Figure S1

Secondary structure prediction of FasX RNA using a covariance analysis approach. (**A**) To enhance the possibility of correct secondary structure prediction via *in silico* analysis we utilized the program RNAalifold, which uses covariance analysis to predict secondary structure (Hofacker *et al.*, 2002). Our input FasX RNA sequences were the four FasX molecules shown in figure 1B, which are representative of FasX from four different streptococcal species. The two conserved regions of FasX are highlighted with numbered green bars. (**B**) GAS FasX secondary structure as predicted by the bioinformatic program RNAfold (Hofacker, 2003). Note the high level of similarity between the predicted secondary structures using RNAalifold and RNAfold. The two conserved regions of FasX are highlighted with numbered green bars. We will use the secondary structure predicted by RNAfold throughout the rest of the manuscript.

Figure S2

Southern blot confirmation of *fasX* mutation in strain 2221ΔFasX. A Southern blot was constructed using *Eco*RV-digested genomic DNAs from parental strain MGAS2221 and putative isogenic *fasX* mutant 2221ΔFasX. The probed blot generated bands of expected size for both the wild-type (~1.6 kb) and mutant (~5.8 kb) strains.

Figure S3

Nucleotide variation between GAS and *S. zooepidemicus fasX* alleles. Nucleotides that differ between the MGAS2221 and *S. zooepidemicus* FasX molecules are highlighted

with blue (SNPs) or purple (additions or deletions) text. The two conserved regions of FasX are highlighted with numbered green bars.

Figure S4

The FASX RNA transcript, and not a protein encoded within the transcript, is the regulatory molecule. (**A**) FASX nucleotide sequence showing the locations of the five ORFs (colored lines; A-E). The three nucleotides in bolded italics are those deleted in the plasmids pΔ1, pΔ2, and pΔ3. Inverted arrows highlight the terminator hairpin. (**B**) The nucleotide and corresponding protein sequences of ORF A are shown at the top. At the bottom are the mutant ORF A nucleotide and protein sequences from the *fasX* allele that is present in plasmid pΔ1. Black shading highlights nucleotide sequence identity between the two ORF A sequences, with the white gap between shading highlighting the deleted base in plasmid pΔ1. The start (green) and stop (red) codons are highlighted. (**C**) Indirect assay of SKA activity showing that none of the *fasX* mutations created in plasmids pΔ1-3 prevented complementation of isogenic mutant strain 2221ΔFASX. The experiment was performed in triplicate with mean values (± standard deviation) shown.

Figure S5

Construction of plasmid-encoded mutant *fasX* alleles. Shown is the overlap PCR mutagenesis protocol used to create plasmid-encoded mutant alleles of *fasX*. The sequences of the primers used to create each mutant allele are listed in table S2.

Figure S6

Inhibition of CvfA and PNPase ribonuclease activity is not the mechanism by which FasX enhances *ska* mRNA stability. (**A**) Quantitative RT-PCR analysis of *ska* (blue lines) and *proS* (red lines) mRNA levels over time after addition of rifampicin to cultures of the strains indicated. Note that strains ending "*cvfA*::pKO" have a plasmid integrated into the *cvfA* gene that disrupts this gene, while strains ending "*cvfA*::pOK" have a plasmid inserted into the *cvfA* gene that does not disrupt this gene. Note that the stability of *proS* mRNA, but not *ska* mRNA, is influenced by the presence/absence of an intact *cvfA* gene. (**B**) Western blot analysis of SKA protein levels in the culture supernatants of MGAS2221 and 2221ΔFasX derivatives containing disrupted or non-disrupted *cvfA* genes. The non-FasX regulated secreted protein Spd3 was assayed for use as a loading control. (**C**) Western blot analysis of SKA protein levels in the culture supernatants of MGAS2221 and 2221ΔFasX derivatives containing a disrupted *pnpA* gene or empty vector. The non-FasX regulated secreted protein Spd3 was assayed for use as a loading control.

Figure S7

Method used to determine the *ska* transcriptional start site. The red block arrow represents the *ska* mRNA with unknown 5' end. Small black arrows represent PCR primers used in the protocol.

References

Hofacker, I. L., (2003) Vienna RNA secondary structure server. *Nucleic Acids Res* **31**: 3429-3431.

Hofacker, I. L., M. Fekete & P. F. Stadler, (2002) Secondary structure prediction for aligned RNA sequences. *J Mol Biol* **319**: 1059-1066.

