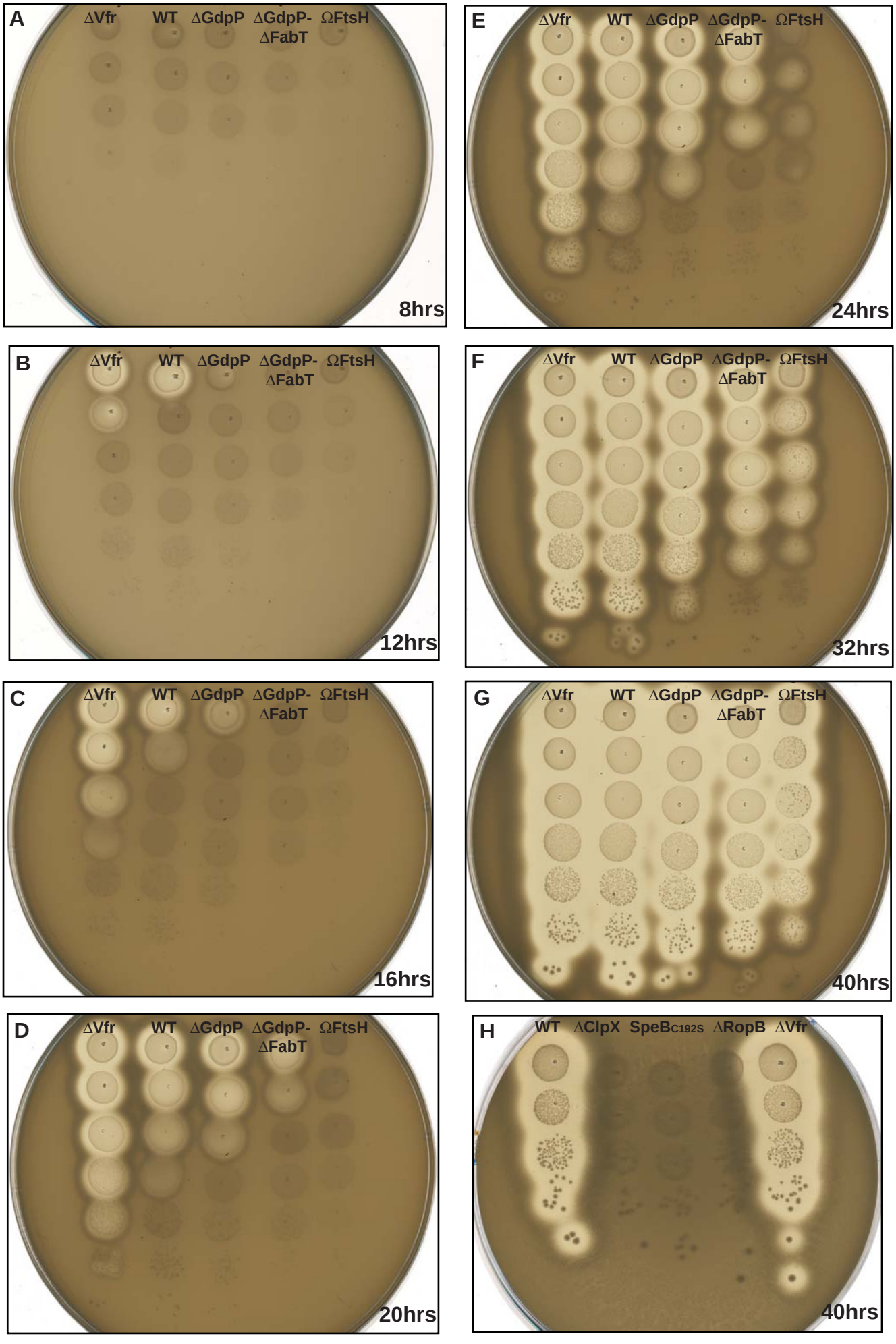
A
ΔVfr
WT
ΔGdpP
ΔGdpP-ΔFabT
ΩFtsH
8hrs
B
12hrs
C
16hrs
D
20hrs
E
24hrs
F
32hrs
G
40hrs
H
WT
ΔClpX
SpeBC192S
ΔRopB
ΔVfr
40hrs

Figure S1

Figure S1. Use of Protease indicator plates to monitor SpeB expression. SpeB protease activity was determined for various strains by plating 10-fold serial dilutions onto protease indicator plates and monitoring for protease activity every 4 hours. **(A-G)** A hyperactive SpeB expresser (ΔVfr) exhibits SpeB activity earlier than wild type (WT) or various PBr mutants including ΔGdpP, ΔGdpP-ΔFabT, and ΩFtsH. **(H)** A ΔClpX mutant displays no SpeB activity even upon extended incubation, which is comparable to SpeB-null mutants including the catalytically inactive $SpeB_{C192S}$ mutant, or the ΔRopB transcriptional activator mutant.

