation as in the case of *Aa* CDT and EBV.[59]

Orogastric administration of culture supernatants of CDT-producing isolates to suckling mice induced overt inflammation in stomach, small intestine and large intestine, which was not seen in the experiments with CDT-negative *Cj* strains,[66] demonstrating that CDT plays a crucial role in gastrointestinal pathology in this model. In NF-κB-deficient mice, inoculation of the *cdtB* mutant strain did not induce gastroenteritis and gastric hyperplasia/dysplasia comparable to that developed in the WT-infected mice.[67] Furthermore, inoculation of CDT-negative Hh mutant showed a significantly diminished capacity to induce colitis lesions in an interleukin-10 deficient mouse model for inflammatory bowel disease.[68] Shen et al. tested mutant and WT CDT from zoonotic *H. cinaedi* using the same mouse model. Both mutant and WT colonized to a similar extent, but the mice infected with the mutant showed attenuated colitis and hyperplasia but no dysplasia or intra-mucosal carcinoma, compared to those infected with WT, which exhibited elevated mRNA expression of tumor necrosis factor alpha, inducible nitric oxide synthase, and gamma interferon in the cecum.[69] Another newly found CDT-positive *H. japonicum* induced the histopathology characterized by moderate to severe inflammation, mild edema, epithelial defects, mild to severe hyperplasia, dysplasia and carcinoma in the lower bowel of the same mouse model.[70] In an immunocompromised mouse model, inoculation of CDT-deficient *Hh* mutant led to less severe cecal pathology without inducing pre-neoplastic lesions, while WT-infected animals developed pre-neoplastic dysplasia and cancer, with concomitant upregulation of cecal Il-6 and Tnfα.[71] In a mouse xenograft model with *Hh cdtB*-transfected human hepatic and intestinal cancer cell lines, both types of the CdtB-derived tumors showed increased apoptosis, senescence, p21 and Ki-67 nuclear antigen expression, an overexpression of cytokeratins at the invasive front of the tumor and an increase in ploidy, while no difference in proliferating cells undergoing mitosis. All these features were considered hallmarks of endoreplication and aggressiveness in cancer.[72] More recently, oral infection of the $Apc^{Min/+}$ mice with CDT-producing *Cj* together with dextran sulfate sodium administration has been reported to induce significantly larger number and size of tumors when compared with a *ctdB* mutant *Cj*. WT *Cj* infection also induced expression of hundreds of colonic genes, with 22 genes dependent on the presence of *cdtB*, as well as altered microbial gene expression, which could also modulate colorectal carcinogenesis.[73]

2.5 Clinical and epidemiological studies concerning CDT

The direct evidence to link CDTs or CDT-positive bacteria to human cancer are still limited. To our knowledge, the presence of CDT itself in human tumor tissue or in surrounding

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mucosa has not been demonstrated. Alternatively, CDT-positive (by PCR) *Ec* has been reported to be more commonly present in colorectal cancer tissue than in colorectal mucosa from diverticulosis patients (16% vs 0%).[33] However, these CDT-positive strains also harbored other known virulence genes and thus it was difficult to disentangle the effect of CDT itself. Another routinely used method to assess the past exposure is serological assays to detect specific antibodies. Although anti-CDT antibody assays have been available, demonstrating increased titers in diarrhea type of irritable bowel syndrome (IRS) compared with control subjects,[74,75] their association with cancer or premalignant pathologies has not been studied. More consistent evidence from human populations has been complied with regard to hepatobiliary tract cancers. It should be noted that these studies evaluated the exposure to bacterial species with known high carriage rates of CDT, without confirmation of CDT status. The best studied is gallbladder cancer and its predisposing condition, cholelithiasis, in relation to infection with *Se* serovar Typhi or *Hh*. *Se* serovar Typhi status has been most often assessed by antibodies against a capsular polysaccharide (Vi). The summary odds ratio (OR) from a meta-analysis based on such serological studies was 4.6, consistent with the summary ORs based on stool or bile culture (4.7 and 5.5, respectively). A recent study in India also reported an equivalent difference in serology using the same assay between cancer and benign conditions, but more positive samples (74%) were detected using PCR for *S. typhi fli*C genes, whereas PCR-positivity in healthy gallbladder samples was very rare.[76] Serological assays against a *Hh* antigen have revealed a significantly higher antibody prevalence in bile duct/gallbladder cancer patients (39%) and even higher prevalence in patients with viral hepatitis (60%) or viral liver cirrhosis (68%), compared with cholelithiasis (13%) or healthy donors (27%), respectively.[77,78] Given low antibody prevalence in non-viral hepatitis and cirrhosis reported at the same time,[78] these findings are also be indicative of bacterial-viral interaction. Alternatively, using PCR, in situ hybridization for16S rRNA and Western blot for *Hh* antigens Hamada et al. reported high *Hh* carriage rates (40%) in cholelithiasis patients with or without gastric cancer, compared to the patients with other benign conditions (13%).[79]

3. Dentilisin (chymotrypsin-like protease (CTLP))

3.1 Biochemical properties of CTLP

Dentilisin belongs to only two known lipoproteins in the subtilase family of subtilisin-like serine proteases. Its activity is suggested to be based on an active seryl residue, on an active imidazole group, and on an active carboxyl group but not on metal cations.[80] The enzyme hydrolyzes specifically N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroaniline (SAAPFNA, a typical chymotrypsin substrate) and various host proteins and peptides, including basement membrane components (type IV collagen, laminin, and fibronectin), serum proteins (transferrin, fibrinogen, IgG, IgA, and α1-antitrypsin), and bioactive peptides (cytokines, and chemokines).[81,82] These biochemical properties are considered to be instrumental in bacterial invasion, immune evasion and cytotoxicity.[81,82] The matured dentilisin is present as a complex, composed of an active enzyme, PrtP (~65 kDa), and two auxiliary proteins, PrcA (~70 kDa) and PrcB (~22 kDa).[83] The two auxiliary proteins are postulated to be required for stabilization of the active enzyme unit.[84,85] PrtP protease activity facilitates maturation

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of PrtP and PrcA, by releasing a 16 kDa N-terminal of the premature PrtP and cleaving PrcA to A1 (~30 kDa) and A2 (~40 kDa).[85,86]

3.2 Microbiological characteristics of dentilisin synthesis

Dentilisin is produced primarily by mammalian oral *Treponema* species,[83,87,88] represented by *T. denticola* (*Td*), a key pathogen of periodontitis, which are Gram-negative obligate anaerobic bacteria, spirochaetes uniquely lacking LPS. It is important to note that substantial inter-strain variability exist in this enzymatic activity, which is not entirely explained by the presence or sequence variabilities of encoding genes.[83,87,88] Typically, the dentilisin complex is encoded by a three-gene operon, consisting of *prcB*, *prcA* and *prtP*, with a putative promoter upstream of *prcB*.[85,86] These gene sequences are recently annotated,[83] demonstrating that *prcB* is the most conserved (89% out of 202 amino acids identical among the strains). *prcA* encoding 639 amino acids was 77% identical among the strains, and much of the variability was in three regions (151–180, 328–362 and 512–545). In *prtP* that transcribes 766 amino acids, the predicted catalytic triad (Asp^{203}, His^{258}, Ser^{447}) was conserved in all strains, and most *prtP* inter-strain variations were concentrated in the C-terminal 270 residues (up to 20%).[83] However, variability in proteolytic activities was not correlated with overall homology of *prtP*, suggesting that other unidentified sensing and signaling mechanisms modulate expression of these operons.

In *Td*, the dentilisin complex is located on bacterial outer membrane and extracellularly.[89] However, the secretion system by which the three subunits are transported through inner and outer membranes has not been well understood, Yet, studies have demonstrated that this complex is co-localized with another major *Td* virulence factor, Msp (major outer sheath protein),[83,85] encoded by *msp* and playing a major role in host adhesion.[90] MSP locates within the outer membrane, but is predominantly periplasmic, with only limited surface exposure.[91] Importantly, there is close interplay between PrtP and outer membrane segment of Msp,[92] and, as a result, *msp* mutants lack CTLP activity while *prtP* mutants show aberrant Msp expression.[93] In addition to cell surface expression, a recent mass-spectrometry-based study revealed that the three dentilisin subunits were enriched in outer membrane vesicles,[89] which enable remote delivery of the toxin.

3.3 Dentilisin in vitro activities

Cytopathic effects of isolated dentilisin, including membrane blebbing, vacuolization, inhibition of motility, loss of epithelial cell contacts and release of a cytosolic enzyme have been demonstrated in multi-layer epithelial cell culture system.[94,95] In this culture model, *Td* did not invade the cells of epithelial multilayers, but evidently shed dentilisin, which rapidly penetrated the cell layers, was transported into large intracellular vacuoles and led to increased epithelial permeability.[94] Using reconstructed basement membrane (Matrigel),[96] Grenier et al. also reported the migration of *Td* through the basement membrane, which was facilitated by protease activity of dentilisin to degrade the basement membrane components. Other investigators employed dentilisin mutant strains and confirmed that dentilisin was responsible for the increase in epithelial permeability and also found an evidence that dentilisin facilitated degradation of the tight junction protein, ZO-1.[97,98]

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In addition, higher O_2^- production from polymorphonuclear leukocytes (PMNs) was observed in a dentilisin-positive wild-type strain in comparison to a dentilisin-negative mutant, suggesting another possible mechanism mediating the cytotoxicity of this enzyme.[99] The O_2^- production was medicated through C3 complement activation, which was associated with matrix metalloproteinase (MMP) 9 release from PMNs. On the other hand, gingival epithelial cells co-cultured with wild-type or dentilisin mutant *Td* showed dentilisin-dependent preferential degradation of tumor necrosis factor α, which resulted in substantial reduction in both IL-8 protein and mRNA levels.[100] These results were indicative of host immune evasion.

An additional potentially oncogenic property of dentilisin is its ability to induce MMP-2, a key enzyme both in tissue homeostasis and tissue destruction, which has been linked to tumor invasion, progression as well as tumorigenesis.[101–103] In periodontal ligament cell (PDL) culture, purified dentilisin protease induced MMP-2 activation, mediated through fragmentation of fibronectin in extracellular matrix.[104] Subsequently, it was found that *Td* plays a key role in the transcriptional regulation of MMP-2 and its activating complex MT1-MMP/TIMP-2 through downregulation of several chromatin modification enzymes, specifically histone phosphorylases (aurora kinases) and histone deacetylase. Inhibition of these enzymes that mediated epigenetic modifications prevented *Td*-mediated increases in MMP-2, MT1-MMP, and TIMP-2 in PDL cells.[105] While specific mechanisms by which dentilisin modulates the expression of these chromatin modification enzymes remain to be studied, this has substantial implication in transcriptional control of a wide range of tumor suppressor and oncogenes. Finally it is intriguing that a low dose exposure to *Td* (5×10^7 cells/mL) increased DNA synthesis (^{3}H-thymidine uptake) via activation of MAP kinase signal pathways in PDL cells, while infection at higher concentrations reduced DNA synthesis, exerting cytotoxic effects. Western blot analysis showed that *Td* strongly but transiently activated ERK1 and ERK2, signals mediating cell proliferation, and JNK and p38, kinases mediating apoptosis.[106] These results underscore the ability of *Td* to modulate cell proliferation and cell survival.