Figure S1

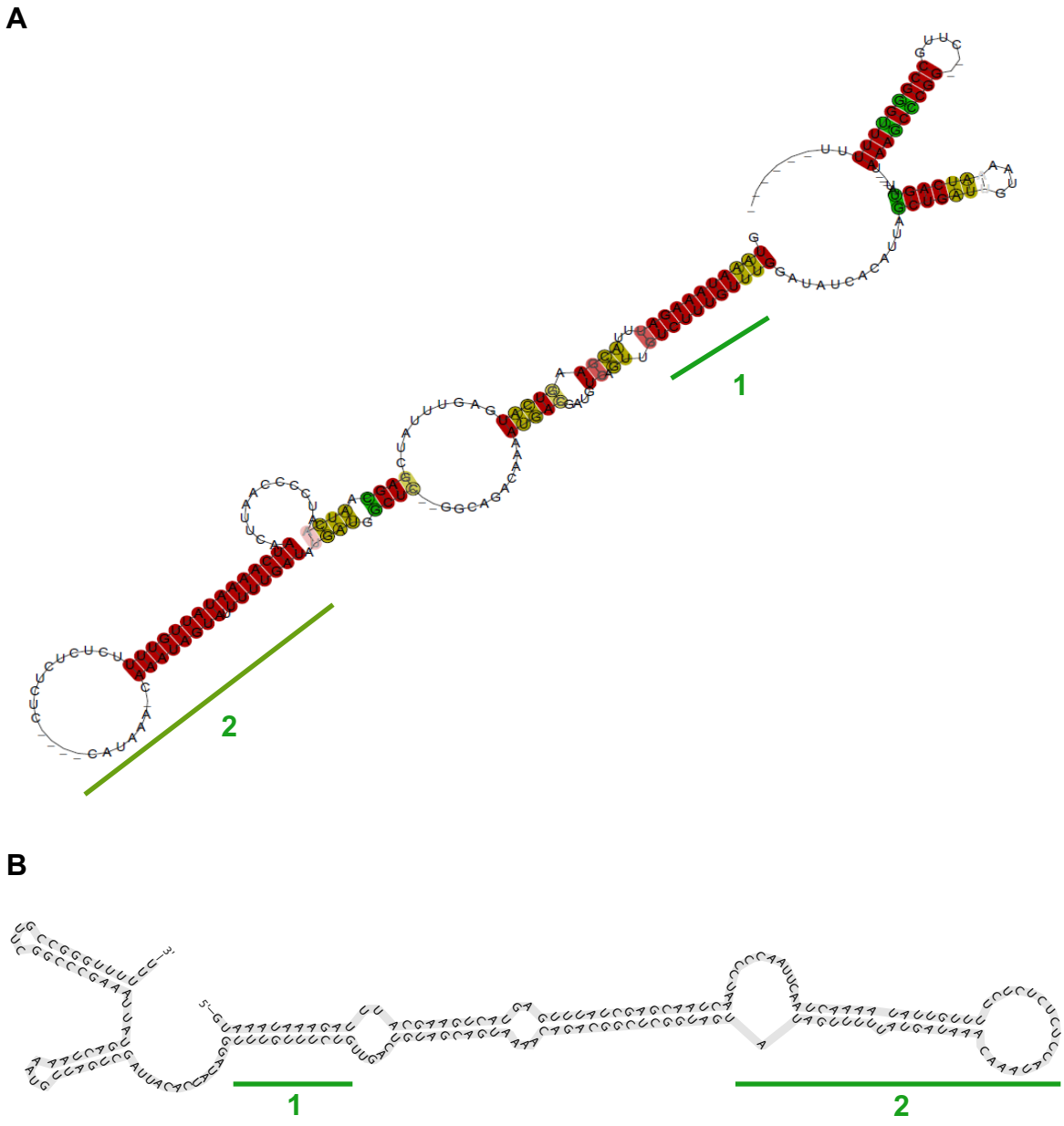


Figure S2

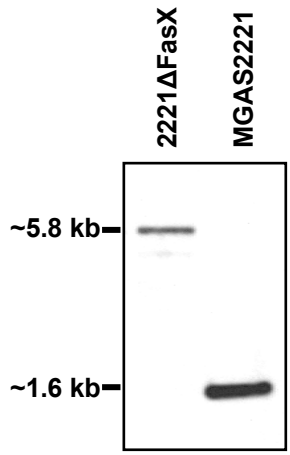


Figure S3

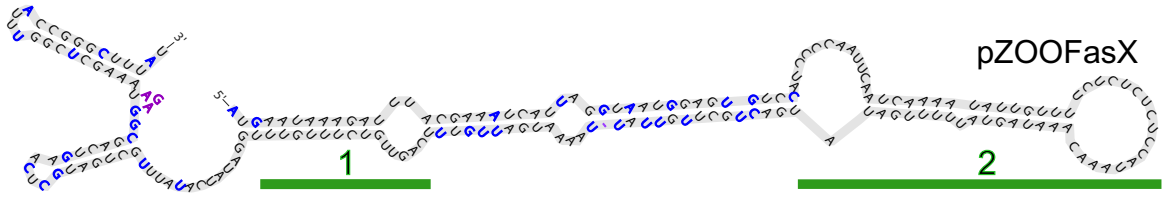


Figure S4

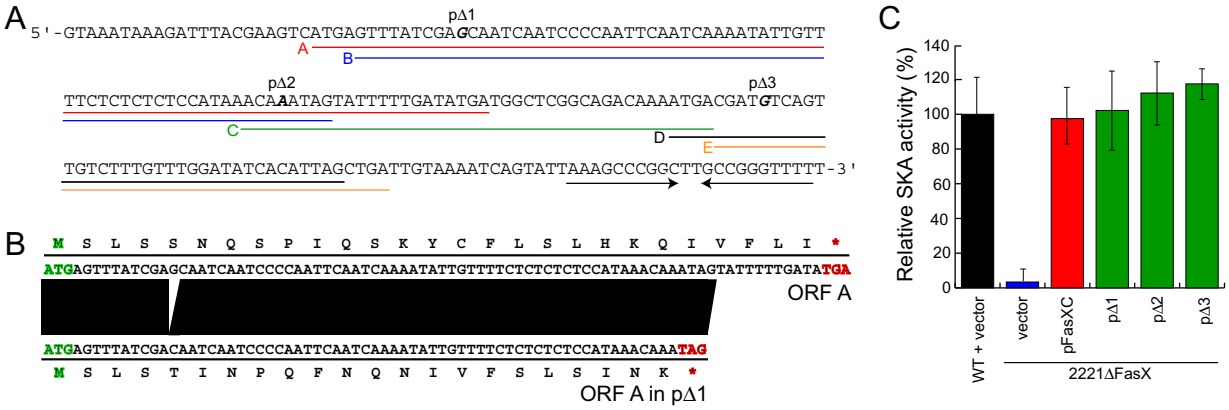

A
pΔ1
5'-GTAAATAAAGATTTACGAAGTCATGAGTTTATCGAGCAATCAATCCCCAATTCAATCAAAATATTGTT
A
B
pΔ2
pΔ3
TTCTCTCTCTCCATAAACAAATAGTATTTTTGATATGATGGCTCGGCAGACAAAATGACGATGTCAGT
C
D
E
TGTCTTTGTTTGGATATCACATTAGCTGATTGTAAAATCAGTATTAAAGCCCGGCTTGCCGGGTTTTT-3'
B
M S L S S N Q S P I Q S K Y C F L S L H K Q I V F L I *
ATGAGTTTATCGAGCAATCAATCCCCAATTCAATCAAAATATTGTTTTCTCTCTCTCCATAAACAAATAGTATTTTTGATATGA
ORF A
ATGAGTTTATCGACAATCAATCCCCAATTCAATCAAAATATTGTTTTCTCTCTCTCCATAAACAAATAG
M S L S T I N P Q F N Q N I V F S L S I N K *
ORF A in pΔ1
C
Relative SKA activity (%)
0
20
40
60
80
100
120
140
WT + vector
vector
pFasXC
pΔ1
pΔ2
pΔ3
2221ΔFasX

