

REVIEW

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Reexamining the role of *Fusobacterium nucleatum* subspecies in clinical and experimental studies

Madeline Krieger ^{a,b}, Mingzhe Guo ^{a,c}, and Justin Merritt ^{a,c}

^aDivision of Biomaterial and Biomedical Sciences, School of Dentistry, Oregon Health & Science University (OHSU), Portland, OR, USA; ^bCancer Early Detection Advanced Research Center, Knight Cancer Institute, Oregon Health & Science University (OHSU), Portland, OR, USA;

^cDepartment of Molecular Microbiology and Immunology, Oregon Health & Science University (OHSU), Portland, OR, USA

ABSTRACT

The Gram-negative anaerobic species *Fusobacterium nucleatum* was originally described as a commensal organism from the human oral microbiome. However, it is now widely recognized as a key inflammophilic pathobiont associated with a wide variety of oral and extraoral diseases. Historically, *F. nucleatum* has been classified into four subspecies that have been generally considered as functionally interchangeable in their pathogenic potential. Recent studies have challenged this notion, as clinical data reveal a highly biased distribution of *F. nucleatum* subspecies within disease sites of both inflammatory oral diseases and various malignancies. This review details the historical basis for the *F. nucleatum* subspecies designations and summarizes our current understanding of the similarities and distinctions between these organisms to provide important context for future clinical and laboratory studies of *F. nucleatum*.

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Introduction

Fusobacterium nucleatum (*Fn*) was originally described as being a ubiquitous commensal of the human oral microbiome, where its long, fusiform morphology and promiscuous coaggregation ability are central to its role as a bridge species in the oral biofilm.¹ However, increasing evidence reveals a more sinister side to *Fn*, as a large number of clinical studies strongly implicate it as a key player in a wide variety of pathological conditions.^{1–14} As with other members of the human microbiome, the mere presence of *Fn* is not necessarily indicative of active disease. In fact, the species is normally found in both healthy and diseased sites of the oral cavity and GI tract. While *Fn* typically exists as a low abundance species in sites of mucosal health, it is evident from numerous studies that elevated levels of *Fn* frequently associate with a variety of adverse health conditions, especially those associated with active inflammation.^{1,15} Although it is still debated whether *Fn* is the causative agent of these conditions (i.e., driver) or if its prominence at sites of inflammation is simply due to a preference for such environments (i.e., passenger), recent molecular work shows that *Fn*

has a mechanistic role in disease progression, and it is clear that *Fn* exhibits the typical features of an inflammophilic pathobiont.^{16–18}

For both clinical and laboratory investigations, *Fn* is frequently treated as a singular species, even though multiple distinct subspecies of *Fn* have been recognized for decades: *Fn* subsp. *nucleatum*, *Fn* subsp. *animalis*, *Fn* subsp. *polymorphum*, *Fn* subsp. *fusiforme*, and *Fn* subsp. *vincentii* (henceforth referred to as *Fnn*, *Fna*, *Fnp*, *Fnf*, and *Fnv*, respectively).^{19–22} In the early literature, *Fnv* and *Fnf* were described as separate subspecies, but they are now known to be genetically identical.^{21,23} *Fnn* has served as the principal laboratory workhorse for the vast majority of *Fn* genetic research, largely due to both historical factors and a common assumption that *Fn* subspecies are relatively synonymous. However, recent evidence examining the subspecies-level distribution of *Fn* at sites of health and disease have challenged this notion. Furthermore, several comparative genomic studies have provided compelling evidence that *Fn* subspecies are actually distinct fusobacterial species.^{13,24–28} However, we will maintain use of the term

CONTACT Justin Merritt  merrittj@ohsu.edu  Division of Biomaterial and Biomedical Sciences, School of Dentistry, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd. MRB424, Portland, OR 97239, USA

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“subspecies” purely as a historical convention. This review will focus specifically upon our current knowledge of the differences between *Fn* subspecies to help inform future investigations of these ubiquitous human pathobionts.

The history of *Fusobacterium nucleatum* subspeciation – how did we get here?

Subspecies in the literature

Although members of the genus *Fusobacterium* have been the subject of scientific investigation since the 1930s,²⁹ the first reference to *Fn* specifically in published literature was from a 1922 German publication.³⁰ It is worth noting that the official origin of the “*nucleatum*” species name as well as the *Fn* subspecies names are vague or undocumented in the literature. The next appearance of *Fn* in the scientific record did not occur until 1968 in a study investigating *Fn*’s capacity for amino acid fermentation (Figure 1).³¹ *Fn* was studied sporadically for the next several decades,^{32–41} but it was not until 1990 that a subspecies classification scheme was proposed for *Fn*, when DNA homology and whole-cell protein electrophoretic migration patterns were used to subdivide this species into three newly named subspecies: *Fnp*, *Fnn*, and *Fnv* (Figures 1 and 2).²³ Despite subsequent unsuccessful attempts to recreate this three-subspecies classification designation using the amino acid utilization profiles of 120 *Fn* strains,⁴² this nomenclature persisted as the accepted classification scheme for *Fn* subspecies.