3.4 Dentilisin activities in animal models and clinical studies

To our knowledge, there have been no animal carcinogenesis models using dentilisin itself or *Td* inoculation, as the primary endpoint in animal models have been periodontal disease. However the effects of wild-type and dentilisin mutant strains have been tested in a mouse subcutaneous abscess model, which showed a significantly reduction in size of the lesion (abscess) with the mutant.[107] Despite the lack of in vivo data in animals, several recent clinical studies suggest potential carcinogenic effects of dentilisin in humans. IgG antibodies against CTLP have been shown to be detectable in human sera,[108] but no seroepidemiological studies have examined the associations between orodigestive cancers and *Td* or dentilisin. In saliva of periodontitis patients and healthy controls, the concentration of *Td* was correlated with activated forms of MMP-8, which was cleaved from its inactive precursor form and which exerts both tumorigenic and anti-tumorigenic effects.[109] In 2004, based on 16S rRNA gene sequencing, Narikiyo et al. reported that *Td* was significantly enriched in esophageal cancer tissue compared with adjacent normal mucosa

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and it was absent in saliva from healthy volunteers.[110] More recently, Nieminen et al. demonstrated using immunehitochemical staining the presence of CTLP in several types of orodigestive cancer tissue. CTLP was positive in 19 out of 29 tongue, 20 out of 25 tonsillar, 3 out of 3 esophageal, 21 out of 32 gastric, 6 out of 6 pancreatic and 25 out of 54 colon cancer cases.[111] The investigators also reported that CTLP degraded several MMP inhibitors, including TIMP-1, TIMP-2, hypothesizing that CTLP may contribute to carcinogenesis through immunomodulation.[111] Subsequently, the same group of investigators analyzed more tissue samples of mobile tongue squamous cell carcinoma. CTLP was present in 95% out of 141 tumors, of which many (40.4%) showed high immunopositivity. CTLP positivity was significantly associated with invasion depth, tumor size and the expression of TLR-7, TLR-9 and c-Myc. Furthermore high CTLP immunopositivity in younger patients (60 years old) predicted early relapse.[112] Kylmä et al. also evaluated the presence of CTLP by immunohistochemistry in a series of 201 unselected consecutive oropharyngeal squamous cell carcinoma patients and reported 81% overall positivity, which was significantly more pronounced in HPV-negative than positive tumors. Among those HPV-negative, higher TLR 5 and lower TLR 7 expression associated with high CTLP expression, which resulted in poor disease-specific survival. No similar association emerged among HPV-positive subgroup.[113] In a prospective cohort study that characterized microbial compositions of oral rinse samples of cancer-free individuals, the risk of subsequent colorectal cancer statistically significantly increased in those who were *Td*-positive, yielding the OR of 1.8, which was highest among ORs for all other periodontal pathogens.[114] In order to substantiate oncogenic potentials of *Td*-CTLP, intrigued by the recent clinical observations, more mechanistic studies are warranted to delineate specific molecular pathways.

4. *Pasteurella multocida* toxin (PMT)

4.1 Biochemical properties of PMT

PMT belongs to a large group of deamidating toxins/effectors, deamidases, which remove the amide functional group from a key glutamine residue of the protein substrate and provoke significant pathophysiological changes of target cells. Other bacterial toxins structurally and functionally related to PMT include the cytotoxic necrotizing factors from *E. coli* and *Yersinia pseudotuberculosis* and the dermonecrotic toxin from *Bordetella* spp.[115] PMT selectively targets the α-subunit of host heterotrimeric G proteins and stimulate its substrates, $G\alpha_q$, $G\alpha_{13}$ and the $G\alpha_i$-family proteins, leading to activation of various host signal transduction pathways.[116,117] As a result, it acts as a highly potent mitogen at picomolar concentrations that stimulates quiescent cells to grow and divide.[118,119]

PMT is an AB toxin, consisting of a receptor binding and translocation domain (B) and a biologically active (A) domain. This AB toxin binds to host cell receptors through their binding B domains and facilitate the cellular uptake and delivery (translocation) of their toxic activity A domains into the host cell cytosol, where the A domains then interact with and modify their cellular G protein targets to cause cellular toxicity.[120] PMT consists of 1285 amino acid residues resulting in a mass of 146 kDa. The receptor binding domain is located in the N-terminal part of the protein, including the amino acid residues 1–580.

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Within this domain a putative translocation domain is located between residues 402–457 in the C-terminus.[116]

The C-terminal part of PMT further contains three domains designated C1, C2 and C3. Of these, the C3 is the major biologically active domain which harbors the catalytic activity of the toxin to modify intracellular targets. The C1-domain (feet), encompassing amino acid residues 575–719, consists of seven helices, four of which serve as a plasma membrane targeting signal in toxin B. Because the primary target proteins of PMT are plasma membrane-bound heterotrimeric G proteins, dysfunctional C1 impairs localization to the substrate and diminishes PMT toxicity. The largest domain in the C-terminal part of PMT is the so-called body or C2 domain (amino acid residues 720–1104), consisting of 18 helices and nine beta-strands and exhibit a structure typical of nucleotide-binding proteins. This C3 domain (amino acid residues 1105–1285) is separated into two subdomains, providing the catalytic cleft for the enzymatic function of the toxin. It has been further clarified that Cys-1165, His-1205, His-1223, Asp-1220 and Gln-1225 are essential for PMT activity.[116]

4.2 Microbiological characteristics of PMT

PMT is produced by toxinogenic strains of *Pasteurella multocida*, mostly serogroups D and some A, which are classified based on capsular polysaccharides. These strains contain a unique 18-kbp region carrying 14 genes (PMCN06_2106 to PMCN06_2119), including the *toxA* gene for PMT flanked with several phage-related genes. *Pm* is a small, pleomorphic, Gram-negative, nonflagellated facultatively anaerobic coccobacillus and a multihost animal, zoonotic and opportunistic pathogen that is capable of causing respiratory and multisystemic diseases, bacteremia, and bite wound infections, while it is part of the normal flora in many animals.[121] The sequence analysis of *toxA*, indicating lower GC content than the rest of the genome, suggests its acquisition through horizontal transmission. Further sequence analysis has revealed that PMT is encoded within a lysogenic bacteriophage and that phage regulatory elements are present upstream and downstream of *toxA*, which control *toxA* expression.[122]

Specific secretion systems that translocate PMT from cytoplasm through bacterial external membrane surface have not been identified in *Pm*. Instead, it has been speculated that stress response induced by environmental factors encountered during host infections leads to induction of the phage lytic cycle, with resultant bacterial cell lysis and toxin release.[122] Knowledge of the uptake of PMT into eukaryotic cells has been still limited. Three major steps are generally acknowledged: (i) Binding of the toxin to a host cell membrane receptor; (ii) Internalization by endocytosis; and (iii) Release of the biologically active toxin into the host cytosol. To date specific host PMT receptors have not been well characterized. However, surface plasmon resonance analysis indicates that PMT initially binds with low affinity to a wide range of abundant membrane lipid components, which is followed by a more specific binding to sphingomyelin and possibly additional putative proteinaceous receptors, which would induce endocytosis.[116,120] Further trafficking within host cells is facilitated by interaction with transferrin receptors and a small regulatory G protein Art6.[116,120] It has been also clarified that PMT N-terminal mediates cytosolic delivery of its native C-terminal cargo as a single polypeptide (C1-C2-C3), without cleavage between

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subdomains.[123] However, cellular uptake process may be cell-type specific. While no endocytic uptake was detectable in enterocytes, mast cells in the lamina propria distinctly accumulated PMT in their secretory granules.[124]

4.3 Cellular activities of PMT

The incubation of mammalian cells with purified recombinant PMT induces strong mitogenic and anti-apoptotic effects in the cell-type specific manner. Myogenic effects have been reported in fibroblasts, preadipocytes, osteoblasts and embryonic kidney cells, while cytopathic responses have been noted in lung cells and cardiomyocytes.[115] Mitogenic responses are mediated through increased intracellular Ca^{2+} and inositol phosphate levels as a result of activation of phospholipase Cβ (PLCβ) and Rho-dependent cytoskeletal signaling.[115] PMT induces Ras-dependent activation of extracellular signal-regulated serine/threonine protein kinase[125] mitogen-activated protein kinase (MAPK) via G_q-dependent, protein kinase C (PKC)-independent transactivation of the epidermal growth factor receptor in a human embryonic kidney cell line.[118,126] The MAPK pathway, also known as the RAS-RAF-MEK-ERK cascade, is often activated in many human cancers.[127] Additionally, PMT has been shown in fibroblast culture to stimulate the mammalian target of rapamycin complex 1, which is also known to be often activated in human cancer,[128] through the $G\alpha_{q/11}$/PLCβ/PKC pathway.[129] PMT induces anchorage-independent growth in rat fibroblast culture with greater potency than that achieved by epidermal growth factor or platelet-derived growth factor.[119] PMT also activates anti-apoptotic pathways, e.g., protein kinase D signaling in both cardiac fibroblasts and cardiomyocytes, which leads to the phosphorylation of the transcription factor, cAMP response element binding protein (CREB), and upregulation of CREB target genes, including the anti-apoptotic Bcl-2 protein.[130,131] These cells exposed to PMT also exhibit increased cell migration capacity,[130,131] which may be instrumental for tumor progression. Additional evidence pointing to the oncogenic potential of PMT is the finding that PMT stimulates Janus kinases (JAK)[132]/signal transducer and activator of transcription (STAT) signaling in embryonic kidney cells and fibroblasts,[133,134] through G_q-dependent phosphorylation and activation of the JAK1 and JAK2 and increased expression of proto-oncogene serine/threonine kinase Pim-1. This is followed by activation of STAT1, STAT3 and STAT5 transcriptional factors, the upregulation of cyclooxygenase 2, a pro-inflammatory protein that is upregulated in many cancers and downregulation of the transcription factor suppressor of cytokine signaling-3 (SOCS-3).[115] Similarly to NF-κB, it has been acknowledged that STAT3 can act as a non-classical oncogene and plays a role in inflammation-associated cancer.[135] Through the STAT activation, PMT exposure in vitro induces proliferation of native T cells and the differentiation of Th17 cells/IL-17 production,[136,137] which is linked to excessive inflammation such as inflammatory bowel disease. PMT also initiates cytoskeletal rearrangements, including focal adhesion assembly and actin stress fiber development in the RhoA-dependent manner,[115] which may underlie increased motility and tumor-aggressive behavior.[138] In summary, these in vitro data support PMT's ability to manipulate host signaling pathways just like oncoviruses.

