Novel roles for pirin proteins and a 2-ketoglutarate:ferredoxin oxidoreductase ortholog in Bacteroides fragilis central metabolism and comparison of metabolic mutant susceptibility to metronidazole and amixicile

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Abstract

The regulation of the central metabolism and fermentation pathways and its effect on antimicrobial susceptibility in the anaerobic pathogen Bacteroides fragilis is not completely understood. In this study, we show that B. fragilis encodes for two iron-dependent redox-sensitive regulatory pirin protein genes, pir1 and pir2, whose mRNA expression is upregulated following oxygen exposure and growth in iron-limiting conditions. Pir1 and Pir2 modulate short-chain fatty acids production and alter susceptibility to metronidazole (MTZ), and to amixicile (AMIX), a novel inhibitor of pyruvate:ferredoxin oxidoreductase (PFOR) in anaerobes. Consistent with this, we showed that Pir1 forms direct protein-protein interactions with PFOR as determined by two-hybrid system assays. In addition, AlphaFold2-based structural analysis predicts that Pir1 and Pir2 form stable interactions with several enzymes of the central metabolism including the 2-ketoglutarate:ferredoxin oxidoreductases Kor1AB and Kor2CDAEBG. A series of metabolic mutants and electron transport chain inhibitors were used to show a wide-ranging effect of bacterial metabolism on MTZ and AMIX susceptibility. Furthermore, we show that AMIX is an effective antimicrobial against B. fragilis in an experimental model of intra-abdominal infection. This investigation led to the discovery that the kor2AEBG genes are essential for growth, and we present evidence that kor2AEBG genes have dual functions including the reductive synthesis of 2-ketoglutarate via reverse TCA cycle. However, the metabolic activity that bypasses KorAEBG function remains to be defined. Collectively our investigation reveals new information on B. fragilis central metabolism and its modulatory control by pirin proteins which may be leveraged for the future development of new narrow-spectrum antimicrobials

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Table 1. Bacterial strains and plasmids used in this study.

