

# Genetic- and culture-based tools for studying *Bacteroides fragilis*

Matthew K. Schnizlein,<sup>1</sup> Abhishek A. Dubey,<sup>2</sup> Aretha Fiebig,<sup>1</sup> Sean Crosson<sup>1</sup>

**AUTHOR AFFILIATIONS** See affiliation list on p. 3.

**ABSTRACT** The relatively limited availability of genetic tools has hampered mechanistic studies of *Bacteroides fragilis*, an opportunistic anaerobe that constitutes 1%–5% of the gut microbiota in healthy humans. Here we describe novel vectors for *B. fragilis* gene deletion and expression as well as a semi-defined media for cultivation of *B. fragilis* str. P207.

**KEYWORDS** *Bacteroides*, molecular genetics, metabolic modeling, anaerobes

Limited genetic tractability of newly isolated bacteria has hindered their study. Here we describe additional tools for genetic manipulation of the gut microbe *Bacteroides fragilis*, specifically the strain P207 which was isolated from an inflamed ileoanal pouch (1–5). In this announcement, we build on work by other researchers (6–13) and report the construction of a semi-defined growth medium as well as novel plasmids for mechanistic studies in *B. fragilis*, which include vectors for constitutive and inducible expression that have versatile multiple cloning sites as well as options for C-terminal 3xFLAG tagging and/or N- and C-terminal fluorescent protein fusion.

## Genetic-based tools

We enhanced the multiple cloning cassette (mcs) of the allelic exchange vector created by García-Bayona et al. (Fig. 1A and B) using a derivation of the mcs regions in the pCOLADuet1 vector (Novagen, Millipore Sigma) (6). We also generated a series of replicating (RepA) and integrating vectors (IntN1; Fig. S1B through D) (14, 15). Each vector series has optional C-terminal 3xFLAG tag fusion (Table 1) as well as options for N- and C-terminal fusion with the fluorescent proteins mScarlet-I, mNeonGreen, and iLOV. Since *B. fragilis* is an anaerobic organism, fluorophores such as mNeonGreen and mScarlet-I require aerobic recovery to stimulate maturation (16). While we had some success with obtaining mNeonGreen fluorescence after 1 hour of aerobic recovery, mScarlet-I, which has a longer maturation time, requires further troubleshooting. The anaerobic fluorescent protein iLOV also requires optimization due to low fluorescent signal.

## Culture-based tools

Due to poor growth of *B. fragilis* str. P207 in an established defined medium (i.e., M9S; see supplemental methods), we optimized growth media composition by predicting auxotrophies using the fba\_tools application in KBase (17–19). This identified putative auxotrophies for siroheme, L-valine, and putrescine. We also found predicted auxotrophies for *B. fragilis* strains NCTC9343 and 638R which included L-valine as well as other amino acids. Since supplementing with single branched-chain amino acids suppresses growth (20), we supplemented M9S with 1% casamino acids, which significantly improved culture density across all strains (Fig. 1C).

We provide primer sequences to substitute antibiotic resistance genes as well as expression promoters, if others wish to expand upon these vectors. The fluorescent proteins can also be transferred into any of the other promoter vectors using

**Editor** Irene L. G. Newton, Indiana University  
Bloomington, Bloomington, Indiana, USA

Address correspondence to Sean Crosson,  
crosson4@msu.edu.

The authors declare no conflict of interest.

See the funding table on p. 3.