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4.4 Animal, epidemiological and clinical data of PMT and *Pm*

There have been no animal carcinogenesis models using PMT to our knowledge. However, a limited number of experimental animal studies for PMT-induced atrophic rhinitis have reported the development of epithelial squamous metaplasia in some of the PMT-injected animals.[139,140] This seems compatible to *H. pylori*-induced gastric carcinogenesis where atrophic gastritis and intestinal metaplasia precede gastric cancer.[141,142]

Likewise, a few studies have reported carriage of *Pm* in human populations using serology or oropharyngeal swab culture. Serological studies were designed to target capsular antigen types A and D or non-specific non-PMT somatic antigens. These studies have revealed that *Pm* carriage is quite common in people routinely exposed to animals, such as meat industry workers and pet owners and the highest rate was found in pig breeders (~77%, serology and culture combined).[143–145] Nevertheless, information concerning prevalence of toxigenic *Pm* has been limited, but it is estimated to be very low in human populations (<10%).[146] Because toxigenic activities were tested biochemically in earlier studies, it was not clear whether strains negative to PMT did not carry the *toxA* gene or whether its expression was transcriptionally repressed. There have also been case reports of *Pm* infection diagnosed in various types of cancer patients.[147,148] Yet, as *Pm* was primarily isolated from non-cancerous infectious lesions or blood, it is difficult to infer any causal associations from these reports.

5. *Fusobacterium nucleatum* toxins

5.1 *Fusobacterium nucleatum* background

The oral microbiota contains one of the highest diversities of bacteria in the human body. Many of these bacteria are believed to contribute to the development of cancer. *Fusobacterium nucleatum* (*F. nucleatum*) is one such bacteria and is considered an oncobacterium.[149] *F. nucleatum* is a Gram-negative anaerobic bacteria that is found primarily in the oral mucosa under normal conditions. However, *F. nucleatum* has been implicated in several disease states including colorectal cancer.[150] *F. nucleatum* is more abundantly found in diseased lesions.[150] Multiple studies have shown that *F. nucleatum* is found to be present in potentially high numbers in cancerous tissues.[149] There has been a renewed interest in studying the pathogenicity of *F. nucleatum* given its prominent placement in diseased states. *F. nucleatum* featured prominently in a review of the bacteria associated with the oral microbiome and their potential contribution to systemic disease and cancer.[16] Two *F. nucleatum* exotoxins with potential links to carcinogenesis are FadA and Fap2. In this review we will focus on the FadA and Fap2 adhesins which may play significant roles in tumor formation and cancer.

5.2 *Fusobacterium* adhesin A (FadA)

5.2.1 FadA molecular and chemical characteristics—*Fusobacterium* adhesin A (FadA) was discovered and found to be conserved among *Fusobacterium* genera that inhabit the oral mucosa and is important for cell binding.[151] FadA is a 129 amino acid protein with an 18 amino acid signaling peptide.[151] It has a secreted form that was shown to cause an upregulation of the β-catenin pathway and Wnt gene expression, both important

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developmental pathways that when dysregulated are leading causes of carcinogenesis.[152] The crystal structure of FadA revealed a unique "leucine chain" structure which, when mutated, abrogated the binding of host cells.[153] Although they did not identify the receptor binding site for FadA they suggest that FadA functions as a filament and therapeutic targets in this region might be suitable for future clinical importance.[153]

5.2.2 Microbial characteristics of FadA—FadA is coded by the fadA gene and shown to be expressed on the cell surface.[151,153] FadA contains a signaling peptide that would be used for its secretory pathway out of the cell and studies have shown that it also has a secreted form.[151]

5.2.3 FadA in vitro studies—Colorectal cancer cell lines HCT116, DLD1, SW480 and HT29 exposed to wild-type *F. nucleatum* demonstrated proliferation but those exposed to deleted FadA mutants did not have the same result.[152] FadA was shown to promote the activation of the β-catenin pathway and Wnt signaling seen by Western blotting suggesting a pathway that potentially contributes to carcinogenesis.[152] When *F. nucleatum* is introduced to E-cadherin expressing CRC cells there is an increase of cell proliferation but not in CRC cells that do not express E-cadherin indicating that FadA promotes tumor growth.[152] Thus, FadA acts like a ligand of E-cadherin and when it binds E-cadherin it reduces its tumor suppressor activity.[152] Work by this same group showed that Annexin A1, a modulator of the Wnt/β-catenin molecular pathway, is also upregulated by FadA when acting on the E-cadherin molecular pathway.[154] This upregulation enters into a positive feedback loop thereby promoting cancer progression by this microbe.[154]

FadA was also shown to stimulate inflammatory responses in these cells to the same extent as *F. nucleatum* pointing to its significance as an oncogenic microorganism.[152] FadA might represent a pathway whereby *F. nucleatum* might attach and invade. When FadA was deleted it was shown that *F. nucleatum* was unable to attach and invade but FadA was shown to trigger a signaling cascade inside HCT116 cell lines promoting lymphoid enhancing factor expression.[152] Collectively, these results suggest a strong contribution to colorectal cancer progression by FadA.

5.2.4 FadA activities in animal models—Both FadA and *F. nucleatum* were studied in vivo by subcutaneously injecting them into nude mice.[152] When HCT116 cells with FadA were injected subcutaneously in nude mice this promoted growth of tumors.[152] Tumor growth increased in nude mice 20% when compared to controls after treatment with FadA protein.[152] Rubinstein et al. also reported positive correlation between fadA gene copy numbers and *ANXA1* mRNA levels in a F. *nucleatum*-induced $Apc^{min/+}$ mouse model.[154] Further studies in mice also conclusively demonstrated that *F. nucleatum*, resulted in increased tumor growth and enriched populations in cancer tissues.[155]

5.2.5 FadA clinical and epidemiological results—Expression of the FadA gene was statistically significantly higher in colorectal cancer tissues than in normal mucosa or adenoma tissues.[152] Presence of FadA in colon tissue from patients with colorectal cancer showed that it was 10–100 times the normal level.[152] However, we are just starting to understand the mechanistic roles that *F. nucleatum* may be contributing to the progression of

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cancer or if it is merely a "hitch hiker" found at tumor tissues. Rubinstein et al. also demonstrated co-localization of FadA and Annexin A1 proteins as well as positive correlation between fadA gene copy numbers and *ANXA1* mRNA levels in human colon cancer tissue.[154] This group suggests that Annexin A1 presents as a novel marker of cancer prognosis based on the results of disease-free survival of 466 CRC cancer patients.[154] On the other hand, a recent study measured the antibody responses to 11 *F. nucleatum* antigens, including FadA, in pre-diagnostic serum samples of colorectal cancer patients and did not find that FadA was statistically significant as an indication of colorectal cancer risk.[156]

5.3 *Fusobacterium* apoptosis protein (Fap2)

5.3.1 Fap2 molecular and chemical characteristics—Fap2 was shown to have a predicted molecular mass of 389.8 kDa and a size of 11.3 kb.[157] This corresponds to a 3692 amino acid protein with no cysteine residues.[157] Using site-directed mutagenesis it was shown that Fap2 contains three domains: the C-terminal autotransporter domain, the central domain containing the repeats seen in other filamentous hemagglutinins and adhesins, and the N-terminal domain.[158] The 3596th to 3606th amino acids have been identified to be an immunogenic epitope of the Fap2 protein.[159] Using an ELISA-based analysis; the immunogenic region: "TELAYKHYFGT," in the Fap2 protein was identified as the specific region.[159]

5.3.2 Microbial characteristics of Fap2—Fap2 was previously identified as a potential apoptosis inducing protein that was treated with proteases and heat which suggested that Fap2 (unnamed at that time) was probably a membrane-bound protein.[160] Further studies by the same group elucidated that the gene product of fap2 codes for the large outer membrane protein with similar parallel β-helix structures and highly conserved C-terminal barrel autotransporter domains seen in other homologues.[161] The fap2 gene codes for the membrane-bound Fap2 protein and was identified as an important binding and galactose sensitive adhesin that was not able to hemagglutinate.[157]

5.3.3 Fap2 in vitro studies—*Fusobacterium* apoptosis protein (Fap2) was initially isolated as a potentially important membrane-bound protein that could play a role in apoptosis.[160] *F. nucleatum* was shown to activate apoptosis in human peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs).[160] More recently it was shown that Fap2 has the capacity to bind to galactose residues and fap2 mutants failed to coaggregate with human embryonic kidney[92] 293 T cells presumably due to lack of galactose binding.[157] Fap2 is able to bind to TIGIT, an inhibitory receptor present on all human natural killer cells that is then able to abrogate the immune cell action against *F. nucleatum*.[162] Human cell lines HCT116, RKO and HT29 were used to demonstrate the binding of *F. nucleatum* to colorectal cancer cells via Fap2, while the binding was absent with Fap2 deletion mutants.[163] When colorectal cancer cells (CRC) were treated with O-glycanase, the binding of *F. nucleatum* and CRC cells was reduced.[163] This could be as a result of loss of sugar residues Gal-Gal-NAc which are needed to bind the Fap2 lectin expressed by *F. nucleatum*. It was also shown that *F. nucleatum* strains that lacked Fap2 or had Fap2 mutant proteins showed reduced binding to Gal-Gal-NAc expressing CRC cells.[163] The binding between Fap2 and Gal-Gal-NAc provides an important mechanism by which *F.*

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nucleatum is able to alter the tumor microenvironment to promote cancer. Given the capacity for binding to a widely present sugar residue, Fap2 presents an important target of studies aimed at uncovering its contribution to cancer.[159,163]

5.3.4 Fap2 activities in animal models—

Use of Fap2 mutants that lack Fap2 show decreased colorectal cancer cells in mice.[163] The mice model showed that established colorectal cancer tumors when inoculated with *F. nucleatum* had more colonization than with the Fap2 deletion mutant and *F. nucleatum* localized to mouse tumor tissues in a Fap2-dependent manner.[163] Consistent with the observations in human tissue described below, Gal-GalNAc (measured using FITC-labeled PNA) was overexpressed in the mouse CRC sections compared with sections prepared from adjacent normal colon tissues.[163]

5.3.5 Fap2 clinical and epidemiological studies—

Fap2 and Gal-GalNAc was shown to potentially give rise to the presence of *F. nucleatum* in cancer and its precursor lesions and metastasis in human.[163] When Gal-GalNAc levels on healthy human colorectal tissues, human colonic adenomas, and human colorectal adenocarcinomas was assessed, its levels were significantly higher in adenocarcinomas compared with adenomas.[163] Within the adenoma group, there were statistically significant trends by histology, i.e., the highest levels of Gal-GalNAc expression on villous adenomas, followed by tubulous villous adenomas.[163]

When 37 human patient colorectal cancer cases were tested immuno- and seroreactivity to Fap2 protein, investigators found a 100% seroreactivity with the colorectal cancer samples and a 32% seroreactivity with healthy controls.[159] The Fap2 peptide mimotope proved to be immunogenic in both animal and human models of colorectal cancer giving some indication of its potential use as a biomarker for cancer progression. However, in a study using 11 *F. nucleatum* antigens (including Fap2 and FadA) in pre-diagnostic serum samples from 485 colorectal cancer patients the results indicated that these proteins did not implicate a statistically significant risk to contract colorectal cancer.[156] The approach used differs from other studies in that they had access to pre-diagnostic serum samples, which were not taken before cancer diagnosis and matched to controls who did not develop colorectal cancer.[156]