Strains	Genotype	References
B. fragilis 638R	clinical isolate, Rif^r	Privitera et al., 1979
BER-2	638R *ΔfurA::cfxA*, Rif^r Cfx^r	Robertson et al., 2006
BER-63	638R *ΔftnA::tetQ*, Rif^r Tet^r	Gauss et al., 2012
BER-74	638R *Δbfr::cfxA*, Rif^r Cfx^r	Gauss et al., 2012
BER-75	638R *ΔftnA::tetQ Δbfr::cfxA*, Rif^r Tet^r Cfx^r	Gauss et al., 2012
BER-150	638R *ΔfnrA::tetQ*, Rif^r Tet^r	This study
BER-156	638R *ΔnrfA::tetQ*, Rif^r Tet^r	Lab strain
BER-164	638R *Δpir1::tetQ*, Rif^r Tet^r	This study
BER-165	638R *Δpir2::cfxA*, Rif^r Cfx^r	This study
BER-166	638R *Δpir1::tetQ Δpir2::cfxA* Rif^r Tet^r Cfx^r	This study
BER-173	BER-164 carrying pER-287, *pir1*$^+$, Rif^r Tet^r Erm^r	This study
BER-174	BER-165 carrying pER-288, *pir2*$^+$, Rif^r Cfx^r Erm^r	This study
BER-175	BER-166 carrying pER-287, *pir1*$^+$, Rif^r Tet^r Cfx^r Erm^r	This study
BER-176	BER-166 carrying pER-288, *pir2*$^+$, Rif^r Tet^r Cfx^r Erm^r	This study
BER-183	638R *Δtdk*, Rif^r FUdR^r	Parker et al., 2022
BER-231	638R ΔPFOR::tetQ, Rif^r Tet^r	This study
BER-282	638R *pyc*::pFD516, Rif^r Erm^r	This study
BER-285	638R made metronidazole resistant at 2 μg/ml	This study
BER-286	638R made metronidazole resistant at 4 μg/ml	This study
BER-287	638R made metronidazole resistant at 8 μg/ml	This study
BER-289	638R made metronidazole resistant at 16 μg/ml	This study
BER-293	638R made metronidazole resistant at 32 μg/ml	This study
BER-294	638R *Δkor1AB*, Rif^r	This study
BER-295	638R *Δkor2ABG*, Rif^r	This study
BER-296	638R *ΔPFOR::tetQ Δkor1AB*, Rif^r	This study
BER-298	638R *ΔpoxB*, Rif^r	This study
BER-299	638R *ΔPFOR::tetQ ΔpoxB*, Rif^r	This study
BER-300	638R *Δkor1AB ΔpoxB*, Rif^r	This study
BER-301	638R *ΔPFOR::tetQ Δkor1AB ΔpoxB*, Rif^r	This study
BER-302	638R Δ*PFOR::tetQ Δkor2ABG*, Rif^r	This study
BER-303	638R *ΔPFOR::tetQ Δkor1AB Δkor2ABG*, Rif^r	This study
BER-308	638R *ΔcitS Δicd ΔacnA*, Rif^r	This study
BER-309	638R *Δkor2ABG ΔcitS Δicd ΔacnA*, Rif^r	This study
BER-310	638R *ΔPFOR::tetQ Δkor2ABG ΔcitS Δicd ΔacnA*, Rif^r	This study
BER-311	638R *ΔPFOR::tetQ Δkor1AB Δkor2ABG ΔcitS Δicd ΔacnA*, Rif^r	This study
BER-323	BER-183 *ΔfnrC Δtdk*, Rif^r FUdR^r	This study
IB260	638R *ΔkatB::tetQ*, Rif^r Tet^r	Rocha et al., 1996
IB263	638R hydrogen peroxide resistant, *hpr*, Rif^r	Rocha & Smith, 1998
IB274	638R *ahpCF::*pFD516, Rif^r Erm^r	Rocha & Smith, 1999
IB276	638R *ahpF:;*pFD516, Rif^r Erm^r	Rocha & Smith, 1999
IB336	638R *Δdps::tetQ*, Rif^r Tet^r	Rocha & Smith, 2004
IB370	638R *ΔtrxB*::*cfxA*, Rif^r Cfx^r	Rocha et al., 2007
IB430	638R *ΔahpC::tetQ*, Rif^r Tet^r	Lab strain
IB500	IB483 Δ*oxyR::tetQ* Rif^r Cfx^r Tet^r	Reott et al., 2009
IB542	IB336 *Δbfr::cfxA*, Rif^r Tet^r Cfx^r	Betteken et al., 2015