The first attempt to categorize *Fn* subspecies as either health- or disease-associated occurred in 1990. A study utilizing 32 human oral isolates sorted them into one of the three established

subspecies categories based on physiological tests, enzymatic electrophoretic profiles, and DNA hybridization/base composition tests.⁴³ This investigation found that a majority of *Fnn* isolates were disease associated, whereas *Fnp* and *Fnv* were more abundant at sites of oral health. Of the three subspecies examined, *Fnv* was found to be the least abundant in either site. Additional early work on *Fn* subspecies also found evidence of subspecies dissimilarities, such as different adhesive properties among strains of *Fnv*, *Fnp*, and *Fnn*.⁴⁴ Amino acid uptake of *Fnf*, *Fnn*, and *Fnp* was found to differ significantly between subspecies, with *Fnf* using the most limited range of amino acid substrates, whereas *Fnn* was found to utilize fewer amino acids than *Fnp* but in higher concentrations.⁴⁵

Although previous work by Gharbia and Shah in 1990 referenced the existence of *Fnf*,⁴³ it was not officially added to the classification ranks until 1992 by the same group, along with the newly identified and named organism *Fna* (Figures 1 and 2).¹⁹ The same work illustrated that biochemical, physiological, and molecular tests could discriminate between these two subspecies. However, this paper also noted an inability to distinguish *Fnv* from *Fnn*. A review published soon after the official classification of *Fna* and *Fnv* provided an early summary of the evidence supporting these changes in nomenclature as well as specific laboratory tests for subspecies discrimination.⁴⁶

As scientists began to scrutinize the newly established four *Fn* subspecies, further questions arose about the appropriate mechanisms needed to distinguish among them, leading some to even question the fundamental clinical and/or biological utility of *Fn* subspecies distinctions. A 1996 study characterized 18 *Fn* isolates utilizing the allozyme

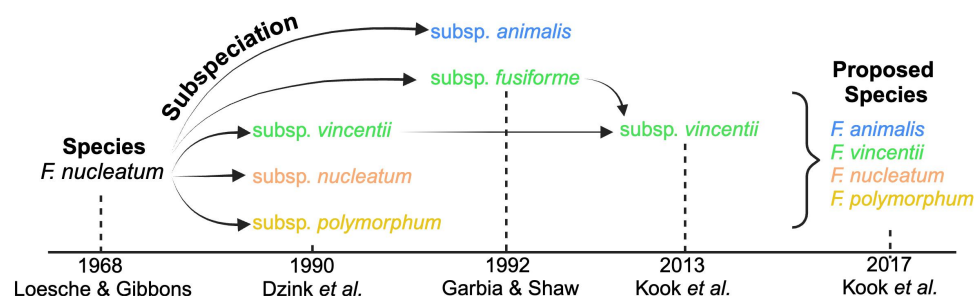


Figure 1. Evolution of species and subspecies level nomenclature assignments for *Fn*. First author and year of publication are shown on the timeline, while the emergence and changes in proposed species and subspecies names are listed above.

Year	Type	Short Description and PMID Number	<i>Fna</i>	<i>Fnn</i>	<i>Fnp</i>	<i>Fnv</i>	<i>Fnf</i>
1990	G	Proposal of <i>Fnn</i> , <i>Fnp</i> , and <i>Fnv</i> as subspecies (2223601)					
1992	G	Proposal of <i>Fnf</i> and <i>Fna</i> as subspecies (1581188)					
2002	G	Genome sequence of <i>Fnn</i> ATCC 25586 (11889109)					
2003	G	Genome analysis of <i>Fnv</i> and comparison with <i>Fnn</i> (12799352)					
2007	G	Genome sequence of <i>Fnp</i> (17668047)					
2010	S	Use of <i>rpoB</i> and zinc protease gene sequences for subspecies discrimination (19955278)					
2012	G	Genome sequence of <i>Fnf</i> (22965077)					
2013	G	<i>Fnf</i> and <i>Fnv</i> are the same subspecies (23263257)					
	G	Genome sequence of <i>Fna</i> (24336380)					
2017	M	<i>Fna</i> influences monocyte activation in human colorectal cancer (CRC) (28483840)					
	M	<i>Fnn</i> increases CRC proliferation and tumor development through signaling (27876571)					
	M	<i>Fnn</i> promotes chemoresistance in CRC by modulating autophagy (28753429)					
	S	<i>Fn</i> subspecies should be reclassified as species (28687946)					
2018	M	Enzyme increases <i>Fnn</i> survival within macrophages (29500439)					
2019	D	The same <i>Fn</i> strains are found in oral cavity and CRC tumors (29934439)					
	M	<i>Fnn</i> promotes CRC through Annexin A1 (30833345)					
2020	S	CRC <i>Fn</i> may migrate from oral cavity to colon (32850497)					
2021	S	PCR-based subspecies identification (34309194)					
	S	Comparative analysis of CRC-derived <i>Fn</i> subspecies (35130731)					
	M	<i>Fnn</i> confers chemoresistance in oesophageal cancer by modulating autophagy (33299132)					
	M	<i>Fnn</i> enhances PD-L1 blockade in CRC (34795206)					
	M	<i>Fnn</i> coaggregation modulates inflammatory cytotoxicity of epithelial cells (33816348)					
2022	S	<i>Fn</i> subspecies detection using mass-spectrometry (34929558)					
	M	<i>Fnn</i> promotes esophageal cancer via immune signaling pathway (35033591)					
	M	<i>Fnn</i> includes colon anastomosis leak through metalloprotease activation (36590433)					
2023	G	<i>Fna</i> and <i>Fnv</i> should be reclassified as species (37889007)					
	M	A novel virulence factor of <i>Fnn</i> promotes CRC metastasis (36693067)					
	M	<i>Fnn</i> progresses OSCC through modulation of glucose transport and metabolism (36709580)					
	M	Intracellular <i>Fnn</i> impacts esophageal SCC through signaling modulation (37723150)					
2024	S	<i>Fn</i> subspecies are heterogeneously distributed in oral disease (38479393)					
	S	A specific <i>Fna</i> clade dominates CRC niche (38509359)					

Legend

M Mechanistic studies
D Disease association

G Genomic studies and classification
S Subspecies-specific identification and study

Figure 2. Major milestones in *Fusobacterium nucleatum* subspecies research. Study type is indicated by the color and letter marker in the second column as described in the figure legend. The specific subspecies mentioned in each study is indicated by the colored circles on the right panel (*Fn. animalis* in blue, *Fn. nucleatum* in orange, *Fn. polymorphum* in yellow, and *Fn. fusiforme/vincentii* in green).