5.4 FadA and Fap2 potential mechanistic roles in carcinogenesis

FadA and Fap2 are two adhesins produced by *F. nucleatum* that are of significant interest to cancer researchers. *F. nucleatum* has the ability to adhere to multiple cell types creating a platform for invasion and persistence of other cell types. *F. nucleatum* is considered an important “bridge” organism paving the way for early colonizers of the oral microbiome such as *Streptococcus*.[132,149] In this regard *F. nucleatum* might be looked at as an ecosystem engineer, a term first coined by Jones.[164] An ecosystem engineer has the ability to transform the environment to suit its need for survival. Studies looking at cancer formation has already implicated cancer cells as being ecosystem engineers.[165] This understanding could help form future research decisions and possible medical interventions. The human oral microbiome contains a very diverse collection of bacteria in addition to other constituents such as viruses and fungi.[16] The mouth has been described as an island with many biogeographical features which result in multiple unique ways for bacteria to colonize the different micro niches.[132] These adaptations may include the ability for *F. nucleatum* to

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signal to other microbial and host cells (as in the case of FadA which acts as a ligand to E-cadherin-expressing colorectal cancer cell types) and adhere to other cells (as Fap2 can bind to sugar residues found on colorectal cancer cells). Under dysbiosis, bacteria, including their toxins, can make their way to other parts of the body and result in many diseased states.[16] These same adaptations might serve to disrupt homeostasis leading to severe conditions such as cancer. The role of *F. nucleatum* in adhering to other cells and creating a stable habitat for other colonizers has already been shown but we are suggesting that it plays an additional role as an ecosystem engineer in altering the tumor environment to facilitate proliferation and colonization. In Fig. 1B *F. nucleatum* functions as a putative ecosystem engineer by signaling to other cell types such as colorectal cancer cells via FadA which in turns leads to proliferation and tumor growth. This would have significant impacts to the tumor microenvironment such as invasion and increase in size. *F. nucleatum* also has the ability to adhere to other cell types via Fap2 binding to sugar residues on the cell surface and this would result in an increased recruitment of *F. nucleatum*. This contributes to the modification of the tumor microenvironment thus implicating *F. nucleatum* as an ecosystem engineer.

6. *Bacteroides fragilis* toxin (Bft)

6.1 Chemical and molecular characteristics of Bft

Bacteroides fragilis toxin (Bft) is a zinc-dependent metalloprotease toxin with three isotypes (BFT-1, BFT-2, and BFT-3).[166] While this enterotoxin has been known for several decades, it was not until the early 1990s that Bft was purified and characterized extensively. At this point it was established through the use of SDS-PAGE that Bft had a molecular weight of approximately 19,000,[167] a value similar to previous estimates that predicted a value of 19,500.[168] Bft was further characterized to have an isoelectric point in the range of 4.4–4.6 and to be stable within a pH range of 5–10.[167] Interestingly, Bft appears to be stable at temperatures ranging from −20 °C to 20 °C, but shows marked inactivation at higher temperatures. Specifically when incubated for 1 h at 55 °C over 90% of the toxin activity was lost, and when incubated for 1 h at 65 °C over 99% of the toxin activity was lost.[167]

At this time the enzyme susceptibility of Bft was also tested, revealing that the toxin is resistant to trypsin, chymotrypsin, dispase, bacterial amylase and bacterial lipase, yet sensitive to proteinase K and *Streptomyces* protease.[167] Antiserum specific to the toxigenic strain of *B. fragilis* has been shown to neutralize the activity of the toxin, while exposure to antiserum specific to nontoxigenic strains does not produce the same effect.[167] In addition to these physical characteristics, the molecular structure of Bft was also established at this time, with the first 20 amino acids from the N-terminal known to be Ala-Val-Pro-Ser-Glu-Pro-Lys-Thr-Val-Tyr-Val-Ile-Xxx-Leu-Arg-Glu-Asn-Gly-Ser-Thr.[167]

6.2 Microbial characteristics of Bft

Bacteroides Fragilis is an obligate anaerobe, Gram-negative bacteria that is a part of the normal biome of the human colon. Functionally, there are two distinct groups of *Bacteroides fragilis*. Strains that secrete a zinc-dependent metalloprotease toxin (Bft) which is encoded from the *B. fragilis* pathogenicity island are referred to as enterotoxigenic *B. fragilis*

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(ETBF). Strains that lack this ability are called nontoxigenic *B. fragilis* (NTBF).[169] While *Bacteroides fragilis* normally comprises only 1–2% of colon flora,[170] a value 10–100 times smaller than some other species of *Bacteroides* found in human intestines, it is the species most frequently isolated from clinical specimens, and as such is often viewed as the most virulent species of *Bacteroides*.[171] *B. fragilis* is so clinically prevalent due to its propensity to cause abscesses, soft-tissue infections, and bacteremias.[172]

Moncrief et al. generated a *Bft* recombinant PCR product that, when cloned into pUC19 and sequenced was determined to be 538 nucleotides in length,[173] providing insight into *B. fragilis'* enterotoxin encoding gene. This PCR product, which they designated as rBF107, was further analyzed and found to contain sequence identity to a number of metalloproteases, including *Pseudomonas aeruginosa* alkaline protease, and matrix metalloproteases.[173] Additionally, the sequence HELGHILGAEH, found in rBF107, exhibits consistency with the zinc-binding motif HEXXHXXGXXH, which is characteristic of the metzincin family.[173] This discovery gave strong support to the theory that Bft is a zinc-dependent metalloprotease.

Kling et al. did additional analysis of the encoding gene for the enterotoxin belonging to *B. fragilis*. This gene, termed *BftP* was found to be 1191 nucleotides long and code for a 44.4 kDa protein that is comprised of 397 amino acids.[174] BftP has a signal peptide 18 amino acids in length that is immediately succeeded by a protoxin that is 379 amino acids in length, while the protoxin found in stool samples begins at the 212th amino acid.[174] Three potential AUG start codons have been identified in the promoter region of the *B. fragilis* enterotoxin, with the middle start codon being followed by the amino acid sequence typical of a signal peptide. The two additional start codons are located 24 nucleotides upstream and 25 nucleotides downstream relative to the middle start codon. BftP has a signal I peptidase cleavage site located between two alanine residues at amino acid 17 and lacks an obvious ribosome-binding site.[174]

B. fragilis does not appear to utilize types III, IV, autotransporter or two-partner secretion systems,[175] yet does employ the type VI secretion system.[176] More specifically, *B. fragilis* expresses the GA3 subtype of T6SS (type VI secretion system), which is not highly conserved among other bacterial species.[177] The type VI secretion system exports the Bft exotoxin directly into eukaryotic host cells or other co-growing bacterial cells. This is accomplished through the T6 apparatus, a multiprotein, cell envelope spanning complex comprised of a core of Tss proteins.[177] This complex is characterized by a needle-like structure that becomes inundated with toxins in the cytoplasm.[178] The needle is then driven from the cell via contraction of the surrounding sheath in order to implant the toxins into extracellular matter.[177] Additionally, *B. fragilis* also appears to use type I secretory system, as it was determined to have three Tolc-like proteins that are encoded adjacent to genes that have previously been shown to be associated with type I secretion systems.[175]

6.3 In vitro toxicity of Bft

In vitro experiments have helped shed light on the potential mechanisms of action used by Bft. On a biochemical level Bft has been shown to utilize its zinc-dependent protease activity to cleave the extracellular domain of E-cadherin, which is followed by further

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intracellular degradation.[179] This is relevant because E-cadherin is not only the primary protein of zonula adherens, which function to maintain tight epithelial junctions, but also has tumor suppressor functions. Bft has been shown to provoke cell rounding, increase in volume, and effacement of microvilli and apical junctional complexes in intestinal and renal cells in culture.[180] This supports the idea that cadherins are functionally involved. This is thought to lead to actin rearrangement, and consequently pro-inflammatory cytokines, which diminish epithelial barrier function.[180] In addition, this leads to an increase in cellular proliferation mediated by elevated expression of the c-Myc oncogene.[181] Interestingly, these consequences appear to be reversible, as cells appear normal 2–3 days after toxin treatment.[180]

Additional experiments have shown that exposure to 15 ng of Bft per mL resulted in formation of blebs on the cell surface and complete disappearance of stress fibers along with F-actin condensation at the cell periphery.[182] These results were more pronounced at increased doses.[182] Cell organelles (including mitochondria and nucleus) appear to be unaffected by exposure to Bft,[182] as the toxin's effects seem to be localized to the actin cytoskeleton and associated structures. Additionally, Bft has been shown to stimulate intestinal epithelial shedding, which is dependent, in part, on E-cadherin degradation and β-catenin–T cell-factor nuclear signaling[183] and to stimulate proliferation and migration of human colon cancer cells in vitro.[181]

Incubation of human colorectal cancer cells with BFT led to upregulation of spermine oxidase, a polyamine catabolic, resulting in increased reactive oxygen species (ROS) and DNA damage measured by γ-H2A induction.[184] Exposure to Bft has also been shown to induce changes in gene expression and chromatin availability in colonic epithelial cells.[185] Eight genes have been identified in colonic epithelial cells that are significantly differentially expressed 24 h post-Bft exposure, but not 48 h post-exposure. It has also been demonstrated that while Bft does induce changes in chromatin accessibility in colonic epithelial cells, these changes are transient, and greatly decline by 48 h after Bft treatment.[185] Specifically, there is a noted increase in chromatin accessibility at transcription factor binding sites, particularly those belonging to the AP-1/ATF family, many of which function downstream of mitogen-activated protein kinase (MAPK) pathways. Critically, while an increase in chromatin accessibility has been shown to have an association with differential DNA methylation and DNA mutation, these differences are not statistically significant.[185]

6.4 Toxicity of Bft in animal models

Mouse models have been used to demonstrate a link between Bft and colorectal cancer.[186] Mice colonized by the enterotoxigenic *B. fragilis* experienced a rapid onset colitis followed by tumors of the distal colon.[186] This was in contrast to mice exposed to nontoxigenic *B. fragilis*, which did not induce tumor formation at a higher rate than the control. Mice inoculated with the toxigenic strain developed observable tumors as early as 4 weeks after exposure, and microadenomas as early as 1 week after exposure.[186] Bft also induced a prominent lamina propria intrusion of IL-17-producing $CD4^+$T cells (Th17) and γδ-T cells with a dominant signal transducer activator of transcription-3 (STAT-3) pathway.[186] The importance of IL-17 in the tumor-inducing response to Bft was demonstrated by the

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decrease in colonic tumors when the mice were treated with IL-17 blocking antibodies.[186] IL-17 has been shown to induce tumor growth both in vitro and in vivo via an IL-6-stat3 signaling pathway.[187] *B. fragilis* has also been found in increased quantity in the intestinal lumen of the 1,2-dimethylhydrazine (DMH)-induced rat colorectal cancer model.[188]