Figure S5

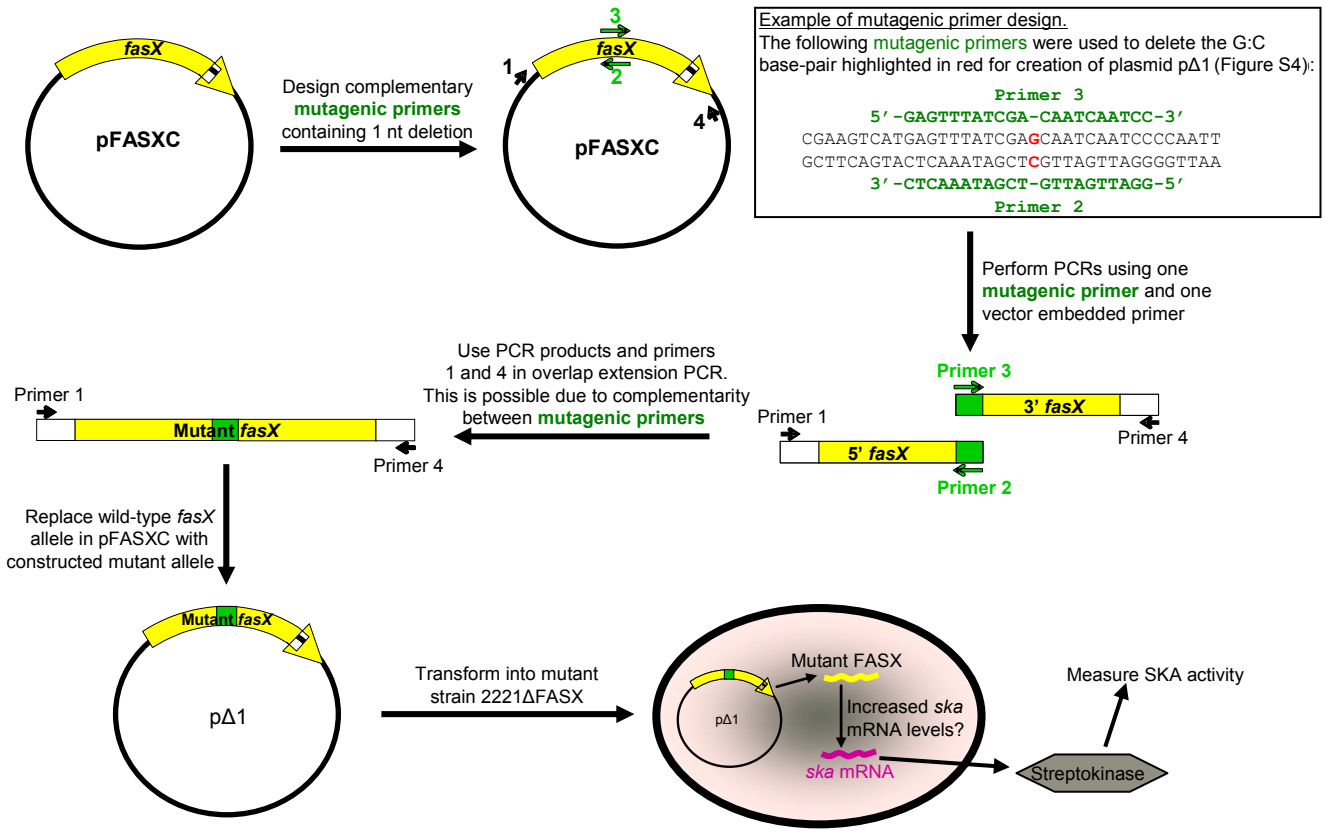
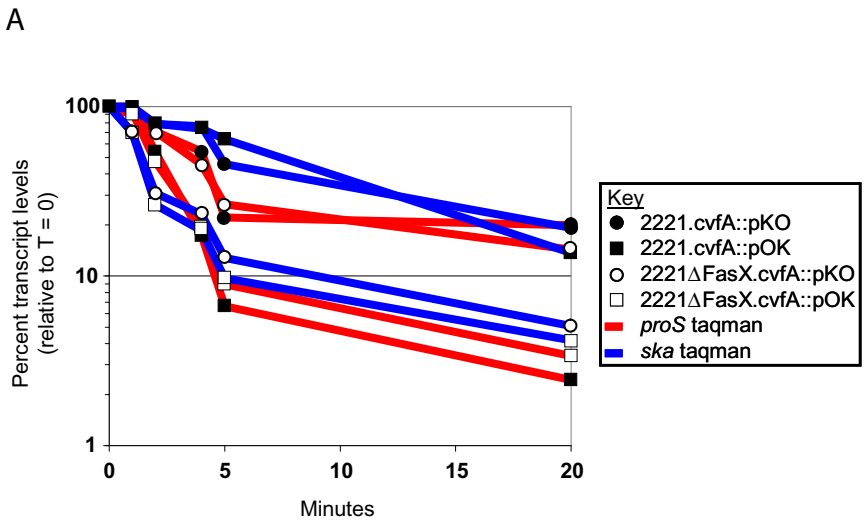
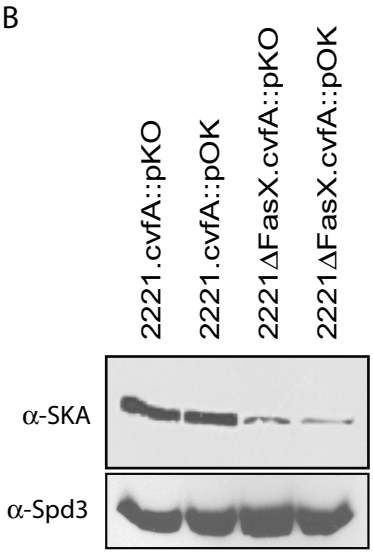