electrophoretic profiles of 21 enzyme loci, which revealed two distinct groups inhabiting similar oral niches along with 82.5% similarity among the tested loci.⁴⁷ The following year, the same group used this approach along with physiological and metabolic tests to classify an additional 44 isolates into three categories based upon their isolation site (oral, extraoral, or single unique oral isolate) but not their subspecies.⁴⁸ This paper explicitly argued against the subspecies classification scheme, suggesting that minimal differences exist between *Fna*, *Fnn*, and *Fnv* in terms of physiological and metabolic capabilities, while also asserting a lack of evidence for differences in pathogenicity between the subspecies. Around the same time, a metabolic

study of *Fn* isolates grown in chemically defined media concluded that the subspecies classification of *Fn* was unreliable due to the variable results of the recent allozyme electrophoretic enzyme panels.⁴⁹ Similarly, a 1997 review described the subspecies classification scheme as being on “shaky ground”.⁵⁰ Consequently, these publications cast doubt upon the validity of the new four subspecies classification scheme, but in retrospect, this also highlights the challenges often encountered with bacterial taxonomy during the era preceding Next-Generation Sequencing technologies.

Despite the ongoing debate about the validity of *Fn* subspecies classification, proponents of this new four subspecies designation soon outpaced out the

early critics. Each of the *Fn* subspecies were mentioned in a 1997 study of clinically relevant anaerobes analyzed using arbitrarily primed PCR (AP-PCR) fingerprinting.⁵¹ Three *Fn* subspecies were also described as part of a core group of subgingival plaque flora.⁵² The genome sequence of *Fnn* strain ATCC 25586 was first published in 2002 (Figure 2)⁵³ and was later employed in a subsequent comparative genomic analysis with the newly identified genome of *Fnv* strain ATCC 49256, resulting in the identification of 118 unique *Fnv* open reading frames (ORFs) (Figure 2).⁵⁴ Furthermore, the July 2002 minutes of the International Committee on Systematics of Prokaryotes Subcommittee clearly indicate that *Fn* subspecies were recognized as distinct organisms, despite prior questions about the validity of subspecies-level classification.⁵⁵ The first draft genome sequence of *Fnf* was published in 2012⁵⁶ before it was later recognized to be synonymous with *Fnv* (Figures 1 and 2).²¹ The first draft genome sequence of *Fna* was subsequently published in 2013 using the strain ChDC F324, which was originally isolated from a subgingival plaque sample (Figure 2).⁵⁷

Subspecies identification methods

Early DNA-based *Fn* subspecies classification approaches

As advancements in DNA sequencing technologies accelerated in the late 1990s and into the early 2000s, an increased number of clinical studies began differentiating between *Fn* subspecies using genotypic comparisons. One such early attempt employed an AP-PCR approach.⁵¹ While this

study lacked the phylogenetic analysis needed to potentially group the isolates into specific subspecies, the authors concluded that AP-PCR may be a suitable approach to do so in future studies. *SacII* digestion was also tested as a method to determine subspecies, but this restriction digest proved to be unreliable with clinical isolates. In 2003, Kook *et al.* developed a set of DNA–DNA hybridization probes specifically designed to categorize the subspecies of *Fn*.⁵⁸ Four out of the 96 probes they tested had subspecies specificity, providing an unambiguous diagnostic screen for subspecies identifications.

16S rRNA gene sequence-based *Fn* subspecies classification approaches

Since its discovery as a species-level determinate in the 1980s,⁵⁹ the 16S rRNA gene sequence has been utilized as a gold standard for bacterial taxonomic classification.^{60,61} Accordingly, 16S-based sequencing methodologies have also been applied to distinguish among *Fn* subspecies, albeit with moderate success due to the low number of 16S rRNA gene single nucleotide polymorphisms (SNPs) among *Fn* subspecies.⁶² In 2002, the first sequences of different fusobacterial 16S–23S internal transcribed spacer (ITS) regions were reported.⁶³ These data were used to develop the first phylogenetic tree of *Fn* subspecies in comparison to other *Fusobacterium* species (Table 1). Notably, the tree placed *F. simiae*, *F. naviforme*, and *F. periodonticum* within the *Fn* subspecies cluster, suggesting that the variations between these *Fn* subspecies may be more akin to species-level distinctions. Consequently, the study

Table 1. Published phylogenetic trees of *Fusobacterium nucleatum* subspecies. All trees showed monophyletic isolation of all *Fn* subspecies.

First Author	Year	PMID	Tree Method	Alignment method	Marker
Gmür, R.	2006	16464693	PHYLP	ClustalX	16S (bases 54–938)
Strauss, J.	2008	19114111	MEGA4	ClustalW	16S (bases 8–907) and <i>rpoB</i>
Kim, H. S.	2010	19955278	PHYLP	ClustalW	16S, <i>rpoB</i> , and <i>znpA</i>
McGuire, M.	2014	25370491	Unlisted	Unlisted	498 orthologous genes conserved across all strains
Ang, M. Y.	2016	27540086	MEGA	Clustal Omega	16S, core-genome SNPs alignments
Kook, J. K.	2017	28687946	MEGA	ClustalW	16S
Richardson, M.	2020	33054632	FastTree	PyNAST	3' end 16S gene (starting around bp 785) with downstream ITS region removed
Queen, J.	2021	35130731	FastTree	MUGSY	Core genes
Ma, X.	2023	37042769	RAxML-NG	MAFFT	Core proteins determined with OrthoFinder
Krieger, M.	2024	38479393	FastTree within Anvi'o	MUSCLE within Anvi'o	275 functionally divergent core genes present in all 72 genomes
Zepeda-Rivera, M.	2024	38509359	kSNP3	MUSCLE using MEGA X	<i>rpoB</i> alone; combination of <i>rpoB</i> , 16S, <i>znpA</i> , <i>nusA</i> , <i>nusG</i>

questioned the appropriateness of the subspecies-level classification scheme originally proposed by Gharbia and Shah in 1992,¹⁹ but it also refrained from proposing any substantive changes, since the authors concurrently concluded that their ITS data supported the existence of *Fn* subspecies. In a follow up study, a sixth *Fn* subspecies named *Fn* subsp. *canifelium*⁶⁴ was proposed based upon metabolic and biochemical tests of isolates derived from human bite wounds from cats and dogs.⁶⁵ Today, *F. canifelium* is recognized as a distinct *Fusobacterium* species.^{66,67}