6.5 Clinical and epidemiological evidence to support in vivo toxicity in humans

In addition to animal models, there is now substantial indication that Bft is involved in the development of colorectal cancer in humans. In addition to being the most commonly isolated Bacteroides species found in clinical specimens,[171] enterotoxigenic *B. fragilis* has been shown to have an increased prevalence in samples from cases involving colorectal cancer when compared to a control group.[166] There has also been shown to be a difference in Bft detection depending on the stage in the course of the disease, with late-stage colorectal cancer patients having an increased rate of Bft detection in comparison to colorectal cancer patients at earlier stages of the disease.[166] This observation becomes more pronounced as the disease progresses, to the point where Bft was identified in 100% of late-stage colorectal cancer patients in one study.[166] It should be noted that this study still found Bft in approximately 54% of the controls when one-sided colon samples were obtained, yet this number was notably lower that the approximately 87% of colorectal cancer cases that tested positive for Bft.[166]

This link is reinforced by additional studies, which have demonstrated that stools from colorectal cancer patients were shown to test positive for Bft at significantly higher rates than controls.[189] Interestingly, *B. fragilis* has been reported to be found in more abundance in mucosal as opposed to luminal samples taken from colorectal cancer patients.[190] Here it is once again important to differentiate between toxigenic *B. fragilis* and nontoxigenic *B. fragilis*. One study determined that *B. fragilis* as a whole was found in 77% of colorectal cancer patients yet 68% of healthy controls, a difference that was not significant.[189] However, the Bft gene was detected in 38% of the *B. fragilis* isolated from the cancer patients, but only 12% from the control, a statistically significant difference.[189] Additionally, individuals with familial adenomatous polyposis, a disease that leads to digestive tract polyp formation at a young age due to mutation of tumor suppression gene APC, has been shown to be associated with enterotoxic *B. fragilis*.[191] In one study 60% of patients with familial adenomatous polyposis had gut mucosa with enterotoxic *B. fragilis* present, compared to only 30% of controls.[191]

7. *Escherichia* (*E*) *coli* toxins

E. coli secretes a number of protein exotoxins, some of them are lineage-specific, and among those, three toxins: colibactin, cytotoxic necrotizing factors (CNF) and α-hemolysin, which are pertinent to human cancer, are reviewed here in Sections 7.1–7.3.

7.1 Colibactin

7.1.1 Chemical and molecular characteristics—Colibactin is a genotoxin causing DNA double strand breaks in a contact-dependent manner. Colibactin is synthesized by a hybrid non-ribosomal peptide synthetase-polyketide synthase (NRPS-PKS) assembly line,

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[192] a sequence of tailoring and editing enzymes, and a critical phosphopantetheinyl transferase that mediates NRPS and PKS activation.[193] Despite multiple attempts, colibactin had not been able to be isolated successfully due to its' extreme instability.[194] Finally, in September 2019, through the interdisciplinary approach encompassing chemical synthesis, metabolomics, and probe-mediated natural product capture, a group of scientists from Yale University claimed a complete elucidation of colibactin structure.[195] It has a nearly symmetrical structure that contains two electrophilic spirocyclopropyldihydro-2-pyrrolone and the hydrolytically labile C36–C37 α-dicarbonyl.[195] These two electrophilic cyclopropane residues undergoes ring-opening through nucleotide addition,[195] consistent with earlier observation that colibactin-producing bacteria cross-link DNA.[196–198] Using genome-editing techniques, the group also showed the production of colibactin's precursor, precolibactin 1489, that requires every biosynthetic gene in the colibactin gene cluster, demonstrating it as being the first reported product derived from the long-elusive and completed biosynthetic pathway.[195] However, precolibactin 1489 was considered a stable product arising from oxidation and macrocyclization of a putative linear direct precursor of colibactin, precolibactin 1491.[195]

7.1.2 Microbial characteristics of colibactin—

Colibactin is encoded by the *pks* genomic island, and is often found on *E. coli* strains belonging to the B2 phylogenetic group.[194] The bacteria *Klebsiella pneumoniae* is also known to carry the colibactin-producing *pks* island, with one study finding 16.7% of *K. pneumoniae* isolates were *pks*+.[190] It is estimated that roughly 20% of healthy individuals are colonized by *pks*+ *E. coli*.[199] In terms of *E. coli* prevalence, the *pks* genomic island is found in 30–40% of all B2 strains.[200] Colibactin-producing *E. coli* has also been shown to be relatively resistant to human macrophages and to not influence COX-2 expression.[201]

The *pks* genomic island is 54-kb in length and directs the synthesis of colibactin via specific cellular machinery.[202] It consists of 19 protein producing genes named *clbA* to *clbS*, which are arranged in sequence as *clbA*, *clbR*, *clbC* to *clbQ* and *clbS*.[192]

The first step in the assembly line of colibactin synthesis consists of the ClbA protein, a phosphopantetheinyl (PPant) transferase, activating the NRPS and PKS enzymes via the addition of a PPant onto the NRPS and PKS carrier protein domains.[192] ClbN and ClbB serve as the NRPS enzymes, with ClbN producing *N*-myristoyl-D-Asn, which is then accepted by ClbB. ClbB then proceeds to add either Ala or Val and incorporate a malonyl-CoA. ClbH and ClbI have been shown to be essential for the formation of cyclopropane, which is responsible for colibactin-induced DNA alkylation.[192] ClbsD-G are required for the synthesis of the unique PKS extender aminomalonyl unit (AM), which is necessary for genotoxicity. ClbG transfers AM to ClbK, which then incorporates AM into colibactin. While less is knows about the off-loading process, it is thought that ClbQ is involved in this process, and therefore controlling the flux of colibactin production.[192] Finally, ClbM (a MATE transporter) is responsible for precolibactin release into the periplasmic space, where ClbP generates the mature colibactin via removal of the *N*-myristoyl-D-Asn side chain.[192] ClbS is also produced by *pks*+ *E. coli* where it is thought to sequester colibactin and therefore protect the *E. coli* from damage from its own product.[192] Additionally, clbM has

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been shown to play a role in the transport of colibactin,[203] but to date the precise mechanism of how colibactin is secreted and transported to host cell cytosol is poorly understood.

7.1.3 In vitro toxicity data of colibactin—

Colibactin induces cellular damage in eukaryotic cells via introduction of a double-stranded break into the DNA of the host cell.[193] This action requires the presence of live bacteria, not being observed with bacteria culture supernatants or cell lysates. This damage serves to activate a signaling pathway that eventually leads to cell cycle arrest, enlargement of cell bodies, and ultimately death of the cell.[193] Cells exposed to *pks*+ *E. coli* strains have been shown to demonstrate many of the defining characteristics of cell death, including chronic DNA double strand breaks, enhanced senescence-associated β-galactosidase (SA-β-Gal) activity, expansion of promyelocytic leukemia nuclear foci and senescence-associated heterochromatin foci.[204] Additionally, exposed cells increase their reactive oxygen species production and pro-inflammatory cytokines, chemokines and proteases secretion.[204] Importantly, the presence of colibactin-producing *E. coli* strains in fibroblasts in vitro has been shown to promote the growth of epithelial human lung adenocarcinoma and colon carcinoma tumor cells.[204] *Pks*+ *E. coli* infection has also been associated with the induction of IL-6 production, which is known to induce epithelial cell invasion, and MMP production, which has been shown to have direct tumor-promoting effects.[204] Taken together this suggests a mechanism for *pks*+ *E. coli* reprogramming fibroblast expression profiles in a way that promotes growth of cancer cells.[204]

Brief exposure to low doses of *pks*+ *E. coli* strains has been shown to transiently damage DNA in mammalian epithelial cells, followed by cellular division with incomplete DNA repair.[205] Increased rates of chromosomal aberrations, instability, and gene mutations were also observed in cells post-exposure.[205] Due to the difficulty in isolating colibactin, alternative methods have been turned to in order to study this toxin's exact mechanism of action. Production and observation of synthetic adenine-colibactin adducts mimicking the results of *pks*+ *E. coli* incubation in human cells demonstrated that colibactin breaks DNA strands by utilizing alkylation via a cyclopropane "warhead."[197] The degradation products observed in this experiment consisted of two diastereomeric adducts that both contain a 5-hyfroxypyrrolidin-2-one ring system with an attached N3-substituted adenine ring. Due to the small size of these adducts, it is likely that they are actually derived from a larger, yet unstable colibactin-DNA interstrand cross-link.[197]

7.1.4 Colibactin toxicity in animal models—

In vivo experimentation has shown that *pks*+ *E. coli* strains induced the formation of phosphorylated H2AX foci in mouse enterocytes and indicates DNA damage to colonic epithelial cells.[205] *Pks*+ *E. coli* strains have also been found to induce a higher degree of lymphopenia in septicemic mice as compared to strain that did not produce colibactin.[206] Perhaps most notably, *Pks*+ *E. coli* has been observed to promote tumorigenesis in the $Apc^{Min/+}$; $Il10^{-/-}$ mouse model in a colibactin-dependent manner, suggesting colibactin is a driver of carcinogenesis.[207] A rat model has also demonstrated the link between *pks*+ *E. coli* strains and DNA damage, with observable signs of genotoxin stress including anaphase bridges, higher occurrence of crypt fission and accelerated renewal of the mature epithelium detected in adult rats.[208] This same

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model revealed that colibactin-producing *E. coli* strains increased intestinal epithelial cell apoptosis and proliferation, as well as amplified goblet, enteroendocrine and paneth cells.[208] This serves to support the connection between *Pks*+ *E. coli* and intestinal tumorigenesis.[208]

Notably, even transient exposure to *Pks*+ *E. coli* has been shown to increase tumor growth.[209] This was shown when intestinal epithelial cells HCT116 that had been mixed with *Pks*+ *E. coli* were injected into nude mice. 3 h post injection the mice were given a broad-spectrum antibiotic (imipenem) in order to kill the bacteria. This short exposure resulted in significantly increased tumor growth, while injection of epithelial cells that had been mixed with *Pks* – *E. coli* had no effect on tumor growth, similarly to uninfected cells.[209] This tumor-promoting effects was mediated by induction of a senescence-associated secretory phenotype via overexpression of microRNA 20a-5p, which led to accumulation of SUMO conjugated p53.[209] The previously mentioned adenosine-colibactin adducts that was shown in vitro mechanistic study of colibactin action have also been observed in the colonic epithelial cells of mice that have been monocolonized with *pks*+ *E. coli*.[197]

7.1.5 Clinical and epidemiological data to support in vivo toxicity in humans

—Among B2 *E. coli* isolated from colorectal cancer and diverticulosis patients, colibactin-producing variants were found to be prevalent at a high rate in the colorectal cancer patients (55.3%), especially when compared with the 19.3% seen among the diverticulosis patients.[33] This observation corroborates additional data showing colibactin-producing *E. coli* strains are associated with colorectal tumors, with 55–66.7% of cancer patient biopsies testing positive while only 19–21% of intestinal control tissue did.[199] Recently, PCR analysis has been used to demonstrate that the *clbA* gene is found at an increased prevalence in the stools of patients with colorectal cancer.[210] In this study 56.4% of stool from colorectal cancer patients was found to have *clbA*, as compared to only 18.5% seen in the controls.[210]