fasX
pFASXC
Design complementary
mutagenic primers
containing 1 nt deletion
3
fasX
1
2
4
pFASXC
Example of mutagenic primer design.
The following mutagenic primers were used to delete the G:C
base-pair highlighted in red for creation of plasmid pΔ1 (Figure S4):
Primer 3
5′-GAGTTTATCGA-CAATCAATCC-3′
CGAAGTCATGAGTTTATCGAGCAATCAATCCCCAATT
GCTTCAGTACTCAAATAGCTCGTTAGTTAGGGGTTAA
3′-CTCAAATAGCT-GTTAGTTAGG-5′
Primer 2
Perform PCRs using one
mutagenic primer and one
vector embedded primer
Primer 3
3' fasX
Primer 1
Primer 4
5' fasX
Primer 2
Use PCR products and primers
1 and 4 in overlap extension PCR.
This is possible due to complementarity
between mutagenic primers
Primer 1
Mutant fasX
Primer 4
Replace wild-type fasX
allele in pFASXC with
constructed mutant allele
Mutant fasX
pΔ1
Transform into mutant
strain 2221ΔFASX
Mutant FASX
pΔ1
Increased ska
mRNA levels?
ska mRNA
Streptokinase
Measure SKA activity

Figure S6


A
100
10
1
0
5
10
15
20
Minutes
Percent transcript levels
(relative to T = 0)
Key
2221.cvfA::pKO
2221.cvfA::pOK
2221ΔFasX.cvfA::pKO
2221ΔFasX.cvfA::pOK
proS taqman
ska taqman


B
2221.cvfA::pKO
2221.cvfA::pOK
2221ΔFasX.cvfA::pKO
2221ΔFasX.cvfA::pOK
α-SKA
α-Spd3

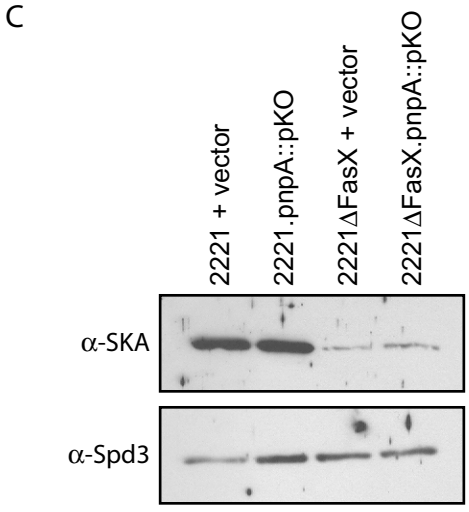
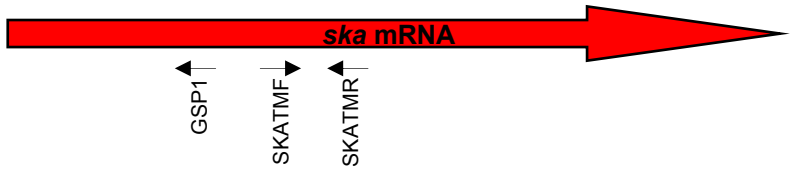

C
2221 + vector
2221.pnpA::pKO
2221ΔFasX + vector
2221ΔFasX.pnpA::pKO
α-SKA
α-Spd3

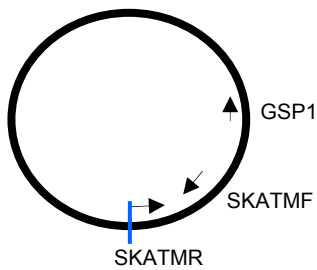
Figure S7



(A) Make cDNA using GAS total RNA and primer SKATMR.

(B) Remove RNA and purify cDNA. Add 5' phosphate to cDNA using T4 polynucleotide kinase.

(C) Ligate cDNA to self (circularize) using T4 RNA ligase 1.



(D) PCR using primers SKATMF and GSP1 using the ligated cDNA as template.

(E) Gel extract PCR, TA-clone, transform *E. coli*, and sequence inserts from a dozen transformants.