IB567	IB542 pFD288::*bfr*$^+$, Rif^r Tet^r Cfx^r Erm^r *bfr*$^+$	Betteken et al., 2015
IB573	IB542 pFD288::*dps*$^+$, Rif^r Tet^r Cfx^r Erm^r *dps*$^+$	Betteken et al., 2015
ADB77	638R isogenic *ΔthyA* Rif^r Tp^r	Baughn & Malamy, 2002
ADB247	ADB77 *ΔfrdC247* reverted to *thyA*$^+$, Rif^r	Baughn & Malamy, 2002
ADB260	ADB77 *ΔfrdB260 ΔthyA* Rif^r Tp^r	Baughn & Malamy, 2002
BER-274	ADB77 *ΔrelA ΔspoT* reverted to *thyA*$^+$, Rif^r	Lab strain
BER-278	ADB247 carrying pER-364, *frdC*$^+$, Rif^r Erm^r	This study
BER-283	ADB260 carrying pER-365, *frdB*$^+$, *ΔthyA* Rif^r Tp^r Erm^r	This study
IB483	ADB77 reverted to *thyA*$^+$, Δ*trxC* Δ*trxD*::*cfxA* Δ*trxE* Δ*trxF ΔtrxG* Rif^r Cfx^r	Reott et al., 2009
B. fragilis BF8	chromosomal *nimB* Ni^r	Haggoud et al., 1994
C. difficile	ATCC 43255	ATCC
P. gingivalis	ATCC 33277	ATCC
Pr. melaninogenica	ATCC 25845	ATCC
E. coli		
DH10B	cloning host strain	Invitrogen
HB101::RK231	HB101 containing RK231, (Km^r) (Tet^r) (Str^r)	Guiney et al., 1984
S17-1 λpir	Strain with the RK2 *tra* genes for conjugative transfer integrated in the chromosome *(RP4-2-Tc::Mu-Km::Tn7, pro, res*$^-$*mod*$^+$, Tp^r Sm^r) λ*pir* lysogen)	Simon et al., 1983
XL1-Blue MRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q} *ZΔM15* Tn*5* (Kan^r)].	Stratagene
BacterioMatch II Reporter Stain	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *lacI*^{q} *HIS3 aadA Kan*^{r}]	Stratagene
Plasmids		
pTRG	BacterioMatch II two-hybrid system target plasmid,	Stratagene
pBT	BacterioMatch II two-hybrid system bait plasmid,	Stratagene
pNBU2-*bla-ermGb*	NBU2 integrase (*intN2*) based genomic insertion vector derived from pKNOCK-*bla-ermGb* inserts into NBU2 *att1* or *att2* sites of tRNA^{ser}. (Amp^r) Erm^r	Koropatkin et al., 2008
pNBU2-*bla-tetQb*	NBU2 integrase (*intN2*) based genomic insertion vector derived from pKNOCK-*bla-tetQb* inserts into NBU2 *att1* or *att2* sites of tRNA^{ser}. (Amp^r) Tet^r	Martens et al., 2008
pExchange-*tdk*	Derivative of pKNOCK-*bla-ermGb* carrying cloned *tdk* gene for counter-selection. (Amp^r) Erm^r	Koropatkin et al., 2008
pFD288	*Bacteroides-E. coli* shuttle vector, *oriT*, pUC19::pBI143 chimera, (Sp^r) Erm^r	Smith et al., 1995
pFD340	*Bacteroides-E. coli* expression shuttle vector. (Amp^r) Erm^r	Smith et al., 1992

pFD516	suicide vector, derived from deletion of pBI143 in pFD288. (Sp^r) Erm^r.	Smith et al., 1995
pLGB36	Suicide vector for allelic replacement in *Bacteroides* species including *B. fragilis* strain 638R, Erm^r selection and aTC-inducible ss-Bfe3 counterselection.	Ito et al., 2020
pER-63	A 2.1 kb BamHI/EcoRI DNA fragment containing *cfxA* cassette gene was cloned into the BamHI/EcoRI sites of pFD516. (Sp^r), Erm^r.	This study
pER-65	A 2.4 kb BamHI/SacI DNA fragment containing *tetQ* gene cassette was cloned into the BamHI/SacI sites of pFD516. (Sp^r), Tet^r.	This study
pER-283	A 1.9 bp N-terminal BglII/BamHI and a 1.935 bp SstI C-terminal DNA fragments flanking BF638R_3039 gene was cloned, respectively, into BamHI and SacI sites of the suicide-vector pER-65 to construct *Δpir1::tetQ* deletion mutant. (Sp^r).	This study
pER-284	A 1.240 bp N-terminal BglII/BamHI and a 1.830 bp EcoRI C-terminal DNA fragments flanking BF638R_1469 gene was cloned, respectively, into the BamHI and EcoRI sites of the suicide-vector pER-63 to construct *Δpir2::cfxA* deletion mutant (Sp^r)	This study
pER-287	An 0.808 kb DNA fragment containing promoterless *pir1* gene was cloned into the BamHI/SacI sites of the expression vector pFD340. (Amp^r) Erm^r	This study
pER-288	A 0.746 kb kb DNA fragment containing promoterless *pir2* gene was cloned into the BamHI/SacI sites of the expression vector pFD340. (Amp^r) Erm^r	This study
pER-364	A 0.96 kb DNA fragment containing promoterless *frdC* gene was cloned into the BamHI/KpnI sites of the expression vector pFD340. (Amp^r) Erm^r	This study
pER-365	A 1.040 kb DNA fragment containing promoterless *frdB* gene was cloned into the BamHI/SacI sites of the expression vector pFD340. (Amp^r) Erm^r	This study
pER-372	A 2,461 DNA fragment containing 2,435 bp in-frame null deletion of *kor1AB* genes (BF638R_4321-4322) was cloned into the BamHI/SalI sites of pLGB36 vector. (Amp^r) Erm^r	This study
pER-373	A 2,040 DNA fragment containing 2,354 bp in-frame null deletion of *kor2ABG* genes (BF638R_1655-1658)	This study