The presence of the *pks* island has also been shown to enhance the ability of *E. coli* to persist in the human gut microbiota.[211] One study on this subject showed that long-term colonizers of the gut were significantly more likely to have the *pks* island than either intermediate-term colonizers or transient strains.[211] This may help explain why B2 *E. coli* strains are represented to a large degree in the gut microbiome.[211] Additionally, individuals with familial adenomatous polyposis, a disease that leads to digestive tract polyp formation at a young age due to mutation of tumor suppression gene APC, has been shown to be associated with *pks*+ *E. coli*.[191] In one study 68% of patients with familial adenomatous polyposis had gut mucosa with *pks*+*E. coli* present, compared to only 22% of controls.[191]

7.2 CNFs (cytotoxic necrotizing factors)

7.2.1 Chemical and molecular characteristics of CNFs

—CNFs belong to a family of deamidating toxins that deamidate glutamine 63/61 in the switch II region of Rho GTPases, leading to constitutive activation of Rho GTPases.[212] There are four identified variants of cytotoxic necrotizing factor (CNF), CNF1, CNF2 and CNF3, which are secreted by *E. coli* and CNFY, which is secreted by *Yersinia*. CNF1 is the best characterized of all CNFs, while CNF2 shares 85% sequence identity with CNF1, CNF3 shares 70% sequence identity with CNF1 and CNFY shares 61% sequence identity with CNF1.[212] While there is a

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great deal of similarity among the CNFs, they differ in terms of their substrate specificity. CNFY exclusively acts on RhoA, B and C and has no activity for Rac or Cdc42.[213] CNF2 preferentially modifies RhoA and Rac,[214] whereas CNF1 and CNF3 deamidate RhoA, Rac and Cdc42.[212]

All CNFs are identical in length at 1013/1014 amino acids long and posses an N-terminal receptor binding domain.[212] Amino acids 710–1014 in CNF1 have been shown to be responsible for the catalytic activity of this protein, while amino acids 53–190 are responsible for cellular uptake.[212]

CNF1 is a protein with a molecular weight of 115 kDa.[215] Experimentation has determined that CNF is stable within a pH range of 6–10.5.[216] The toxic activity of CNF was destroyed when exposed both to a temperature of 75 °C for 15 min and 60 °C for 1 h.[216] As CNF is primarily a protein, it has a low carbohydrate content of no more than 25 μg.[216]

7.2.2 Microbial characteristics of CNFs—

Compared to the *pks* island, *CNF1* is relatively less common, with one study determining that, of 78 *E. coli* strains isolated from adults with urinary tract infections, 13% were positive for the *CNF* gene.[217] The nucleotide sequence of the *CNF* gene has been identified, and notably the start codon is located at nucleotide 856 while the TGA termination codon is located at nucleotide 3900.[218] Interestingly, the C+G content of the *CNF1* is lower than that of the E. coli genome as a whole at 36.5%.[218]

For CNF1 specifically, ferredoxin is required for secretion at the step of crossing the cytoplasmic membrane.[219] CNF1 is transported in its active form by outer membrane vesicles.[220] This is supported by the fact that the histone-like nucleoid structuring protein H-NS has been shown to down regulate CNF1 production by affecting outer membrane vesicle release.[220] *CNFY* utilizes the type III secretory pathway, which supports *CNF1* using the same pathway.[221]

7.2.3 In vitro toxicity data of CNFs—

CNF induces actin assembly and multinucleation in mouse embryonic fibroblasts that were incubated with the purified toxin.[222] CNF has been shown to inhibit the intrinsic GTPase activity of RhoA and completely blocks GTPase activity stimulated by the Rho-GTPase-activating protein (rhoGAP).[222] CNF has been observed to reduce function at the basolateral membrane of intercellular epithelial junctions.[125] This is accomplished through displacement of the proteins occludin and zonula occludens-1 (ZO-1), and reorganization of junction adhesion molecule-1 (JAM-1).[125] CNF1 has also been shown to increase F-actin content in cytoskeletons of polymorphonuclear leukocytes.[223] CNF1 also increases superoxide generation and adherence on epithelial monolayers, but significantly decreases their phagocytic function.[223] This implies a possible role of CNF1 in intestinal infections by way of by stimulating polymorphonuclear leukocyte cytotoxicity.[223]

It has also been shown that treatment with CNF1 initially inhibits mitosis and cytokinesis and elicits endoreplication and polyploidization in human colon cancer cells in culture.[224] These outcomes were found to be followed by depolyploidization-associated survival of

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cells. An important finding from this study was the discovery that progeny derived from cells treated with CNF1 exhibited marked genomic instability, which was clearly seen by an increased rate of aneuploidy.[224] These progeny cells were noted to be more resistant to CNF1, but not to commonly used chemotherapy agents 5-fluorouracil and oxaliplatin.[224] There is also evidence that CNF1 induces COX2 and NF-κB expression in mouse fibroblasts and a human squamous cancer cell line, respectively.[225,226] CNF1 has also been shown to act in endothelial cells to both inhibit apoptosis and promote pro-inflammatory cytokine release, with the later effect observed in both epithelial and endothelial cells.[227]

7.2.4 CNF toxicity in animal models—

The majority of CNF-related research using animal models to date has focused primarily on inflammation rather than cancer, yet is still relevant for the purposes of this chapter. *CNF* positive *E. coli* has been shown to cause more inflammation-mediated morphological and histological tissue damage in a rat prostate model than an isogenic mutant.[228] Interestingly, this occurred despite the fact that total bacterial counts for each strain were nearly identical. A mouse bladder model demonstrated that mice infected with *CNF1*-positive strains consistently showed deeper and more extensive inflammation than those infected with the isogenic mutants.[229]

CNF1 has also been shown to have an association with spread of prostate cancer in a mouse model.[230] In this experiment induced overexpression of CNF1 was observed to promote both cellular invasion and migration.[230] Injection of CNF1 expressing cells into the tails of mice was observed to lead to a higher level of lung metastasis when compared to mice receiving a control vector.[230]

7.2.5 Clinical and epidemiological data to support carcinogenic potential in humans—

Similar to colibactin, *cnf*+ B2 *E. coli* strains have been shown to have an association with colorectal cancer.[33] Of total *E. coli* strains isolated from colorectal cancer patients, *cnf*+ B2 *E. coli* strains comprised 18%.[231] Once again, this is more prevalent that what is seen in patients with diverticulitis.[231] One study found that 39.5% of biopsies from patients with colorectal cancer contained CNF1-producing *E. coli*, whereas 12.9% of biopsies from diverticulitis patients were positive for CNF1-producing *E. coli*.[33] Notably, All *cnf1*-harboring strains belonged to the B2 phylogroup.[33]

It is important to note that an individual *E. coli* strain is not limited to producing only one exotoxin. In fact, the previous study found that 33% of B2 strains possessed both the *pks* island and the *cnf1* gene.[33] Additional studies have shown that both the *cnf1* gene and the *hlyC* (discussed below) are detectable in significantly lager numbers from *E. coli* blood isolates from cancer patients than in fecal isolates from healthy volunteers.[33] Cnf1-producing *E. coli* has also been associated with pathological conditions in other body systems. Strains of *E. coli* that possess the cnf1 gene have been shown to be associated with urosepsis,[232] possible indicating that the cnf1 gene is indicative of higher bacterial virulence. This higher virulence may lead to more severe inflammation and increases risk of carcinogenesis.

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7.3 α-Hemolysin

7.3.1 Chemical and molecular characteristics of α-hemolysin—α-Hemolysin is a cytotoxic protein that is a member of the repeats in toxin (RTX) group. This group is characterized by glycine and aspartate-rich repeats located at the C-terminus of these toxin proteins.[233] This nonapeptide sequence is GGXGXDXUX, where U represents a large, hydrophobic residue.[233] α-Hemolysin requires calcium to function optimally, and it is this repeating domain that is responsible for calcium binding.[233] Specifically, Asp-863 has been shown to be of special importance in calcium binding, and therefore HlyA conformational change and ability to breakdown the bilayer permeability layer.[233] HlyA possesses 15 of these nonapeptide repeats, implying the presence of a large number of calcium binding sites.[233]

HlyA consists of both protein (95% of weight) and carbohydrates, but lipids have not been detected.[234] The majority of hlyA is not heat-stable as it is inactivated at 56 °C,[234] but when exposed to temperatures of 37 °C inactivation did not occur for upwards of 18 h.[235] HlyA appears to be generally more stable at a lower pH, as it is stable at pH 3.0 for 6 h, but inactivates at pH 7.0–10.0 within 6 h.[236] This is not an unexpected result due to the acidity of the gut, a frequent location of *E. coli* colonization. Functionally, α-hemolysin of *E. coli* is a pore-forming toxin that acts by generating small cation-permeable channels in the membrane of a host cell.[237]

7.3.2 Microbial characteristics of α-hemolysin—The hemolysin gene (*hly*) was identified on 23% of 78 *E. coli* strains taken from adults with UTI's.[217] This study also concluded that *hly* was more prevalent than *CNF* in this population, with *hly* being present on 18 *E. coli* strains while *CNF* was present on 10.[217] Four defined genes encode for α-hemolysin. *HlyA* codes for a 107,000-kDa protein that is thought to be the inactive precursor of hemolysin.[238] *HlyC*, codes for a 20,000 polypeptide that is crucial for post-translational modification of the protein encoded by *hlyA*, rendering this protein hemolytically active. *HlyB* and *hlyD* are necessary for hemolysin secretion.[239] The gene for all four *hly* citrons has been localized to an 8211-base-pair region.[240] Of note, there is an ATG codon preceded by a potential ribosome-binding site (GCGG) at base-pair 5317.[240]

The secretory pathway of hly is well known, as it is both the prototypical and best characterized type I secretion system.[241] HlyA reaches the extracellular space in a soluble form without the formation of periplasmic intermediates.[242] Interestingly, a fraction of hlyA appears to be very tightly associated with outer membrane vesicles.[242] This corresponds with the observation that hlyA localized in outer membrane vesicles is frequently found among hemolytic *E. coli* strains.[242]

7.3.3 In vitro toxicity data of α-hemolysin—*E. coli* hemolysin has a pronounced effect on human monocytes, with nanomolar concentrations (250–2000 ng/mL) resulting in rapid and irreversible depletion of cellular ATP to levels below 20% of controls within 60 min.[223] Exposure of human monocytes to lower doses (10–200 ng/mL) of hemolysin results in the release of large amounts of interleukin 1 beta within 1–2 h.[223] 50% of cellular ATP is observed to be depleted within 90 min when incubated with only 0.3–3.0 colonyforming

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units of *E. coli* per monocyte.[223] In neutrophils, hemolysin causes defects in membrane permeability, resulting in ATP efflux and propidium iodide influx.[243] These cellular membrane lesions do not appear to be repaired.[243] Human serum albumin, lipoproteins and IgG protect erythrocytes and platelets, but not neutrophils, from the effects of hemolysin.[243] The binding of hemolysin to neutrophils results in granule exocytosis and loss of cellular phagocytic killing ability.[243] This is expected to impair or destroy the typical immunoprotective properties of these cells.