Table S1. Bacterial strains containing engineered and complemented mutations used in this study

Strains (alternate name)	Relevant Genotype	Mutated Loci[a]	Plasmid(s)[b]	Comment[c]	Reference
HSC5	wild type (WT)	NA			(1)
GCP688	ΔClpX	03620	pGCP666		This study
GCP751	ΔGdpP	09125	pGCP723		This study
GCP754	ΔPstS	04725	pGCP733		This study
GCP766	ΔGdpP-ΔFabT	04725, 07215	pGCP733; pGCP760		This study
GCP767	ΔClpX-ΔFabT	03620, 07215	pGCP666; pGCP760		This study
GCP771	ΔManLMN	07135-45	pGCP761		This study
GCP784	ΔEbsA	03260	pGCP774		This study
GCP953	ΔPtsI::*cat*	05585	pGCP793	CamR, allelic replacement of *ptsI* with *cat*	This study
HSC5-Spc (GCP292)	ΩControl (ΩCon)	09210-15 intergenic	pSPC18::'*recF*	SpcR, plasmid insertion downstream of *recF*	(2,3)
GCP859	ΩHpt	00060	pGCP856	SpcR, plasmid insertion within *hpt*	This study
GCP862	ΩFtsH	00065	pGCP857	SpcR, plasmid insertion within *ftsH*	This study
GCP682	ΔVfr	03630	pGCP661		This study
JWR100 (GCP057)	$SpeB_{C192S}$	08645		Enzymatically inactive SpeB	(4)
MNN100 (GCP543)	ΔRopB	08655			(5)
GCP920	ΔGdpP	09125	pGdpP	KanR, GdpP expressed on multicopy plasmid	This study
GCP1230	ΔGdpP-ΔFabT	04725, 07215	pGdpP	KanR, GdpP expressed on multicopy plasmid	This study
GCP695	ΔClpX	03620	pClpX	KanR, ClpX expressed on multicopy plasmid	This study
GCP1232	ΔClpX-ΔFabT	03620, 07215	pClpX	KanR, ClpX expressed on multicopy plasmid	This study

[a]Loci are based on the genome of HSC5 (1) and follow the format L897_xxxxx, where xxxxx is the number listed in the Table. NA, not applicable.

[b]Mutagenic plasmid (Table S3) used to delete, disrupt or alter or endogenous gene(s) in HSC5. Complementation plasmid (pGdpP or pClpX, Table S3) used to restore expression of select genes in deletion mutants. See the Experimental Procedures for details.

[c]Antibiotics are abbreviated as follows: chloramphenicol (Cam), spectinomycin (Spc), kanamycin (Kan).

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Table S2. Auxotrophic P_i revertant mutants isolated in this study

Revertant Strain	Parental Strain	Gene	Parental		Auxotrophic P_i Revertant		Consequence versus wild type (WT)
			Mutation[a]	Consequence	Mutation	Codon (Alteration)	
GCP1091	PBr1.1	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1092	PBr2.1	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1093	PBr3.1	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1094	PBr3.3	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1095	PBr3.7	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1096	PBr4.4	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1097	PBr4.5	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1098	PBr10.14	*pstS*	A -> -	Frame Shift: aa6	- -> A	X6K (X:AAx @ 6 -> K:AA)	Restore WT *pstS* allele
GCP1100	PBr10.9	*pstA*	19bp -> -	Frame Shift: aa35	- -> C	X35R (X:xGC @ 35 -> R:cGC)	Restore *pstA* open reading frame: 18bp in frame deletion (ΔG36-41S)
GCP1104	PBr3.2	*pstC*	C -> T	Premature Stop Codon: Q126*	G -> T	*126Y (*:TAG @ 126 -> Y:TAt)	Restore *pstC* open reading frame: missense mutation (Q126Y)

[a]PBr 10.9 19bp deletion is described in Table S4. - indicates absence of nucleotide (deletion).