Despite exhibiting significant homology between *Fn* subspecies, the 16S V2-V4 region has been successfully utilized for subspecies identification of isolates from CRC tumors.¹² Similarly, another recent study examined a larger 792 bp fragment mapping to the second half of the 16S gene to identify *Fn* subspecies in the saliva and gastric sites of irritable bowel disease (IBD) patients, allowing the authors to group sequences into subgroups based upon SNPs.⁶⁸ The 16S gene V4-V5 region has also been successfully employed to quantify the composition of actively growing organisms within endodontic infections, where *Fna* was found to be the dominant *Fn* subspecies within the study cohort.⁶⁹

Other genomic markers used for *Fn* subspecies classification

Although 16S gene sequence analysis has remained as the most popular method of classifying phylogeny, the limited heterogeneity among *Fn* 16S gene sequences yields relatively low confidence in its power to distinguish between subspecies.⁶² This has spurred the development of alternative DNA sequence-based classification approaches. Two genes that have proven to be useful in this regard are *rpoB* (encoding the beta subunit of RNA polymerase) and *znpA* (encoding the fusobacterial zinc protease). Both genes have highly conserved primer binding sites that support workflows analogous to the typical 16S-based approaches, but yield a significantly greater number of SNPs, resulting in much higher confidence subspecies resolution compared to traditional 16S genotyping approaches (Figure 2).⁶² For example, a 2008 study evaluated 26 *Fn* and 7 *F. periodonticum* human intestinal tract isolates using

a combination of phenotypic, genotypic, and biochemical techniques to determine whether the population of oral fusobacteria differed from the community found in the intestine.⁷⁰ Phylogenetic analysis of ~800 bp and 500 bp segments of 16S rRNA and *rpoB* genes, respectively, yielded a tree that grouped isolates into distinct clusters based upon subspecies. Group IV, which clustered *Fna* isolates, contained over half of the collected fecal isolates, implicating *Fna* as the predominant *Fn* subspecies within the gut. More recently, an *rpoB* amplicon-sequencing based approach was utilized to discriminate fusobacterial subspecies within CRC tumor and fecal samples.¹³ This method, termed “Frpo-B seq,” employs a ~370 bp segment of the *rpoB* gene to distinguish between various microbial taxa, including *Fn* subspecies. This methodology was able to profile a surprisingly diverse community of microbiota from tumors and healthy tissue, noting that, among *Fn* subspecies, *Fna* and *Fnp* were the most abundant.

The *znpA* gene encoding the fusobacterial zinc protease has similarly proven to be a reliable marker of *Fn* subspecies.⁶² Recent work describing a comprehensive analysis of *Fn* subspecies abundance utilized three separate approaches to analyze *Fn* subspecies from clinical samples.²⁸ The first is a subspecies-specific set of five traditional PCR primers that produce differently sized, subspecies-specific DNA amplicons for rapid genotyping of clinical isolates. The second approach is a quantitative assay that employs Next-Generation Sequencing (NGS) of a hypervariable segment of the *znpA* gene to directly classify and quantify *Fn* subspecies within complex clinical specimens. Lastly, the utility of the *znpA* NGS approach was independently assessed and verified using a set of newly designed subspecies-specific qPCR primers.

Aside from the *rpoB* and *znpA* gene sequences, other genomic makers have been used to distinguish *Fn* subspecies. A set of fourteen PCR-primers was developed to aid in the identification of *Fn* strains derived from clinical specimens (Figure 2).⁷¹ Intriguingly, when this discriminative primer set was applied to fecal and tumor-tissue samples from CRC patients, a heterogeneous stratification of *Fn* subspecies was detected, with a strong enrichment of *Fna* in the CRC group. Although not

specifically dedicated to subspecies analyses, it is worth noting that an online repository of *Fusobacterium* genomes called “FusoPortal” provides an interactive database of annotated fusobacterial genomes, including multiple genomes from *Fna*, *Fnv*, *Fnn*, and *Fnp*.⁷² A comprehensive transcriptome map of *Fna*, *Fnp*, *Fnv*, and *Fnn* has been curated and is freely accessible, providing the first complete map of both protein-coding and non-coding RNA abundance during early exponential, mid-exponential, and early stationary growth.⁷³ The FusoPortal could be a useful resource to identify additional subspecies-specific genomic and transcriptional markers.

Non-genomic markers for subspecies classification

Despite the popularity of DNA-sequencing technologies for *Fn* subspecies classification, other strategies have proven to be similarly successful as well. In 2015, MALDI-TOF mass spectrometry (MS) data were first shown to effectively distinguish among *Fn* subspecies.⁷⁴ Later in 2022, the MALDI-TOF MS approach was further optimized to classify *Fn* subspecies directly from patient saliva specimens (Figure 2).⁷⁵ In addition to MS, antibodies have been employed to characterize the antigen profiles associated with fusobacteria found within Chinese and European clinical specimens of necrotizing ulcerative gingivitis, gingivitis, and periodontitis.⁷⁶ The Chinese and European cohorts differed in the types of *Fn* they contained, with *Fna* and *F. periodonticum* being predominant in Chinese samples, and *Fnn* and *Fnv* prevailing in European samples. Although the reason for this discrepancy was undetermined, it was suggested that the heterogeneous distribution of *Fn* subspecies across different populations could be due to disease states or dietary factors.