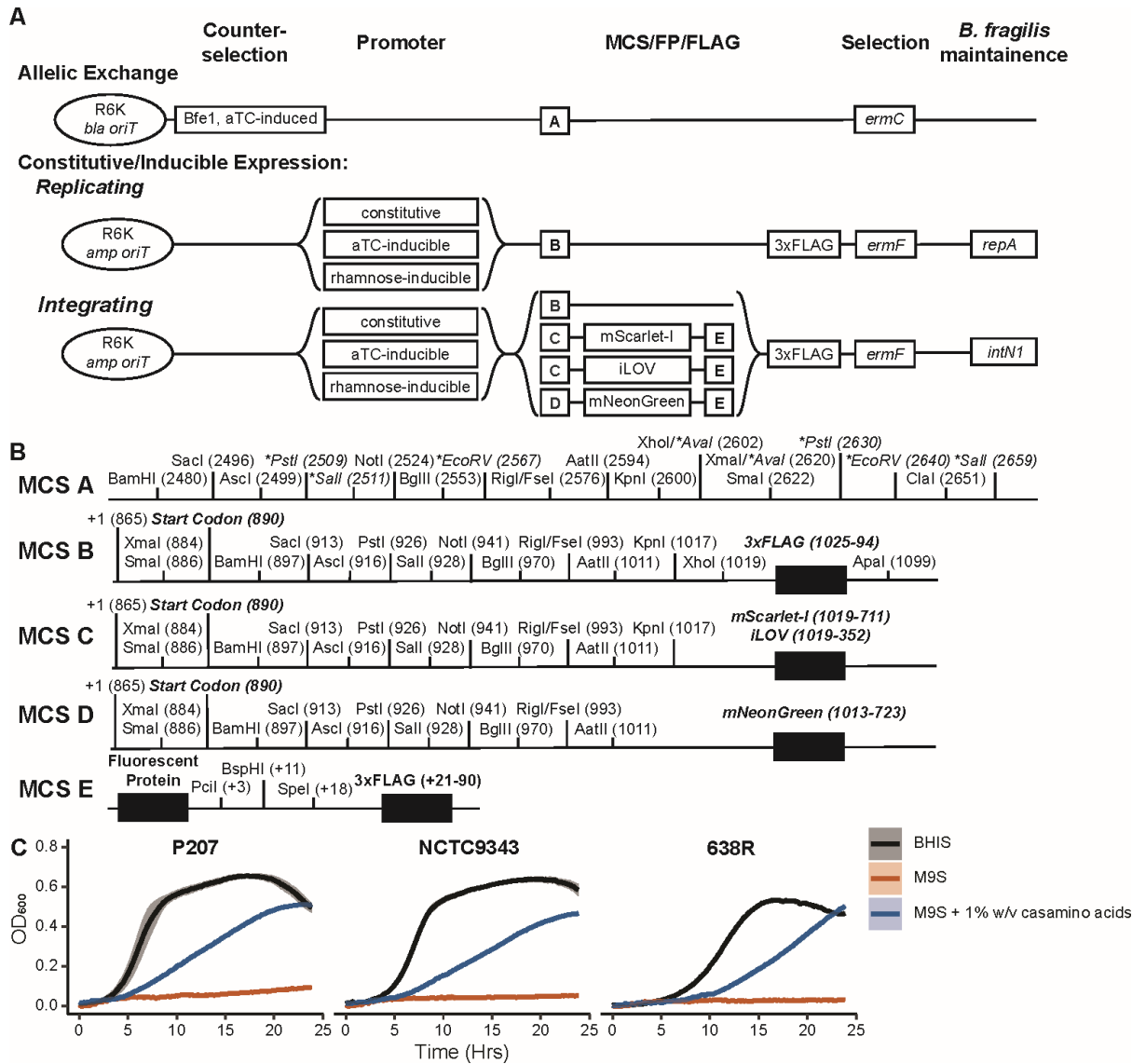
**Received** 15 January 2025

**Accepted** 4 March 2025

**Published** 25 March 2025

[This article was published on 25 March 2025. An  
Addendum with supporting references was included in  
the current version, posted on 21 April 2025.]

Copyright © 2025 Schnizlein et al. This is an  
open-access article distributed under the terms of  
the [Creative Commons Attribution 4.0 International  
license](#).



**FIG 1** (A) A graphical summary of allelic exchange, replicating and integrating vectors designed in this study. (B) A representation of multiple cloning sites A–E. An asterisk/italic font indicates that the site appears more than once in the MCS. (C) OD<sub>600</sub> growth measurements of *B. fragilis* strains P207, NCTC9343, and 638R grown in BHIS, M9S, or M9S + 1% (wt/vol) casamino acids.

**TABLE 1** Vectors and sequencing primers

Vector ID	Maintenance	Promoter	Selection	Features	AddGene ID
pMS01	Allelic exchange	no inserted promoter	<i>ermC</i>	aTC-inducible bfe1 counterselection	230064
pMS02	Integrating	Constitutive	<i>ermF</i>	C-terminal 3x-FLAG	230066
pMS03	Replicating				230937
pMS04	Integrating	aTC-inducible			230068
pMS05	Replicating				230069
pMS06	Integrating	Rhamnose-inducible			230070
pMS07	Replicating				230071
pMS08	Integrating	Constitutive		N- or C-terminal fusion with mScarlet-I; C-terminal 3x-FLAG	230072
pMS09	Integrating	Constitutive		N- or C-terminal fusion with iLOV; C-terminal 3x-FLAG	230073
pMS10	Integrating	Constitutive		N- or C-terminal fusion with mNeonGreen; C-terminal 3x-FLAG	230074

pre-designed primers. As fluorescent protein use continues to be an ongoing challenge in anaerobic organisms, we hope that these vectors will facilitate the further optimization needed.