Table S3. Plasmids used in this study

Plasmid (resistance)[a]	Features	Reference
pABG5 (Kan, Cam)	Expression vector containing *rofA* promoter, source of *cat*	(1)
pJRS233 (Erm)	Low-copy temperature-sensitive shuttle vector used for allelic replacement	(2)
pGCP213 (Erm)	High-copy temperature-sensitive shuttle vector used for allelic replacement	(3)
pSPC18 (Spc)	Integrational vector	(4)
pGCP666 (Erm)	pGCP213::Δ*clpX*, allelic replacement plasmid	This study
pGCP723 (Erm)	pGCP213::Δ*gdpP*, allelic replacement plasmid	This study
pGCP733 (Erm)	pJRS233::Δ*pstS*, allelic replacement plasmid	This study
pGCP760 (Erm)	pJRS233::Δ*fabT*, allelic replacement plasmid	This study
pGCP761 (Erm)	pGCP213::Δ*manLMN*, allelic replacement plasmid	This study
pGCP774 (Erm)	pGCP213::Δ*ebsA*, allelic replacement plasmid	This study
pGCP775 (Erm)	pGCP213::Δ*pstI*, allelic replacement plasmid, used to generate pGCP793	This study
pGCP793 (Erm, Cam)	pGCP213::Δ*pstI*::*cat*, allelic replacement plasmid	This study
pGCP856 (Spc)	pSPC18::Ω*hpt*, insertional disruption plasmid	This study
pGCP857 (Spc)	pSPC18::Ω*ftsH*, insertional disruption plasmid	This study
pGCP661 (Erm)	pGCP213::Δ*vfr*, allelic replacement plasmid	This study
pGdpP (Kan, Cam)	pABG5::GdpP, expression vector	(5)
pClpX (Kan, Cam)	pABG5::ClpX, expression vector	This study

[a]Antibiotics are abbreviated as follows: kanamycin (Kan), chloramphenicol (Cam), spectinomycin (Spc), erythromycin (Erm).

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Table S4. Mutagenesis and complementation primers used in this study