	was cloned into the BamHI/XmaI sites of pLGB36 vector. (Amp^r) Erm^r	
pER-375	A 3,723 DNA fragment containing 1,350 bp in-frame null deletion of *poxB* gene (BF638R_3245) was cloned into the BamHI/SalI sites of pLGB36 vector. (Amp^r) Erm^r	This study
pFD1198	A 1 kb DNA fragment upstream and 1kb DNA fragment downstream of *fnrA* gene (BF638R_0432) were clone into the BamHI/SacI and SacI/EcoRI sites of pFD516. A 2.3 TetQ cassette was cloned into the SacI site to replace an internal 700 bp deleted DNA fragment (Spr) Erm^r.	This study
pFD1245	An 802 bp insertional inactivation fragment of the *pyc* gene (BFR638R_1927) cloned into the BamHI/EcoRI sites of pFD516. (Sp^r) Erm^r	This study
pFD1277	A 2,243 bp DNA fragment containing 566 bp in-frame null deletion of *fnrC* gene (BF638R_1017) was cloned into the PstI/XbaI sites of pExchange-*tdk* vector. (Amp^r) Erm^r	This study
pFD1278	A 2.002 kb N-terminal SphI/BamHI and a 1.819 SacI DNA fragments flanking BF638R_3194 gene was cloned, into the SphI/BamHI and SacI sites of the suicide-vector pER-65 to construct *ΔPFOR::tetQ* deletion mutant. (Sp^r).	This study

Erm^r: erythromycin resistance; Rif^r: rifampicin resistance; Tet^r: tetracycline resistance; Cfx^r: cefoxitin resistance; Ni^r: 5-nitroimidazole resistance; Tp^r: trimethoprim resistance; Amp^r: ampicillin resistance; Sp^r: spectinomycin resistance; Str: streptomycin resistance. FUdR^r: 5-fluor-2'-deoxyuridine resistance; Kan^r: kanamycin resistance. Parenthesis indicates antibiotic resistance expression in *E. coli*. ATCC: American Type Culture Collection.

Table2.Agar dilution determination of minimal inhibitory concentration (MIC μg/ml) of metronidazole (MTZ) and amixicile (AMIX) for *Bacteroides species* and *B. fragilis* 638R mutant strains.