Table S1
Serotype M1 GAS strains used in this study.

Strain	**Location**	**Year**	***emm* allele**	**Disease or source**	***fasX SNPs***
MGAS2221	Australia	1988	1	Scarlet fever	REFERENCE
MGAS5300	W Finland	1988	1.2	Throat	NONE
MGAS5305	N Finland	1988	1	Throat	NONE
MGAS5307	E Finland	1988	1	Blood	NONE
MGAS2081	New Zealand	1989	1	Cellulitis	NONE
MGAS5316	W Finland	1989	1	Blood	NONE
MGAS5322	E Finland	1989	1	Throat	NONE
MGAS5337	W Finland	1989	1	Pus	NONE
MGAS5348	C Finland	1989	1	Blood	NONE
MGAS253	California	1980s	1.3	Invasive	NONE
MGAS571	Montreal	1980s	1.3	Scarlet fever	NONE
MGAS290	Colorado	Late 1980s	1	Invasive	NONE
MGAS294	Washington	Late 1980s	1	Invasive	NONE
MGAS313	Wyoming	Late 1980s	1	STSS	NONE
MGAS337	New York	Late 1980s	1	Throat	NONE
MGAS1284	Denmark	1990	1	-	NONE
MGAS1573	Germany	1990	1	-	NONE
MGAS5393	C Finland	1990	1	Throat	NONE
MGAS5411	C Finland	1990	1	Blood	NONE
MGAS5432	W Finland	1991	1	Blood	NONE
MGAS4943	Ontario	1992	1	Bacteremia	NONE
MGAS5087	Ontario	1993	1	Lower respiratory tract	NONE
MGAS5171	Ontario	1994	1	Necrotizing fasciitis	NONE
MGAS5459	N Finland	1994	1	Throat	NONE
MGAS5475	N Finland	1994	1	Throat	NONE
MGAS5508	S Finland	1994	1	Pus	NONE
MGAS3437	Ontario	1995	1	Soft tissue	NONE
MGAS3680	Virginia	1995	1	-	NONE
MGAS5531	E Finland	1995	1	Throat	NONE
MGAS5579	W Finland	1995	1	Pus	NONE
MGAS6413	Georgia	1995	1	Blood	NONE
MGAS5005	Ontario	1996	1	Cerebral spinal fluid	NONE
MGAS5669	S Finland	1996	1	Pus	NONE
MGAS5682	S Finland	1996	1	Pus	NONE
MGAS5690	C Finland	1996	1	Blood	NONE
MGAS6419	Atlanta	1996	1	-	NONE
MGAS6470	Connecticut	1996	1	-	NONE
MGAS6499	Connecticut	1996	1	Blood	NONE
MGAS5792	W Finland	1997	1	Blood	NONE
MGAS5804	C Finland	1997	1	Blood	NONE
MGAS6617	Minnesota	1997	1	Blood	NONE
MGAS6558	Maryland	1998	1	-	NONE
MGAS6700	San	1998	1	-	NONE

	Francisco				
MGAS6727	E Finland	1998	1	Blood	NONE
MGAS6184	Texas (TDH)	1997-98	1	Invasive	NONE
MGAS9127	Alberta	Late 1990s	1	Invasive	NONE
MGAS9138	Alberta	Late 1990s	1	Invasive	NONE
MGAS9144	Alberta	Late 1990s	1	Invasive	NONE
MGAS9150	Alberta	Late 1990s	1	Invasive	NONE

Table S2
Primer and probe sequences.