Name (description)[a]	Sequence[b]	Template	Plasmid[c]
GP712 (ΔClpX-M13F-F1)	tgtaaaacgacggccagt-GAGGTGTTGACAATGACTAGTATTG	HSC5	pGCP666
GP713 (ΔClpX-R2)	GTTTTGACTTGCCTTCGAC-CTGGCTTTTACCACAAAATG		
GP714 (ΔClpX-F3)	CATTTTGTGGTAAAAGCCAG-GTCGAAGGCAAGTCAAAAC		
GP715 (ΔClpX-M13R-R4)	cacacaggaaacagctatgac-ACCTATCGCTAGACCTGCTC		
GP822 (ΔGdpP-M13F-F1)	tgtaaaacgacggccagt-AAATATTGATTGGGATGCTATTG	HSC5	pGCP723
GP823 (ΔGdpP-R2)	CAATAATAAGTGCTTCGCTTG-CATCATAATCAAATGAATGGTTTC		
GP824 (ΔGdpP-F3)	GAAACCATTCATTTGATTATGATG-CAAGCGAAGCACTTATTATTG		
GP825 (ΔGdpP-M13R-R4)	cacacaggaaacagctatgac-CACGTGTTTTGTCCTCATC		
GP807 (ΔPstS-M13F-F1)	tgtaaaacgacggccagt-CAACAGGCTGGTACCAATTAC	HSC5	pGCP733
GP808 (ΔPstS-R2)	CTTTTTGTTACTTTTCCATCATG-GAAAGTTGATAGGACCAAAAGAC		
GP809 (ΔPstS-F3)	GTCTTTTGGTCCTATCAACTTTC-CATGATGGAAAAGTAACAAAAAG		
GP810 (ΔPstS-M13R-R4)	cacacaggaaacagctatgac-CAAAAAGGATCCTGTAATCATTG		
GP828 (ΔFabT-M13R-F1)	cacacaggaaacagctatgac-CTCTTCAAATTCTTATGCTGG	HSC5	pGCP760
GP802 (ΔFabT-R2)	CCAAAAATTGATGTAGATTCCC-CAAAATCCGGTTGAAAATATC		
GP803 (ΔFabT-F3)	GATATTTTCAACCGGATTTTG-GGGAATCTACATCAATTTTTGG		
GP829 (ΔFabT-M13F-R4)	tgtaaaacgacggccagt-GCAATAGCTGCTTGAACTTTC		
GP846 (ΔManLMN-M13F-F1)	tgtaaaacgacggccagt-GGTGGTTTTTCTTCTGTCAAC	HSC5	pGCP761
GP847 (ΔManLMN-R2)	CAAGGTGAGCAAGGATACC-GCTGGCAATAATAATACCGATAC		
GP848 (ΔManLMN-F3)	GTATCGGTATTATTATTGCCAGC-GGTATCCTTGCTCACCTTG		
GP849 (ΔManLMN-M13R-R4)	cacacaggaaacagctatgac-CTGTCCAAGGAATTTGAATATAATC		
GP865 (ΔEbsA-M13F-F1)	tgtaaaacgacggccagt-AAGAAGTAATGGAAGAGCTTGC	HSC5	pGCP774
GP866 (ΔEbsA-R2)	GGCTTTTTTATCATGCTGATAAAC-CTGCCAGTGATACCTTATTTTACC		
GP867 (ΔEbsA-F3)	GGTAAAATAAGGTATCACTGGCAG-GTTTATCAGCATGATAAAAAAGCC		
GP868 (ΔEbsA-M13R-R4)	cacacaggaaacagctatgac-CGTGTTCGTGTCCCTATAGTATC		
GP871 (ΔPtsI-M13F-F1)	tgtaaaacgacggccagt-GCTTCAAAAGACTTTCACATTG	HSC5	pGCP775
GP872 (ΔPtsI-R2)	CTTCTTCTGCTGTTGAACATTC-GGCTGCAATTCCTTTAAGC		
GP873 (ΔPtsI-F3)	GCTTAAAGGAATTGCAGCC-GAATGTTCAACAGCAGAAGAAG		
GP874 (ΔPtsI-M13R-R4)	cacacaggaaacagctatgac-GATAGTTTCCATTCACCGTTC		
GP886 (ΔPtsI::cat-F1)	CTAAAGAGGGATTGGCATAAGACT-*ATGAACTTTAATAAAATTGATTTAGACAATTG*	pABG5	pGCP793
GP887 (ΔPtsI::cat-R2)	GTTTAAACCAACTCTTTAACCCG-*TTATAAAAGCCAGTCATTAGGCCTATC*		
GP966 (ΩHpt-M13F-F1)	tgtaaaacgacggccagt-GCTTATGATTGGTGTATTAAAAGG	HSC5	pGCP856
GP967 (ΩHpt-M13R-R1)	cacacaggaaacagctatgac-CAGGTTTATCAAACAGTGTTGC		
GP968 (ΩFtsH-M13F-F1)	tgtaaaacgacggccagt-CATCTTAAGGCTGGAGATATAAAATC	HSC5	pGCP857
GP969 (ΩFtsH-M13R-R1)	cacacaggaaacagctatgac-CATCATCATCATGAAAGCAG		
GP690 (ΔVfr-M13F-F1)	tgtaaaacgacggccagt-GGGAAAGATGATCGAAGAATAC	HSC5	pGCP661
GP691 (ΔVfr-R2)	GACGGACTTTTTTTTAGCGC-CAATGACTTTCCTTTAGAGCG		
GP692 (ΔVfr-F3)	CGCTCTAAAGGAAAGTCATTG-GCGCTAAAAAAAAGTCCGTC		
GP693 (ΔVfr-M13R-R4)	cacacaggaaacagctatgac-CGTTATTATTGCTACATCAAATGC		
ZC341 (pClpX-F-EcoRI)	AACT-GAATTC-GTAAGAGAATTATAAGAAATGGC	HSC5	pClpX
ZC343 (pClpX-R-PstI)	AATGAT-CTGCAG-TTAAGCTGTCTCTAAAACGGG		

[a]Primers are categorized as forward (F) or reverse (R) relative to the direction of transcript.

[b]Sequence is shown 5' to 3'. Uppercase sequence anneals to the HSC5 chromosome, uppercase italics sequence anneals to the *cat* gene, lowercase sequence anneals to the M13F and M13R universal primer sequences. Hyphens indicate junctions between contiguous DNA regions, underline indicates restriction sites.

[c]Plasmid that was constructed using the indicated primers.