## ADDENDUM

Although we were unable to cultivate *B. fragilis* strains, including P207, in a defined medium lacking amino acids, prior studies have demonstrated that *B. fragilis* can grow under fully anaerobic conditions in media supplemented with cysteine but without additional amino acids (6, 21–24). *B. fragilis* has also been cultivated in anaerobic defined media supplemented only with methionine and cysteine (25). We expect that strain P207 is similarly capable of growth under these conditions. However, in our experiments, cultures were exposed to oxygen during preparation and only transferred to the anaerobic chamber after inoculation. This likely impaired growth by oxidizing dihydroxyacid dehydratase (23), which is required for branched chain amino acid biosynthesis.

## ACKNOWLEDGMENTS

We thank the Michigan State University Genomics core for their help with Sanger sequencing. Thank you to Maeve McLaughlin for their gifts of several plasmids with the 3xFLAG tag as well as fluorescent proteins. pGEX iLOV was a gift from John Christie (Addgene plasmid # 26587; <http://n2t.net/addgene:26587>).

We also sincerely appreciate the funding provided by NIDDK (RC2DK122394) and NIGMS (R35GM131762). This work is supported as part of the BER Genomic Science Program. The DOE Systems Biology Knowledgebase (KBase) is funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Numbers DE-AC02-05CH11231, DE-AC02-06CH11357, DE-AC05-00OR22725, and DE-AC02-98CH10886.

## AUTHOR AFFILIATIONS

<sup>1</sup>Department of Microbiology, Genetics and Immunology, Michigan State University, East Lansing, Michigan, USA

<sup>2</sup>Leibniz Institute on Aging, Fritz Lipmann Institute, Jena, Thuringia, Germany

## AUTHOR ORCID*s*

Matthew K. Schnizlein  <http://orcid.org/0000-0002-0797-8357>

Aretha Fiebig  <http://orcid.org/0000-0002-0612-5029>

Sean Crosson  <http://orcid.org/0000-0002-1727-322X>

## FUNDING

Funder	Grant(s)	Author(s)
National Institute of Diabetes and Digestive and Kidney Diseases	RC2DK122394	Sean Crosson
National Institute of General Medical Sciences	R35GM131762	Sean Crosson

## AUTHOR CONTRIBUTIONS

Matthew K. Schnizlein, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing | Abhishek A. Dubey, Formal analysis, Investigation, Methodology | Aretha Fiebig, Conceptualization, Supervision | Sean Crosson, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review and editing

## DATA AVAILABILITY

You can access the vectors and their sequences via AddGene (#230064-230066, 230068-230074, and 230937). In addition, you can find vector maps and sequences, primers (Table S1), and methods on GitHub ([https://github.com/mschnizlein/bfrag\\_genetictools](https://github.com/mschnizlein/bfrag_genetictools)).