Name	Sequence (5' - 3')	Information
PROSTMF	GCTGACCGCAAAGTGCAAA	Taqman primer
PROSTMR	TGACTCCTGTCATATGGAAACCA	Taqman primer
PROSTMP	TACCAATGCCGTTGCAGGAGCTAACAA	Taqman probe
SKATMF	CGGCTACTTTGAGGTCATTGATT	Taqman primer
SKATMR	CCGAACCATCTTTGTCAGCAA	Taqman primer
SKATMP	CAAGCGATGCAACCATTACTGATCGAAAC	Taqman probe
FASXTMF	ATTTACGAAGTCATGAGTTTATCGAGC	Taqman primer
FASXTMR	TGTCTGCCGAGCCATCATATC	Taqman primer
FASXTMP	CCCCAATTCAATCAAAATATTGTTTTCTCTCTCTCC	Taqman probe
FASXF	GAAGTCATGAGTTTATCGAG	Northern probe primer
FASXR	CAAACAAAGACAACTGACATCG	Northern probe primer
5SF	GTTAAGTGACGATAGCCTAG	Northern probe primer
5SR	CTTAATACGACTCACTATAGGGAGCTAAGCGACTACCTTATCTC	Northern probe primer
FASXA	TCGTGTGGATATAGCCAAACG	Primer to KO *fasX*
FASXB	GTTATAGTTATTATAACATGTATTCACTAATTATTATAGCGAAAAATG	Primer to KO *fasX*
FASXC	CTATTTAAATAACAGATTAAAAAAATTATAAGGGGTTTTTTGATAGGTAAATAC	Primer to KO *fasX*
FASXD	GGGGTAAGAGACTAGCATAAG	Primer to KO *fasX*
FASXE	GAGACAGCCAAAGGCAGTAGACG	Primer to KO *fasX*
FASXF	GGAATAAGGCAATCATAACTGG	Primer to KO *fasX*
FASXSF	CATTTTTCGCTATAATAATTAGTGAATACATGTTATAATAACTATAAC	Primer to KO *fasX*
FASXSR	GTATTTACCTATCAAAAAACCCCTTATAATTTTTTTAATCTGTTATTTAAATAG	Primer to KO *fasX*
COMPFASF	GCAGATCTCGAATTGATAAGAGTAAGCAGC	Complementation primer
COMPFASR	GCATGCATGACGTCAGCTACTTATCCC	Complementation primer
DC123ECORV	CCTTATTAACATTCACAAC	pDC123-embedded primer
DC123BGLII	TATCATCCACTCAAGACTTTTGAC	pDC123-embedded primer
Δ20F	GATTTACGAATCATGAGTTTATC	Mutant *fasX* allele primer
Δ20R	GATAAACTCATGATTCGTAAATC	Mutant *fasX* allele primer
Δ26F	CGAAGTCATGGTTTATCGAG	Mutant *fasX* allele primer
Δ26R	CTCGATAAACCATGACTTCG	Mutant *fasX* allele primer
Δ36F	GAGTTTATCGACAATCAATCC	Mutant *fasX* allele primer
Δ36R	GGATTGATTGTCGATAAACTC	Mutant *fasX* allele primer
Δ46F	CGAGCAATCAATCCCAATTCAATC	Mutant *fasX* allele primer
Δ46R	GATTGAATTGGGATTGATTGCTCG	Mutant *fasX* allele primer
Δ61F	CAATTCAATCAAATATTGTTTTCTC	Mutant *fasX* allele primer
Δ61R	GAGAAAACAATATTTGATTGAATTG	Mutant *fasX* allele primer
Δ100F	CAAATAGTATTTTTATATGATGGCTC	Mutant *fasX* allele primer