Table S5. Real-time RT-PCR primers used in this study

Name (description)[a]	Sequence
GP1021 (*recA* -F)	AGTGATGCGATTAGGAGAACG
GP1022 (*recA* -R)	TCGTTTTACCGGAAGACTCTG
GP1023 (*speB* -F)	CCAAGGTGTCGGTAAAGTAGG
GP1024 (*speB* -R)	AGAGCTGAAGGGTTTAGTGC
GP1027 (*fabM* -F)	TGACAGGTGAAGGAATTACTGC
GP1028 (*fabM* -R)	CCAGCATAAGAATTTGAAGAGCC
GP1029 (*fabH* -F)	ACGGAATGGTAATAGGTGCAG
GP1030 (*fabH* -R)	CACCAGCTCCATCTCCAAAA
GP1031 (*fabK* -F)	AAGAAATGGGAGCAGGATCG
GP1032 (*fabK* -R)	CTCACAAGCCCTGCAATTTG
GP1033 (*pgsA* -F)	GCGTAAGTGGCATGTAGTCAG
GP1034 (*pgsA* -R)	AGGCACTCATGACAAGCATC
GP1035 (*cls* -F)	ACCTATTACAATTATCGAGATCACCG
GP1036 (*cls* -R)	CCTCAAGCATTAAACCAGCATC
GP1039 (*fabT* -F)	AGACGAGTCAGTTTAGTGATGTC
GP1040 (*fabT* -R)	GCTAGTGGTTACTGTCCCTAAC

[a]Primers are categorized as forward (F) or reverse (R) relative to the direction of transcript.

Table S7. Transitions and Transversions of SNPs

Type (Total)[a]	Event	Number
Transitions (17)	C:G → T:A	16
	A:T → C:G	1
Transversions (14)	A:T → T:A	5
	A:T → C:G	0
	C:G → A:T	9
	C:G → G:C	0

[a]Analysis of 31 total SNPs distributed among 25 PB-resistant mutants.

Table S8. Number of Deleted or Inserted Nucleotides in InDels.

Events (Total)[a]	Number of Altered Nucleotides[b]					
	1	3	12	15	19	167
Deletions (21)	15	1	1	2	1	1
Insertions (3)	3	-	-	-	-	-

[a] Analysis of 24 total InDels distributed among 25 PB-resistant mutants.
[b] Inverse background and font indicates an in-frame InDel (multiple of 3 nucleotides). - indicates a mutation that was not observed.

Table S9. Deletions or Insertions of Repetative Elements.

Type (Total)[a]	Event	Gene(s)	Number
Deletions (19)	9xA → 8xA	*pstS* (8), *yfmH* (1)	9
	8xA → 7xA	*topA, 07750*	2
	7xA → 6xA	*gdpP*	1
	6xA → 5xA	*gdpP*	1
	3xC → 2xC	*topA*	1
	2xC → 1xC	*gdpP*	1
	3x(CGT) → 2x(CGT)	*ftsH*	1
	3x(ATT)[b] → 1x(ATT)	*gdpP*	1
	4x(ATT)[c] → 1x(ATT)	*ebsA*	1
	2x(AAGCCATTG)[d] → 1x(AAGCCATTG)	*clpX*	1
Insertions (3)	6xA → 7xA	*gdpP*	1
	4xC → 5xC	*hpt*	1
	2xC → 3xC	*clpX*	1

[a] Analysis of 24 total InDels distributed among 25 PB-resistant mutants.

[b] PBr 4.4 contains a 12 nucleotide deletion within *gdpP* including loss of a complete and partial copy of the 3-nucleotide repetative element ATT separated by 7-nucleotides, GAAAACT.

[c] PBr 11.1 contains a 15 nucleotide deletion within *ebsA* including loss of three copies of the 3-nucleotide repetative element ATT separated by 1-nucleotide, T and 5-nucleotides, GGTCT.

[d] PBr 9.21 contains a 15 nucleotide deletion within *clpX* including loss of one copy of the 9-nucleotide repetative element AAGCCATTG separated by 6-nucleotides, CCAATA.

Table S10. Distribution of Mutation Type in PBr Isolates.

Class	Gene (Frequency)	Premature Termination		Codon Altering/Loss		RBS	Other (type)
		Frame-shift	Nonsense	Missense	In-frame Deletion		
Core	*pstSCA*[a] (10)	9	1				
	gdpP (7)	4	1	1	1		
	fabT[b] (6)			4		1	1 (stop codon lost)
	clpX (4)	1	2		1		
	hpt[c] (4)	1				3	
	manLN[b, d] (4)			4			
	topA (4)	2	1	1			
	ptsI (2)			2			
	ebsA (2)			1	1		
	ftsH (2)			1	1		
Guanine[e]	*deoB* (1)			1			
	gmk (1)			1			
	guaA (1)			1			
	nupP (1)		1				
Other	*yfmH* (1)	1					
	agaS (1)			1			
	L897_07750 (1)						1 (intergenic)
	nanH (1)						1 (synonymous SNP)
	luxR (1)			1			
	fba (1)			1			
	Total (55)	18	5	21	4	4	3

[a] All *pstS* mutations (8/8) are identical, but were recovered in independent experiments or with different companion mutations. Mutations in *pstC* and *A* are included with *pstS*, as they are in the same putative operon and metabolic pathway.