## REFERENCES

- Wexler HM. 2007. *Bacteroides*: the good, the bad, and the nitty-gritty. Clin Microbiol Rev 20:593–621. <https://doi.org/10.1128/CMR.00008-07>
- Patrick S. 2022. A tale of two habitats: *Bacteroides fragilis*, a lethal pathogen and resident in the human gastrointestinal microbiome. Microbiology (Reading) 168. <https://doi.org/10.1099/mic.0.001156>
- Vineis JH, Ringus DL, Morrison HG, Delmont TO, Dalal S, Raffals LH, Antonopoulos DA, Rubin DT, Eren AM, Chang EB, Sogin ML. 2016. Patient-specific *Bacteroides* genome variants in pouchitis. MBio 7:e01713-16. <https://doi.org/10.1128/mbio.01713-16>
- Mulowney MW, Fiebig A, Schnizlein MK, McMillin M, Rose AR, Koval J, Rubin D, Dalal S, Sogin ML, Chang EB, Sidebottom AM, Crosson S. 2024. Microbially catalyzed conjugation of GABA and tyramine to bile acids. J Bacteriol 206:e0042623. <https://doi.org/10.1128/jb.00426-23>
- Fiebig A, Schnizlein MK, Pena-Rivera S, Trigodet F, Dubey AA, Hennessy MK, Basu A, Pott S, Dalal S, Rubin D, Sogin ML, Eren AM, Chang EB, Crosson S. 2024. Bile acid fitness determinants of a *Bacteroides fragilis* isolate from a human pouchitis patient. MBio 15:e0283023. <https://doi.org/10.1128/mbio.02830-23>
- García-Bayona L, Comstock LE. 2019. Streamlined genetic manipulation of diverse *Bacteroides* and *Parabacteroides* isolates from the human gut microbiota. MBio 10:e01762-19. <https://doi.org/10.1128/mbio.01762-19>
- Lim B, Zimmermann M, Barry NA, Goodman AL. 2017. Engineered regulatory systems modulate gene expression of human commensals in the gut. Cell 169:547–558. <https://doi.org/10.1016/j.cell.2017.03.045>
- Hamady ZZR, Farrar MD, Whitehead TR, Holland KT, Lodge JPA, Carding SR. 2008. Identification and use of the putative *Bacteroides ovatus* xylanase promoter for the inducible production of recombinant human proteins. Microbiology (Reading) 154:3165–3174. <https://doi.org/10.1099/mic.0.2008/019109-0>
- Horn N, Carvalho AL, Overweg K, Wegmann U, Carding SR, Stentz R. 2016. A novel tightly regulated gene expression system for the human intestinal symbiont *Bacteroides thetaiotaomicron*. Front Microbiol 7:1080. <https://doi.org/10.3389/fmicb.2016.01080>
- Parker AC, Jeffrey Smith C. 2012. Development of an IPTG inducible expression vector adapted for *Bacteroides fragilis*. Plasmid 68:86–92. <https://doi.org/10.1016/j.plasmid.2012.03.002>
- Mimee M, Tucker AC, Voigt CA, Lu TK. 2015. Programming a human commensal bacterium, *Bacteroides thetaiotaomicron*, to sense and respond to stimuli in the murine gut microbiota. Cell Syst 1:62–71. <https://doi.org/10.1016/j.cels.2015.06.001>
- Orth P, Schnappinger D, Hillen W, Saenger W, Hinrichs W. 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nat Struct Biol 7:215–219. <https://doi.org/10.1038/73324>
- Lederer T, Takahashi M, Hillen W. 1995. Thermodynamic analysis of tetracycline-mediated induction of Tet repressor by a quantitative methylation protection assay. Anal Biochem 232:190–196. <https://doi.org/10.1006/abio.1995.0006>
- Rajeev L, Segall A, Gardner J. 2007. The *Bacteroides* NBU1 integrase performs a homology-independent strand exchange to form a holliday junction intermediate. J Biol Chem 282:31228–31237. <https://doi.org/10.1074/jbc.M705370200>
- Betteridge T, Yang J, Pittard AJ, Praszkie J. 2003. Interaction of the initiator protein of an IncB plasmid with its origin of DNA replication. J Bacteriol 185:2210–2218. <https://doi.org/10.1128/JB.185.7.2210-2218.2003>
- Zhang C, Xing X-H, Lou K. 2005. Rapid detection of a gfp-marked *Enterobacter aerogenes* under anaerobic conditions by aerobic fluorescence recovery. FEMS Microbiol Lett 249:211–218. <https://doi.org/10.1016/j.femsle.2005.05.051>
- Allen B, Drake M, Harris N, Sullivan T. 2017. Using KBase to assemble and annotate prokaryotic genomes. Curr Protoc Microbiol 46:1E. <https://doi.org/10.1002/cpmc.37>
- Arkin AP, Cottingham RW, Henry CS, Harris NL, Stevens RL, Maslov S, Dehal P, Ware D, Perez F, Canon S, et al. 2018. KBase: the United States department of energy systems biology knowledgebase. Nat Biotechnol 36:566–569. <https://doi.org/10.1038/nbt.4163>
- Allen BH, Gupta N, Edirisinghe JN, Faria JP, Henry CS. 2022. Application of the metabolic modeling pipeline in KBase to categorize reactions, predict essential genes, and predict pathways in an isolate genome, p 291–320. In Navid A (ed), Microbial systems biology: methods and protocols. Springer US, New York, NY. [https://doi.org/10.1007/978-1-0716-1585-0\\_13](https://doi.org/10.1007/978-1-0716-1585-0_13)
- Massey LK, Sokatch JR, Conrad RS. 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol Rev 40:42–54. <https://doi.org/10.1128/b.r.40.1.42-54.1976>
- Baughn AD, Malamy MH. 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. Proc Natl Acad Sci U S A 99:4662–4667.
- García-Bayona L, Coyne MJ, Hantman N, Montero-Llopis P, Von SS, Ito T, Malamy MH, Basler M, Barquera B, Comstock LE. 2020. Nanaerobic growth enables direct visualization of dynamic cellular processes in human gut symbionts. Proc Natl Acad Sci U S A 117:24484–24493.
- Meehan BM, Baughn AD, Gallegos R, Malamy MH. 2012. Inactivation of a single gene enables microaerobic growth of the obligate anaerobe *Bacteroides fragilis*. Proc Natl Acad Sci U S A 109:12153–12158.
- Varel VH, Bryant MP. 1974. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl Microbiol 28:251–257.
- Parker AC, Seals NL, Baccanale CL, Rocha ER. 2022. Analysis of Six tonB Gene Homologs in *Bacteroides fragilis* Revealed That tonB3 Is Essential for Survival in Experimental Intestinal Colonization and Intra-Abdominal Infection Infect Immun 90:e0046921.