Reassessing the current *Fn* subspecies classification scheme

The first detailed comparative genomic analysis of *Fn* subspecies was completed in 2016, when an *Fn* pangenome was constructed using the genome sequence data housed at NCBI together with the Prokaryotic Genome Annotation Pipeline.²⁵ This study revealed a pangenome consisting of 6,666 total gene clusters, 13% (880) of which constituted

core genes and 87% comprised the accessory genome (43% dispensable gene clusters and 44% strain-specific gene clusters). The *Fn* pangenome was subsequently designated as an “open” pangenome by mathematical modeling. This designation, coupled with its large accessory genome, provided compelling evidence that *Fn* subspecies possess much higher genomic diversity than might be expected from closely related subspecies, especially among organisms often treated as relatively synonymous in many clinical and laboratory investigations. Another study investigating the ability of CRC-derived *Fn* subspecies to colonize murine models also profiled the genomic differences between previously sequenced *Fn* genomes (Figure 2).²⁷ Consistent with the previous report, *Fn* subspecies genomes clustered tightly into distinct phylogenomic groups, with strain-level variations being relatively minor in comparison to the large distinctions observed between subspecies. This work described a slightly larger core genome than previously reported (1,291 protein coding genes), but it also found a similarly large subset of genes unique to each subspecies. A subsequent study constructed a pangenome using the Roary Pan Genome Pipeline to compare 30 *Fn* genomes comprised of members from each *Fn* subspecies and a previously unidentified strain.²⁶ The results revealed a much smaller *Fn* core genome consisting of 517 core and soft-core genes present in at least 28 of the 30 isolates, 3,954 “shell” genes found in between 4 and 28 of the isolates, and 8,277 “cloud” genes present in 4 or fewer of the isolates. Despite the different core genome results determined from these studies, they were all consistent in their findings of substantial genomic diversity between *Fn* subspecies. Additionally, this same analysis found large differences in genome sizes between isolates of the four *Fn* subspecies. *Fnp* and *Fna* isolates had the largest genomes (~2.6 and ~2.5 Mb, respectively), while *Fnv* isolates had the smallest (~2.2 Mb).²⁶ This pattern of variable genome size remained consistent for multiple isolates, further supporting the observed genomic distinctions between *Fn* subspecies.

Kook *et al.* were the first group to explicitly argue for a species-level reclassification of *Fn* subspecies (Figures 1 and 2).²⁴ Their 2017 study performed a whole-genome average nucleotide

identity (ANI) analysis that determined an ANI of <93.01% between subspecies, whereas the ANI within subspecies groups was found to be >96%. Compared to the 96.69% ANI of two unequivocally designated subspecies of *Streptococcus constellatus* (subsp. *pharyngis* SKI1060 and subsp. *constellatus* SK53), the weaker ANI of *Fn* subspecies suggested that their genomic differences warranted reclassification as separate species. The results of this study were also consistent with a prior fusobacterial ANI analysis that reported an ANI 89–93% between *Fn* subspecies.⁷⁷ It is worth noting that ANI values <95–96% are commonly accepted as the threshold required for taxonomic speciation.⁷⁸ A recent comprehensive analysis of multiple *Fusobacterium* species similarly advocated for the reclassification of *Fn* subspecies into separate species.¹³ Their analysis of 157 fusobacterial genomes included both ANI analysis and phylogenetic tree constructions using whole genomes, 16S rRNA genes, and *rpoB* gene sequences. The resulting phylogenetic trees placed the four subspecies into separate branches parallel to other recognized *Fusobacterium* species. Likewise, a recent study by Krieger *et al.* provided multiple independent lines of evidence to support *Fn* subspecies reclassification into separate species.²⁸ Their conclusions were derived from a combination of both comprehensive genomic analyses of the four *Fn* subspecies as well as phenotypic evidence derived from paired clinical specimens of patient-matched dental plaque and odontogenic abscesses. The data indicated that the four currently accepted *Fn* subspecies exhibit both genotypic and phenotypic distinctions that are far greater than would be expected from bona fide subspecies. This study and others proposed to retain the current *Fn* subspecies nomenclature as the accepted species designations (i.e., *F. animalis*, *F. nucleatum*, *F. polymorphum*, and *F. vincentii*). Results of this and other phylogenetic tree-based studies on *Fn* subspecies are summarized in Table 1.

***Fn* subspecies in laboratory investigations**

Genetic manipulation

Genetic manipulation within the genus *Fusobacterium* has proven to be quite challenging. The first full genome of *F. periodonticum*, a close

relative of *Fn* and its subspecies, was published in 2007 in a paper that described the organism as “genetically tractable” due to the large number of horizontally transferred genes that were nearly identical to those found in *Clostridium* and *Salmonella* species (Figure 2).²⁰ Despite its genome being theoretically malleable, minimal progress has been reported with *F. periodonticum* genetic manipulation. Recently, a CRISPRi system was successfully employed in *F. periodonticum* and *Fnn*,⁷⁹ potentially paving the way for further gene inactivation studies in *Fn* and related species.

In another promising advance for *Fn* genetic studies, it was recently revealed that *Fnn* exhibits natural competence (exogenous DNA uptake) via its Type IV pili.⁸⁰ This ability potentially provides a convenient strategy to introduce foreign DNA into *Fnn*. It was noted that the efficiency of DNA uptake varied between the two ATCC *Fnn* strains examined. Strain-level variability in transformation efficiency is a common feature of most naturally competent bacteria.⁸¹ It is not yet clear whether this natural competence ability extends to the other relatively understudied *Fn* subspecies, but such an ability could prove to be a boon for genetic studies of these organisms. In 2005, sonoporation was first reported as a successful strategy to construct a double crossover *fadA* mutant in the *Fnp* strain 12230.⁸² This technique was employed in subsequent studies in 2007⁸³ and 2009⁸⁴ to create *recA* deletion and *fadA* complementation strains, respectively. Despite the utility of sonoporation for *Fn* genetic manipulation, electroporation-based transformations remain the most commonly employed technique. This was first documented in 2000 by electroporating the *Escherichia coli*-*Fn* shuttle vector pFN1 into *Fnp* strain ATCC 10953.⁸⁵ Subsequently, several additional shuttle vectors have been developed for electroporation-based plasmid transformations of *Fnn* strain ATCC 23726.⁸⁶ In this same study, a suicide vector pHS17 was successfully employed to engineer the first targeted fusobacterial chromosomal mutation. Several years later, this same system was used to inactivate two *Fnn* membrane proteins involved in triggering lymphocyte cell death.⁸⁷ A recent strategy using methylated plasmid DNA to bypass the *Fnn* restriction-modification systems was able to greatly increase the efficiency of targeted