HlyA has also been shown to play an important role in giving *E. coli* the ability to translocate across cellular monolayers.[237] In this case, translocation was associated with decreased epithelial integrity of colonic cell cultures, which exhibited increased ion permeability. Experimentation has demonstrated that hemolysin secreting *E. coli* strains were able to translocate, whereas non-hemolytic mutants were unable to do so, and did not compromise the epithelial barrier.[237] In human uroepithelial cell lines, incubation with α-hemolysin substantially reduced IL-6 secretion by LPS through degradation of β-catenin and NF-κB subunit RelA.[244] Co-culture of a normal human colon mucosal epithelial cell line with *hly*+ *E. coli* activated expression of HIF1α and the glucose transporter GLUT1, which often upregulated in many types of cancer as an indication of reprogrammed energy metabolism, and repressed expression of the tumor suppressor BIM,[245] suggesting undelaying mechanisms of tumor promotion.

7.3.4 α-Hemolysin toxicity in animal models—
Much like the case of CNF, there has been relatively little cancer-specific research pertaining to animal models and hemolysin exposure. However, some relevant studies on hemolysin toxicity are discussed in this section. In a rat model, *Hly*+ strains provoked higher urinary shedding of leukocytes and erythrocytes, indicating an affect of hemolysin on inflammatory reactions.[246] Rat models have revealed that hemolysin mediates neutrophil toxicity, resulting in in vivo necrosis and lysis.[247] In the same study mentioned above that showed the importance of hemolysin in *E. coli* translocation ability in vitro, hemolysin was also demonstrated to induce focal leaks in native rat colon.[237] The rat colons exposed to hemolytic *E. coli* in this experiment were observed to have both absent nuclei and notable bacterial adherence to the epithelial surface in addition to decreased epithelial integrity.[237]

7.3.5 Clinical and epidemiological data to support carcinogenic potential in humans—
The effect of hemolysin has also been examined using *E. coli* strains taken directly from human patients. α-Hemolysin synthesized by *E. coli* strains isolated from ileal mucosa of patients with Crohn’s disease has been shown to adhere to differentiated intestinal cells, and may disrupt the intestinal barrier.[248] Hemolysin has also been shown to be associated with bacteremia in hospitalized cancer patients.[249] One study found that 26% of blood samples from cancer patients with bacteremia were positive for hemolysin-producing *E. coli*, while only 6% of fecal samples from healthy patients were positive for hemolysin-producing *E. coli*.[249]

In a very interesting outcome, *hly*+ *E. coli* has been implicated in adenomagenesis and colorectal cancer in females but not males.[245] *Hly*+ *E. coli* has been found to be relatively more prevalent in stools from females with adenoma and colorectal cancer, and to be

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correlated with poor survival in female but not male colorectal cancer patients.[245] In this experiment, *hly*+ *E. coli* was detected in 36.1% of female colorectal cancer patients, 39.3% of female adenoma patients as compared to just 6.7% of female healthy controls.[245] The association of *hly*+ *E. coli* with colorectal cancer was shown to be independent of both cancer stage as well as patient age.[245] It is hypothesized that the reason *hly*+ *E. coli* appears to be correlated to increased negative outcomes in female as opposed to males is due to immunological, hormonal or behavioral differences.[245] It is unlikely that genetic factors are causative of this discrepancy due to the fact that in vitro experimentation demonstrated both cytotoxic and carcinogenic effects on male derived cellular lines.[245]

8. *Salmonella enterica* avirulence protein A (AvrA)

8.1 Background *of Salmonella enterica* infection in humans

Salmonella enterica is a Gram-negative, facultative anaerobe, not a symbiotic commensal, but an intracellular pathogen to both humans and animals. *Salmonella* infection poses a major public health concern worldwide as foodborne illness.[250] There has been also a concern that long-standing *Salmonella* infection may increase the risk of colorectal cancer in humans as increased risk of colorectal tumor has been reported in several populations worldwide.[251,252] The *Salmonella* genome contains a remarkably large number (up to 23) of pathogenicity islands (SPI),[253,254] synthesizing over 60 effectors.[255] We describe here Avirulence protein A (AvrA), one of the bacterial type three secretion system effectors as there have been recent significant findings to support its oncogenicity.

8.2 Microbial characteristics of AvrA

AvrA represents the least characterized effector from the SPI1 which is originally thought to be non-virulent.[256] AvrA is directly injected into host cytoplasm by type 3 secretion system (T3SS) with a filament like-needle that is encoded by the same SPI1 in a finely coordinated manner.[257,258] The AvrA gene is present in most *Salmonella enterica* isolates from humans and animals. This is particularly the case for non-typhoid *Salmonella* as its prevalence was reported to be 98–100% (514/523 and 185/185),[259,260] while it was absent in typhoid strains, which has been linked to gallbladder cancer. Natural AvrA genetic variants have been reported for some *Salmonella typhimurium* strains, which lacks an internal leucine residue (L140) and has a profound effect on AvrA function.[261,262] Among non-typhoidal strains, AvrA protein expression is known to vary markedly with clinical presentation. AvrA protein is not often produced by clinical isolates from systemic disease, but it is often detectable in those from limited enteritis.[263] Interestingly, whereas expressions of most effectors from SPI1 are controlled at the transcriptional level,[264] it is now clear that expression of AvrA is regulated in a post-transcriptional manner and that mRNA transcription takes place constitutively in all AvrA-positive strains,[265] as AvrA remains silent when cloned into *E. coli*.[266] In fact, a number of Proteobacteria have evolved global regulation systems of post-transcriptional gene expression for their various virulence factors to be responsive to changes in the environment and to flourish in specialized host niches. *Salmonella enterica* is equipped with the Csr (carbon storage regulator) system, consisting of a small RNA binding protein (CrsA) and non-coding RNAs (CsrB/CsrC).[267] CsrA binding to the 5′ untranslated and/or early coding regions of mRNAs alters mRNA translation,

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turnover and/or transcript elongation, leading to either decay or stabilization of specific mRNA targets, while CsrB/CsrC containing multiple CsrA binding sites bind and sequester CsrA, thereby antagonizing CsrA activities.[267,268] Overexpression of either CsrA or CsrB shuts down AvrA expression, but that constitutional CsrA expression was required for AvrA expression.[265]

8.3 Chemical and molecular characteristics of AvrA

AvrA belong to a superfamily of the Yersinia outer protein J (YopJ) acetyltransferases, consisting of 287 amino acids.[262,269,270] The regulatory regions are located at flanking N- and C-terminal segments (residues 1–45 and 220–287, respectively) and the intervening residues (46–219) form the catalytic domain, which is structurally homologous to the CE clan peptidases.[262,270] In addition, AvrA possesses ubiquitin-like protein protease and deubiquitination activities. These enzymatic activities have been considered crucial for bacterial survival and virulence through post-translational modifications (PTMs) of host proteins to modulate the activity of key proteins on oncogenic and inflammatory pathways. Specific host targets for deubiquitination include IκBα and β-catenin and those for O (threonine)- and N^{e}(lysine)-acetylation include MAPKK[271] and p53.[272,273] p53 acetylation has been shown to increase its stability and transcriptional activities.[274]

8.4 In vitro toxicity data of AvrA

AvrA transfection to HeLa cells led to inhibition of activation of NF-κB, a key pro-inflammatory transcription factor, and augmented epithelial apoptosis.[275] AvrA transfection to human 293 T cells induced repression of c-Jun N-terminal kinase and NF-κB signaling pathways[271] as acetylation of critical amino acid residues in MAPKK blocks MAPKK phosphorylation activities.[272] Colonization of AvrA-positive *Salmonella* with human colon cancer cells corroborated this finding showing inhibition of IκBα degradation.[276] The ubiquitination of β-catenin decreased in the presence of AvrA expression, indicating that the expression of the *Salmonella* AvrA effector stabilizes β-catenin by decreasing the ubiquitination of β-catenin.[276] Further colonization experiments with human colon cancer cell lines demonstrated modulation of several members of the Wnt pathway by AvrA expression, i.e., upregulation of Wnt11 and Wnt2 and downregulation of Wnt1,[277–279] which was also confirmed in vivo with murine models.

8.5 AvrA toxicity in animal models

In both transgenic *Drosophila* and murine models, AvrA was shown to potently inhibit c-Jun N-terminal kinase (JNK) and NF-κB signaling pathways and, in the mouse intestinal mucosa, infection with AvrA-positive *Salmonella* dampened the proapoptotic innate immune response to *Salmonella*, suggesting a survival strategy for intracellular pathogen, where the bacteria elicit transient inflammation but do not destroy epithelial cells.[271] In a murine short-term infection model, epithelial cell proliferation of the large intestine was increased in mice infected with AvrA-positive *Salmonella* than those infected with AvrA-negative *Salmonella*.[276,278] Further studies have revealed that infection with AvrA-expressing *Salmonella* increased the Wnt/β-catenin activity, intestinal stem cell population, and cell proliferation in the infected intestinal mucosa.[280] In their murine chronic infection model, Lu et al. found that AvrA persistently regulated β-catenin post-translational modifications, including

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phosphorylation and acetylation. Moreover, the upstream regulator Akt, transcription factors, T cell factors, nuclear β-catenin, and β-catenin target genes were enhanced in mice infected with *Salmonella*-expressing AvrA, suggesting its functional role in promoting intestinal renewal.[281] The subsequent studies using mouse chemical carcinogenesis models have demonstrated that colorectal tumor incidence indeed markedly increased (almost doubled) in the AvrA+ *Salmonella* infected mice, compared with mice without bacterial gavage or mice infected with AvrA– *Salmonella*.[282] While the Wnt1 protein level in the mouse intestine was decreased in the AvrA+ infected mice compared with those infected with an AvrA– strain,[279] Wint11 expression was enhanced in animals infected with AvrA+ strains.[277] In the same animal model colonized with *Salmonella* AvrA-sufficient or AvrA-deficient bacterial strains, AvrA-expressing bacteria activated the STAT3 pathway, which is predicted to enhance proliferation and promote tumorigenesis, providing new insights regarding a STAT3-dependent mechanism by which the specific bacterial product AvrA enhances the development of infection-associated colon cancer.[283]

8.6 Clinical and epidemiological data to support carcinogenic potential in humans

There have been sparse data from epidemiological and clinical studies to directly support oncogenicity of AvrA in humans. However, Lu et al. repotted that *AvrA*-gene positive *Salmonella* was detectable in human feces and demonstrated that AvrA protein was immunohistochemically detectable in human colorectal mucosa and that its expression in tumor adjacent mucosa in colorectal cancer cases was significantly higher than in normal mucosa of non-cancer patients.[284] Consistent with the results from in vitro and animal studies, decreased Wint1 expression was observed in human colorectal cancer tissue.[279]

9. Concluding remarks

We have conducted an in-depth review on several emerging bacterial toxins that may be involved in pathogenesis of human orodigestive tract cancer. It was clear that these bacterial toxins have biological activities to induce several hallmarks of cancer.[285] Some toxins (CDT, colibactin and CNF) can directly interact with DNA or chromosomes leading to their breakdowns, causing mutations and genome instability, which is considered a crucial step for tumor initiation. Other cancer hallmarks commonly manipulated by bacterial toxins are cell proliferation, replication and death. While sustained proliferation and unlimited replication of epithelial cells are essential for tumor growth, enhanced cell apoptosis and senescence can lead to evasion of immune surveillance if they occur preferentially in hematologic cells, and to tumor stimulatory SASP[45] if they primarily involve epithelial and stromal cells. Manipulation of cell proliferation and death by bacterial toxins often are achieved by induction or degradation of oncogenes or tumor suppressors. The two most often modulated pathways were p53[286] and β-catenin/Wnt pathways,[287,288] while others, such as JAK-STAT, MAPK/ERK, Ras and Myc pathways are also exploited by PMT, Bft and AvrA as discussed above.