[b] A *fabT* mutation (S84L) and a *manL* mutation (N284K) were recovered twice, but in independent experiments and with different companion mutations.

[c] Mutation of the *hpt* RBS also affects *tilS*, as the RBS overlaps the 3' end of *tilS*.

[d] Grouped together as part of the same putative operon and metabolic pathway.

[e] Genes involved in metabolism of guanine, exclusive of *hpt*.

Table S11. Distribution of Core PBr Gene Mutations Isolated from Independent Experiments.

Gene	Experiment [a]							
	1	2	3	4	6	9	10	11
pstSCA [b]	X	X	X	X			X	
gdpP			X				X	
fabT			X			X	X	X
clpX				X		X	X	X
hpt [c]					X		X	X
manLN [b]					X			X
topA			X				X	X
ptsI								X
ebsA			X					X
ftsH			X					
Guanine [d]				X			X	X

[a] Mutations in spontaneous PB-resistant isolates selected from independent cultures. The individual experiments listed were conducted on different days. No PB-resistant colonies were isolated in experiments 5, 7 and 8. The "X" indicates isolation of 1 or more mutants altered in the indicated gene in that experiment.
[b] Genes from the same putative operon and metabolic pathway grouped together.
[c] Due to overlap, mutation of the *hpt* RBS also affects the 3' end of *tilS*.
[d] Genes other than hpt predicted to be involved in guanine metabolism, including *guaA*, *deoB*, *gmk* and *nupP* (*yufP*).

Table S12. Genomic Analysis of *S. pyogenes* HSC5 Sugar Transporters.

Type	Sugar	Gene(s)[a]	Transport Protein(s)	Loci[b]	Comments/Reference[c]
PTS	Glucose	*ptsG*	IIABC	08465	
	Mannose	*manLMN*	IIAB, C, D	07135-45	This Study
	Mannose/Fructose	*manXYZ (ptsBCD)*	IIA, B, C, D	04035-50	
	Fructose	*fruA*	IIABC	03490	
	Galactose	*gatABC.1*	IIA, B, C	07005-15	Lac1 (1)
	Lactose	*lacEF*	IIA, BC	08210-15	Lac2 (1)
	Sucrose	*scrA*	IIABC	07410	
	Trehalose	*treP*	IIABC	08870	
	β-Glucoside	*bglF*	IIABC	02590	
	Cellobiose	*celABC.2*	IIA, B, C	08690-700	
	Cellobiose	*celABC.1 (ptcAB)*	IIA, B, C	05360-75	
	Ascorbate	*sgaTBA.1 (ulaABC)*	IIA, B, C	0980-90	
	Ascorbate/Mannitol	*sgaTB.2 bglG (ulaA)*	IIA, B, C	08335-45	
	GalNAc	*agaFVWD*	IIA, B, C, D	02795-815	
ABC	Neu5Ac	*nanBCDH*	Substrate Binding, Permease 1, 2	01215-35	
	Guanosine	*nupOPQ*	ATP Binding, Permease 1, 2	04645-55	
	Maltose/Maltodextrin	*malEFG*	Substrate Binding, Permease 1, 2	05285-95	(2, 3)
Other	Multiple sugars	*msmK*	ATP Binding	08425	
	Malate	*malP*	Malate/Sodium Symporter	04180	
	Glycerol	*glpF, glpF.2*	Permease 1, 2	06905, 07885	
	Glycerol-3-P	*glpT*	Permease	01935	

[a] Gene names derive from a consensus of fully annotated *S. pyogenes* genomes, while alternative names are listed in parentheses. Inverse background and font indicate genes which were mutated in PBr isolates. HSC5, similar to most sequenced strains (15/20 total sequenced strains), lacks the 9kb region encoding MalCD (maltodextrin ABC transporter permease) as this operon appears to be unique to M1 (all four sequenced M1 strains), M2, M4, and M28.

[b] Loci are in format L897_xxxxx, where xxxxx is numbered.

[c] Sugar transporters are predicted based on gene annotations, or established by experimental analysis as detailed in cited literature.

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