chromosomal gene knockouts and complementation.⁸⁸ Conceivably, this approach could function similarly in a variety of other fusobacteria as well, provided one were to express the relevant restriction methylase(s). Additionally, it is worth noting that *Fn* transformation has so far only been reported in *Fnn* and *Fnp*.^{83,85–87} The genetic tractability of other *Fn* subspecies has yet to be determined.

Coaggregation and biofilm formation

Coaggregation is one of the most famous and well-studied phenotypes of *Fn*. This ability is thought to be a key aspect driving the ecology and structure of oral biofilms due to both the unrivaled breadth of *Fn* coaggregation partners as well as its role in recruiting numerous late colonizing species into the oral biofilm.^{89,90} A 1989 study first examined the characteristics of *Fn* coaggregation with other oral species.⁹¹ This early work was followed by more detailed examinations of *Fn* coaggregation with specific bacterial species, including *Treponema*, *Helicobacter pylori*, *Porphyromonas gingivalis*^{90,92–95} and many others. Co-occurrence of specific *Fn* subspecies with other bacterial species has been similarly noted in multiple studies.^{96,97–99} However, current research suggests that differences in fusobacterial coaggregation profiles are largely determined at the strain level, rather than by species or subspecies levels.¹⁰⁰

While *Fn* has long been characterized by its ability to coaggregate within a complex polymicrobial environment, each *Fn* subspecies appears to possess a unique biofilm-forming capacity. All *Fn* subspecies except *Fnp* have been demonstrated to form monospecies biofilms *in vitro*,¹⁰¹ while *Fnp* is able to form biofilm in the presence of amyloid FadA.¹⁰² Different *Fn* subspecies were also found to uniquely impact the composition of other species present within a mixed species biofilm model.¹⁰³

Fn subspecies in systemic disease

Fn subspecies in oral disease

Our current understanding of *Fn* pathogenesis is primarily derived from laboratory investigations using *Fnn/Fnp* strains.^{16–18,104–111} Due to extensive coaggregations with many other inflammophilic pathobionts,^{90,92–95} *Fn* has been proposed to play

a central role in promoting the formation of dysbiotic oral biofilms.¹¹² For example, early work revealed that *Fn* abundance increases significantly during the onset of gingivitis,⁵² which is an inflammatory condition that precedes the chronic inflammation characterizing periodontitis. Thus far, the principal virulence factors shown to promote host cell binding/invasion are FadA and Fap2.^{84,113,114} Interestingly, *Fnn* ATCC 23726 contains four *fadA* orthologs while *Fnp* 12230 appears to encode only one,¹¹⁴ indicating that different *Fn* subspecies likely utilize distinct adhesins. *Fnp* 12230 also encodes at least one additional FadA paralog.⁸² Similarly, the ability of FadA to compromise the gingival epithelial barrier may be used as a route for *Fnp* and other subspecies to migrate from the oral cavity to various extraoral sites.¹¹⁵ *Fnn*, *Fnp*, and *Fnv* have been shown to significantly increase nuclear factor kappa-B (NF- κ B), IL-8, and tumor necrosis factor alpha (TNF- α) transcription in neutrophils, activating an inflammatory response that can trigger tissue destruction.¹¹⁶ *Fnn/Fnp* also release endotoxins upon decimation, particularly lipopolysaccharide (LPS), which can activate Toll-like receptors on the surface of gingival epithelial cells and fibroblasts to stimulate periodontal inflammation and bone resorption.^{117–119} In addition, *Fnn* and *Fnp* can secrete a serine protease capable of degrading extracellular matrix proteins, leading to the destruction of periodontal connective tissues and the production of immunoglobulins and complement from the host immune system.^{120,121} Through each of these mechanisms, *Fn* may promote a local inflammatory microenvironment that is conducive for periodontal disease progression.¹²² Although all *Fn* strains can deplete neutrophils at the site of infection via necrotic and apoptotic mechanisms,¹¹⁶ it appears that each subspecies varies in their capacities to modulate neutrophil function. For instance, *Fnn* and *Fnp* can completely inhibit superoxide production, while *Fnv* displays only a moderate impact on free radical formation.¹¹⁶ Additionally, *Fnp* appears to be more efficient at inducing neutrophil apoptosis compared to *Fnn* and *Fnv*.¹¹⁶

Fn is frequently detected in inflammatory oral infections, such as gingivitis, pulpitis, peri-implant disease, Vincent's angina, peritonsillar abscesses, periapical abscesses, and leukoplakia, and is a major contributor to halitosis as well.^{123–128} *Fn* has also been identified as a key pathobiont in the

development of periodontitis,^{129–131} and is especially abundant within odontogenic abscesses. While *Fnp* was shown to induce subcutaneous abscesses within an animal model,¹³² until recently, it was unclear whether particular *Fn* subspecies predominate within acute periapical and peritonsillar abscesses.^{124,125,133} This question was recently directly examined using paired dental plaque and odontogenic abscess clinical specimens, revealing a particularly strong bias for *Fna* within the inflammatory abscess environment, whereas it is substantially less prevalent in disease-free dental plaque.²⁸ *Fn* has also been detected in higher amounts on the tongues of patients suffering from halitosis, which could be linked with its secretion of various volatile sulfur compounds, such as hydrogen sulfide.¹²⁶ Previous research indicates that H₂S-producing enzyme profiles differ among *Fn* isolates,¹³⁴ suggesting that the production of sulfur compounds might similarly vary between *Fn* subspecies. While multiple studies have reported an increased abundance of *Fn* and other periodontal pathogens in oral leukoplakia lesions,^{127,135} it is currently unclear whether this association stratifies by *Fn* subspecies. In fact, this is a common theme for many *Fn*-associated oral diseases. Based upon our recent understanding of the important distinctions between *Fn* subspecies and the clear enrichment of *Fna* in oral disease specimens, there is a strong rationale to reexamine the ecological role of *Fn* subspecies in sites of oral health vs. disease.