Strains	MTZ Anaerobic	MTZ O_2 exposure	AMIX Anaerobic	AMIX O_2 exposure
BF638R	0.5	0.5	2	1
BF638R isolated at 2 μg/ml MTZ	4	2	2	1
BF638R isolated at 4 μg/ml MTZ	8	8	4	4
BF638R isolated at 8 μg/ml MTZ	16	16	4	4
BF638R isolated at 16 μg/ml MTZ	32	32	4	4
B. fragilis ADB77 *ΔthyA* (638R isogenic)	0.5	0.5	2	1
B. fragilis BF8	16	2	2	1
B. fragilis ATCC 25285	1	0.5	1	1
B. thetaiotaomicron VPI 5482	1	1	1	1
B. vulgatus ATCC 8482	1	0.5	1	0.0625
B. fragilis 638R mutant strains				
Δpir1::tetQ	0.5	0.5	2	1
Δpir2::cfxA	0.5	0.5	2	1
Δpir1::tetQ Δpir2::cfxA	0.5	0.5	2	1
Δpir1::tetQ/pir1⁺	0.25	0.125	2	0.5
Δpir2::cfxA/pir2⁺	0.25	0.125	2	0.5
Δpir1::tetQ Δpir2::cfxA/pir1⁺	0.25	0.125	2	0.5
Δpir1::tetQ Δpir2::cfxA/pir2⁺	0.25	0.125	2	0.5
BF638R *pir1⁺*	0.5	0.0625	2	1
BF638R *pir2⁺*	0.5	0.0625	2	1
ΔPFOR::tetQ	1	1	2	1
Δkor1AB	0.5	0.5	2	1
Δkor2ADBG	1	1	4	2
ΔPFOR::tetQ Δkor1AB	1	1	2	2
ΔpoxB	1	0.5	4	1
ΔPFOR::tetQ ΔpoxB	1	1	4	1
Δkor1AB ΔpoxB	1	0.5	2	1
ΔPFOR::tetQ Δkor1AB ΔpoxB	1	1	2	2
ΔPFOR::tetQ Δkor2ADBG	1	1	2	2
ΔPFOR::tetQ Δkor1AB Δkor2ADBG	1	1	4	2
pyc::pFD516	0.5	0.5	1	1
ΔfrdC247	0.25	0.25	1	0.5
ΔfrdB260	0.25	0.0625	0.5	0.5
ΔfrdC/frdC⁺	0.5	0.25	2	1
ΔfrdB260/frdB⁺	0.5	0.25	1	1
ΔcitS Δicd ΔacnA	1	1	4	2
Δkor2ADBG ΔcitS Δicd ΔacnA	1	1	2	2
ΔPFOR::tetQ Δkor2ABG ΔcitS Δicd ΔacnA	4	2	2	1
ΔPFOR::tetQ Δkor1AB Δkor2ADBG ΔcitS Δicd ΔacnA	4	2	4	2
Hydrogen peroxide resistant strain, *hpr*	0.5	0.25	2	1
ΔkatB::tetQ	0.5	0.25	2	1
ahpCF:pFD516	0.5	0.25	2	1
ahpF::pFD516	0.5	0.25	2	1
ΔahpC::tetQ	0.5	0.25	2	1

Δftn A:tetQ	0.5	0.25	2	1
Δbfr::cfxA	0.5	0.25	2	1
Δdps::tetQ	0.5	0.25	2	1
Δbfr::cfxA ΔftnA::tetQ	0.5	0.25	2	1
Δbfr Ddps	0.25	0.25	2	0.5
Δbfr::cfxA Δdps::tetQ/bfr^{+}	0.25	0.25	1	0.5
Δbfr::cfxA Δdps::tetQ/dps^{+}	0.25	0.125	1	0.5
ΔfnrA::tetQ	0.5	0.5	2	1
ΔnrfA::tetQ	0.5	0.5	2	1
ΔfnrC	0.5	0.5	1	1
ΔrelA ΔspoT	0.5	0.5	2	1
ΔtrxB::cfxA	0.25	0.25	1	0.5
ΔtrxC ΔtrxD::cfxA ΔtrxE ΔtrxF ΔtrxG	0.5	0.5	2	2
ΔtrxC ΔtrxD::cfxA ΔtrxE ΔtrxF ΔtrxG ΔoxyR::tetQ	0.5	0.25	2	0.5

Anaerobic: Inoculated plates were immediately incubated anaerobically. O_2 exposure: Duplicate plates were incubated for16-20 h in aerobic incubator at 37° C prior to anaerobic incubation at 37° C.