Wnt/β-catenin signaling pathway controls several crucial biological processes, such as cell motility, proliferation and the induction of differentiation. The activation of its canonical pathway is dependent on the binding of Wnt glycoprotein ligands to Frizzled (Fzd) receptors

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and its co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6) on the plasma membrane, forming the ternary complex Wnt–Fzd–LRP5/6. Subsequent recruitment of a scaffolding protein disheveled (Dvl) leads to the inhibition of Axin-mediated β-catenin phosphorylation, and thus β-catenin stabilization, cytoplasmic accumulation, and nuclear translocation. Once in the nucleus, β-catenin activates Wnt-dependent gene expression.[154] Wnt/β-catenin has recently emerged as an important target of several virulence factors produced by bacteria. The mechanisms are diverse and include the repression of Wnt inhibitors' expression by the epigenetic modification of histones, blocking Wnt–Fzd ligand binding, manipulating β-catenin nuclear translocation, down- or upregulation of Wnt family members, post-translational modification of β-catenin, and inhibition of Axin-1 expression, which promotes β-catenin activity,[154,280,287] while others, such as HlyA directly degrades β-catenin through its protease activity and suppresses its activity.[244,288] Accordingly, outcomes of manipulations of the Wnt/β-catenin pathway by these bacterial toxins are diverse. Both *Salmonella* AvrA that deubiquitinates β-catenin to prevent its degradation and *Fusobacterium* FadA that binds E-cadherin to stimulate β-catenin lead to increased cell proliferation and tumor formation,[154,282,288] while *E. coli* HlyA results in immunosuppression.[244,288]

Many bacteria as well as oncoviruses, such as HPV and EBV, are known to repress p53 through direct cleavage or proteasome degradation.[286] However, as we discussed above, other bacterial toxins, such as CDT and colibactin, upregulate P53 due to their genotoxic effects, while AvrA introduces a post-translational modification by acetylation to stabilize and stimulate this pathway.[273] Resulting sustained apoptosis has been speculated to lead eventually to the development of SASP, which in turn promotes tumorigenesis.[45] An additional way to resist cell death has been recognized to be enhancing autophagy, which is considered a pro-survival signal in challenging cellular environment.[285] CDT exploits this strategy.

An emerging hallmark of cancer is reprograming of cellular metabolism and energetics from mitochondrial respiration to aerobic glycolysis, so-called the Warburg effect.[285] While this metabolic shift caused by exposure to LPS/endotoxin has been well documented,[289,290] the effects of the exotoxins reviewed here on cellular energetics have been less well delineated. Yet, there are ample laboratory data to support this possibility. CDT from *Campylobacter* and *Helicobacter* species and HlyA from E coli have been shown to damage mitochondria, which may lead to reduced respiratory function.[291–293] On the other hand, activation of key signaling molecules, such as HIF1α and GLUT1 by HlyA[245] and c-Myc by Bft,[181] on the glycolysis pathway has been noted. Tan et al. directly assessed glycolysis and mitochondria biogenesis in mouse melanoma cells exposed to *E. coli* CNF, reporting an increase in glycolysis and a decrease in mitochondrial biogenesis.[294] However, conflicting data exist, suggesting that explore to CNF enhances mitochondrial volume and function in other cell culture systems,[295,296] and thus the net effect on cellular energetics in human non-transformed cells is unknown.

Many bacterial toxins, such as CDT, CTLP, PMT, FadA, Bft, CFA and HlyA, also exert biological activates to degrade intercellular junction proteins, including tight junction ZO-1 and E-cadherin, as well as extracellular matrix, partly through activation of matrix

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metalloproteinases, which leads to increased mucosal permeability, decreased mucosal defense and disruption of cellular communications, and activates tumor invasion.[285] On the other hand, bacteria effectors, such as AvrA, stabilizes tight junction to accommodate their own survival in the host.[297,298]

However, by far, the cancer hallmark "tumor-promoting inflammation" is most often controlled by bacterial toxins. In fact, all but Fap2 reviewed here act on this pathway, through diverse mechanisms and with distinct outcomes. A common aspect of infection-related cancer is the induction of chronic inflammation through various mechanisms.[1,299] Whereas most bacteria toxins reviewed here and others induce inflammation and often produce tumor-promoting cytokines, such as IL-17 and IL-6,[187] some pathogenic bacteria have evolved to synthesize bacterial toxins, such as AvrA, HlyA and CTLP, with the ability to temper the inflammatory response to create a suitable niche for their survival and proliferation in the host, not killing the host or host cells they infected. Acute excessive inflammation is a short-term response that either clears the pathogenic bacteria from the host through recruitment of immunological cells, or results in fatal consequences (host death). Thus, it does not lead to the development cancer. Accordingly, anti-inflammatory properties are particularly important and common in many T3SS pathogens with numerous virulence factors that induce strong pro-inflammatory reactions, such as *Salmonella* and *E coli*.[272] The NF-κB network is often manipulated by bacterial toxins to both up- and downregulate inflammatory responses.[300]

Table 1 summarizes the strength of evidence from human studies and mechanistic studies to support carcinogenicity of each selected toxin. Causal association has not been established for any of the toxins or bacteria reviewed here and the strength in human studies simply reflect consistency in statistical associations. It is important to note that the vast majority of in vitro studies with pathogens are short-term infections that induce a range of host cell responses. Besides, intestinal cells have short turnover rate of 3–4 days. Thus, the interpretation of transitory cellular signaling in the context of in vitro infection as tumorigenic may not be necessarily warranted. The tumor-promoting effects seen in animal models thus far have been delivered from genetically susceptible or chemically induced carcinogenesis models, and no transgenic animal models for the genes encoding these toxins have been tested. Overall, despite growing laboratory evidence to support oncogenic potential of selected bacterial toxins reviewed here,[3,301,302] evidence from human studies has been rather limited. This is partly because most microbiome research to date have focused on global community composition based on 16S rRNA gene sequencing. More targeted research will certainly help advance establishing the bacterial etiology of orodigestive tract cancer, which includes studies testing various human tissue samples, including not only tumors, but also surrounding mucosa, precursor lesions and healthy mucosa, as well as fecal, oral rinse/plaque and pre-diagnostic blood samples to directly detect bacterial toxins and their molecular signatures and to assess antibody responses. In addition, we need more physiologically relevant experimental animal models, which can reflect chronic infection in vivo in humans as well as take bacterial-bacterial interactions among microbiome into consideration. Such effort may lead to the development of new probiotic, prebiotic or antibiotic approaches or immunotherapies to prevent cancer development and progression.

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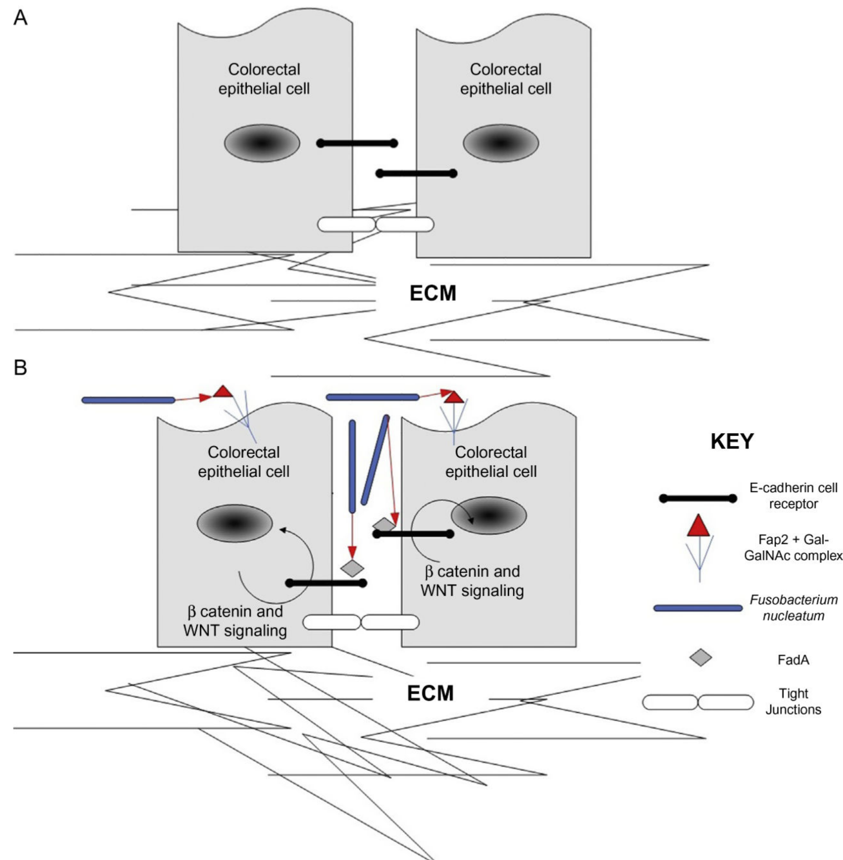

Fig. 1.

F. nucleatum's putative role as an ecosystem engineer. (A) Normal state without the presence of *F. nucleatum*. ECM: extracellular matrix. (B) The presence of rod shaped *F. nucleatum* releases FadA which triggers a signaling cascade in colorectal epithelial cells leading to proliferation.[152,154] Fap2 binds to sugar residues on colorectal epithelial cells which leads to co-aggregation and increased recruitment of *F. nucleatum* thus modifying the tumor microenvironment and behaving as an ecosystem engineer.[163]

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Table 1

Summary of existing evidence to support carcinogenicity of selected bacterial toxins.

Toxins	Mechanistic (in vitro/animal)	Human (clinical/epidemiological)	Target organs
CDT	*a*	*b*	Oral, enterohepatic, genital
CTLP	*c*	*a*	Oro-digestive tract/organ
PMT	*b*	*c*	Ororespiratory tract
FadA	*a*	*c*	Oral, colorectum
Fap2	*c*	*c*	Oral, colorectum
Bft	*a*	*a*	Colorectum
Colibactin	*a*	*a*	Colorectum
CNF	*c*	*b*	Colorectum
α-Hemolysin	*c*	*b*	Colorectum
AvrA	*a*	*b*	Colorectum

[a] Strong mechanisms with animal model/association with cancer in multiple studies.

[b] Strong mechanisms only/association with cancer from single reports.

[c] Suggestive mechanisms only/presence in target organs or in circulation.