***Fn* subspecies translocation from the oral cavity to the gastrointestinal tract**

Although *Fn* was originally thought to be an obligate oral bacterium, studies have since discovered *Fn* at other sites within the body, especially the gut. While early surveys of *Fn* in fecal samples suggested only a limited and/or transient presence within the gastrointestinal (GI) tract,¹³⁶ advancements in sampling methodologies would soon suggest otherwise. A 2008 study examining both oral and GI *Fn* and *F. periodonticum* isolates were the first to note a striking trend: nearly identical fusobacterial strains were found in both the oral cavities and colons of the same individuals.⁷⁰ This result also added further support for a previous speculation of an oral origin for *Fn* strains present in the amniotic fluid of patients experiencing preterm

birth.¹³⁷ A recent study examining fusobacteria from the oral cavity and colon identified 28 unique strains from 381 total sequences (Figure 2).⁶⁸ According to the Chao1 richness index, the oral cavity exhibited a more diverse community of *Fusobacterium* species compared to the gastric or colonic sample sites. A principal component analysis of the fusobacterial populations found in each sample type suggested that the gastrointestinal communities are more closely related than those in saliva. When grouped at the subspecies level, different strains of *Fnv* are significantly enriched in saliva. The levels of *Fna* appear to be maintained from the saliva to the colon, whereas *Fnv* is apparently ineffective at colonizing the colon. This is consistent with previous suggestions that *Fna* is particularly adept at translocating from the oral cavity to other body sites.¹³⁸ The mechanistic basis for this trend has yet to be determined.

Adverse pregnancy outcomes (APO) stratify by *Fn* subspecies

Fn is a predominant bacterial species associated with adverse pregnancy outcomes,¹³⁸ and the majority of *Fn* isolates from intrauterine infections are *Fna*, followed by a much smaller proportion of *Fnp*. The aforementioned translocation capability of *Fn* may also explain its presence in amniotic fluid and umbilical cord blood, which is linked with neonatal sepsis and is a potential causative mechanism of pre-term labor.^{137,139,140} In addition, a subgingival plaque isolate of *Fna* was determined to be identical to a strain isolated from the placenta of a preterm stillbirth infant, and this same strain was not found in the supragingival plaque, vagina, or rectum of the patient.¹⁴¹ In this particular case, the mother had pregnancy-associated gingivitis in addition to a respiratory infection several days prior to the stillbirth. Thus, it was speculated that the respiratory infection weakened the mother's immune system, thereby facilitating *Fna* translocation to the placenta. Like *Fna*, matching strains of *Fnp* have also been isolated from maternal oral cavities and preterm neonatal specimens with no detectable vaginal colonization.¹⁴² For more specific details regarding APO-associated virulence mechanisms of *Fn*, we would refer the reader to the review by Vander Harr *et al.*¹⁴³

Several studies have employed murine models of preterm birth to investigate oral/placental *Fn* translocation. Murine premature birth, stillbirths, and unstained live births were triggered by maternal tail vein injections with *Fnp* strain 12230 at days 16 and 17 of pregnancy (proportional to 28–32 weeks of pregnancy in the human).² Additionally, *Fnp* was shown to establish long-term colonization within the murine placentas despite being efficiently cleared from the liver and spleen within 24 hours. Live *Fnp* cells isolated from the uterus, fetus, and amniotic fluid were determined to have reached these sites via hematogenous transmission. Another study examining the effect of *Fnn*, *Fnp*, *Fnv*, and *Fnf* on parturition in preterm mice similarly found that injections of all *Fn* isolates induced varying degrees of APOs, including pre-term birth and intrauterine growth restriction.¹⁴⁴

Recent studies have shown that *Fn* infiltration was significantly more frequent in endometrial and endometriotic tissues from patients with endometritis, which suggests *Fn* involvement in APO is likely to be multifaceted.¹⁴⁵ *Fn* has also been linked to endometritis cases.¹⁴⁶ However, none of these recent studies of endometritis have provided subspecies level resolution. Thus, it is currently unclear whether particular *Fn* subspecies predominate.

***Fn* subspecies implications in colorectal cancer (CRC)**

In recent years, there has been a rapidly growing interest in the role of *Fn* within malignant tumors, particularly those of CRC. Multiple studies have reported strong associations of *Fn* with CRC tumors sampled from patient specimens (Figure 2).^{18,104,147–}

¹⁵⁴ Furthermore, a comprehensive examination of >1,000 CRC cases across two U.S. cohort studies concluded that the abundance of *Fn* DNA in CRC tumors correlated positively with increased patient mortality.¹⁵² However, a common limitation found among many *Fn* – CRC studies is the lack of taxonomic resolution provided to distinguish among *Fn* subspecies. Among the minority of studies that have examined *Fn* subspecies composition in tumors, there appears to be a distinct bias for the presence of *Fna*. A study of the CRC tumor microbiota found a significant enrichment of *Fusobacterium*, and specifically *Fna*, in tumors (Figure 2).^{12,155} Likewise, a profile of *Fn* in CRC tumors using *nusA*, *nusG*, and 16S rRNA gene sequences from almost 2,000

patients found *Fna* to be the only subspecies significantly associated with CRC mortality.⁴ Another recent study profiling the mucosal microbiome of colonic biopsies from CRC patients also found *Fna* to be the predominant subspecies within *Fn*-positive CRC tumors.¹⁵⁶ A MALDI-TOF based technique has similarly been employed to determine the *Fn* subspecies composition within colorectal cancers.⁷⁵ Of the 196 *Fn* proteins identified, 157 were attributed to specific subspecies, with *Fna* comprising the largest fraction, followed by *Fnv* and *Fnp*. *Fnn* proteins were not detected in the adenoma specimens and were present in only half of the control samples. This finding is consistent with another recent analysis of CRC samples that identified an increased abundance of *Fna* and *Fnp* within tumors, with *Fnn* being the least abundant.¹³ A transcriptomic analysis of 807 CRC tumors reported *Fna* to have the most significant association in mesenchymal CMS4 tumors, which typically have a worse prognosis compared to other subtypes.⁸ *Fna* has also been reported to selectively adhere to CRC cells, presumably promoting its colonization and proliferation.⁹

Several recent prominent studies have highlighted the importance of considering subspecies-specific taxonomic resolution in *Fn* research. Zepeda-Rivera *et al.* found that *Fna* not only comprise the majority of *Fn* subspecies detected within a CRC cohort but a specific clade within *Fna* dominates the CRC niche (Figure 2).¹⁴ Subsequent work by Qin *et al.* found a higher prevalence of *Fna* than other *Fn* subspecies in a CRC cohort, also noting that *Fna* isolates had a higher rate of genome coverage detection within the CRC samples when compared to other *Fn*.¹⁵⁵ It is worth noting that this study adopted a species-level distinction when referring to *Fna* as “*F. animalis*”, whereas all other *Fn* isolates were called “*F. nucleatum*.”

This recent body of work implicates *Fna* as the most clinically relevant subspecies associated with CRC. However, *Fnn* or *Fnp* strains have thus far been employed in the majority of *Fn* laboratory studies investigating CRC initiation and progression. *Fnn* has been shown to activate NF-κB signaling and stimulate chemokine CCL20 secretion through miRNA modulation in CRC cells.¹⁵⁷ Additionally, *Fnn* selectively recruits immunosuppressive myeloid suppressor cells (MDSCs) via activation of the NLRP3

inflammasome, resulting in weakened anti-tumor immunity. Three studies using *Fnp* 12230 have demonstrated that the aforementioned adhesin FadA plays an important role in accelerating the growth of CRC cells. A 2013 study reported that FadA facilitates CRC progression by modulating E-cadherin/ β -catenin signaling.¹⁵⁸ Later in 2019, FadA was shown to work in concert with Annexin A1 to activate β -catenin in CRC cells, thus accelerating cancer growth.¹¹⁰ An excreted amyloid-like form of FadA was also shown to promote CRC progression by serving as a scaffold for biofilm formation and host cell adherence.¹⁵⁹ Additionally, this study found that production of FadA increases acid tolerance, which may be an important mechanism to facilitate GI translocation. It will be interesting to determine whether these same pro-oncogenic mechanisms and others occur similarly with *Fna* or if this organism triggers an altogether distinct response from *Fnn* and *Fnp*.

The role of *Fn* subspecies in other types of tumors is largely unexplored

In addition to CRC, *Fn* appears to have similar associations with various other tumor types throughout the body.¹⁶⁰ Recent studies have identified an enrichment of *Fn* in oral cancer, but there is still an overall dearth of evidence describing *Fn* subspecies associations among head and neck cancers (HNCs).⁵ A recent meta-analysis of 17 publications reported *Fnn* as the most prevalent subspecies within HNC,¹⁶¹ but given the limited amount of supporting evidence, it will be interesting to observe whether this trend holds as additional studies are performed in the future. Similarly, a potential role for *Fn* in breast and pancreatic cancers has been recently reported.^{106,162–164} Additionally, *Fn* associates with both gastric and esophageal cancers and may even serve as a predictor of disease severity.^{150,165,166} Thus far, all of the laboratory studies of *Fn* with these malignancies have employed *Fnn* strains.^{16,106,108,109,162–164}

Discussion

Since *Fn*-associated diseases overwhelmingly occur in a polymicrobial context, a critical question inevitably arises regarding the role of this organism as a driver or passenger. In support of

the former, studies have elucidated specific molecular mechanisms underlying *Fn* pathogenicity^{17,107,110,111,167} as well as its role in chemoresistance^{18,108} and immunomodulation (Figure 2).¹⁰⁹ The majority of such studies have employed model systems incorporating *Fnn*, with a much smaller proportion employing *Fnp*. It is important to note that despite the large number of oral and extraoral conditions associated with *Fn* infection, there is currently limited clinical evidence provided to clearly delineate *Fn* subspecies prevalence in many of these conditions. Current evidence has revealed that substantial biases can exist among the *Fn* subspecies associated with such conditions, which has important implications for the experimental models created to investigate relevant *Fn* pathobiology mechanisms. Furthermore, there is compelling evidence that *Fn* subspecies are in fact distinct *Fusobacterium* species,^{13,24,28,77} and therefore, should be treated as such in laboratory and clinical investigations.

Accordingly, a recent trend toward species-level distinctions has already begun in experimental and taxonomic work, with Kook *et al.* issuing a 2021 correction to their 2017 study to elevate the ranks of *Fna*, *Fnp*, and *Fnv* from the subspecies to species level.¹⁶⁸ The List of Bacterial Names with Standing in Nomenclature (LPSN) database has made updates to reflect this shift toward species-level distinctions as well. However, LPSN still currently lists all historical *Fn* subspecies under species *F. nucleatum*,¹⁶⁹ which can lead to confusion. To better clarify these issues, the current review has summarized the known physiological and genomic distinctions between *Fn* subspecies in hopes of promoting further nuanced investigations of fusobacterial roles in human health and disease.

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ORCID

Madeline Krieger  <http://orcid.org/0000-0001-5209-3905>

Justin Merritt  <http://orcid.org/0000-0002-3464-0490>

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