Δ100R	GAGCCATCATATAAAAATACTATTTG	Mutant *fasX* allele primer
Δ111F	GATATGATGGCCGGCAGACAAAATG	Mutant *fasX* allele primer
Δ111R	CATTTTGTCTGCCGGCCATCATATC	Mutant *fasX* allele primer
Δ121F	CTCGGCAGACAAATGACGATGTC	Mutant *fasX* allele primer
Δ121R	GACATCGTCATTTGTCTGCCGAG	Mutant *fasX* allele primer
Δ144F	CAGTTGTCTTTTTTGGATATCAC	Mutant *fasX* allele primer
Δ144R	GTGATATCCAAAAAAGACAACTG	Mutant *fasX* allele primer
Δ10F	TAGTGTAAATAAAATTTACGAAGTC	Mutant *fasX* allele primer
Δ10R	GACTTCGTAAATTTTATTTACACTA	Mutant *fasX* allele primer
Δ15F	GTAAATAAAGATTTCGAAGTCATGAG	Mutant *fasX* allele primer
Δ15R	CTCATGACTTCGAAATCTTTATTAC	Mutant *fasX* allele primer
Δ29F	GAAGTCATGAGTTATCGAGCAATC	Mutant *fasX* allele primer
Δ29R	GATTGCTCGATAACTCATGACTTC	Mutant *fasX* allele primer
Δ38F	AGTTTATCGAGCATCAATCCCCA	Mutant *fasX* allele primer
Δ38R	TGGGGATTGATGCTCGATAAACT	Mutant *fasX* allele primer
Δ42F	TTTATCGAGCAATCATCCCCAATTC	Mutant *fasX* allele primer
Δ42R	GAATTGGGGATGATTGCTCGATAAA	Mutant *fasX* allele primer
Δ50F	CAATCAATCCCCATTCAATCAAA	Mutant *fasX* allele primer
Δ50R	TTTGATTGAATGGGGATTGATTG	Mutant *fasX* allele primer
Δ54F	CAATCCCCAATTCATCAAAATATTG	Mutant *fasX* allele primer
Δ54R	CAATATTTTGATGAATTGGGGATTG	Mutant *fasX* allele primer
Δ66F	CAATCAAAATATTTTTTCTCTCTCTCC	Mutant *fasX* allele primer
Δ66R	GGAGAGAGAGAAAAAATATTTTGATTG	Mutant *fasX* allele primer
Δ103F	GTATTTTTGATTGATGGCTCGGC	Mutant *fasX* allele primer
Δ103R	GCCGAGCCATCAATCAAAAATAC	Mutant *fasX* allele primer
Δ106F	ATTTTTGATATGTGGCTCGGCAG	Mutant *fasX* allele primer
Δ106R	CTGCCGAGCCACATATCAAAAAT	Mutant *fasX* allele primer
Δ115F	ATGATGGCTCGGAGACAAAATGAC	Mutant *fasX* allele primer
Δ115R	GTCATTTTGTCTCCGAGCCATCAT	Mutant *fasX* allele primer
Δ128F	AGACAAAATGACATGTCAGTTGTC	Mutant *fasX* allele primer
Δ128R	GACAACTGACATGTCATTTTGTCT	Mutant *fasX* allele primer
Δ137F	GACGATGTCAGTGTCTTTGTTTGG	Mutant *fasX* allele primer
Δ137R	CCAAACAAAGACACTGACATCGTC	Mutant *fasX* allele primer
ZOOFASXF	GCAGATCTGAAAATGGTGATTCTTGTATGG	*S. zooepidemicus fasX* primer
ZOOFASXR	GCATGCATCAACGCTTGCTTGGTCATGAATC	*S. zooepidemicus fasX* primer
GSP1	CTAACTGGCTGTTATTGATAGG	Gene-specific primer for *ska*
SKAMUT1	GCATGGATCCACTTTGAGGACTTAGGAATTG	Construction of 2221ΔX.GCSKA
SKAMUT2	CTAAAAGTTAAGTTTCAATCGGCATTATTAGAATAAC	Construction of 2221ΔX.GCSKA
SKAMUT3	GTTATTCTAATAATGCCGATTGAAACTTAACTTTTAG	Construction of 2221ΔX.GCSKA
SKAMUT4	GCATGGATCCCTTGTTTAAGGACGTAATATTTCTC	Construction of 2221ΔX.GCSKA
FXΔ155-205F	GCAGATCTCGAATTGATAAGAGTAAGCAGC	Mutant *fasX* allele primer
FXΔ155-205R	GCTCTAGAAAAAAACCTAACCAGAACTGGTCAGGTTTGATATCCAAACAAAGACAAC	Mutant *fasX* allele primer
FXΔ1-	GCATGCATTTGACTTTATCTACAAGGTGT	Mutant *fasX* allele primer

34,115-205F	GGCATAATGTGTGGTCGAGCAATCAATCCCCAATTC	
FXΔ1-34,115-205R	GCAGATCTAAAAAAACCTAACCAGAACTGGTCAGGTTTCCGAGCCATCATATCAAAAATAC	Mutant *fasX* allele primer
FXC45/46GF	TTTATCGAGCAATCAATGGCCAATTCAATCAAAATATTG	Mutant *fasX* allele primer
FXC45/46GR	CAATATTTTGATTGAATTGGCCATTGATTGCTCGATAAA	Mutant *fasX* allele primer
FXΔ72-93F	TTCAATCAAAATATTGTTTTCATTTTTGATATGATG	Mutant *fasX* allele primer
FXΔ72-93R	CATCATATCAAAAATGAAAACAATATTTTGATTGAA	Mutant *fasX* allele primer
CFVAKOF	CAAATTTTCATCAAGCTCTAGTTCaatctaaacacattgatgagcggc	*cvfA* mutation plasmid
CFVAKOR	TAATTCTCCATTGACTCATTACG	*cvfA* mutation plasmid
CVFAOKF	CAAATTTTCATCAAGCTCTAGTTCaatctaaacacattgatgagcggc	*cvfA* control plasmid
CVFAOKR	ATAAAGAAAAAGAGGATAATTCC	*cvfA* control plasmid
PNPAF	CTGTTATGTCTAAGAAAATGGCAAC	*pnpA* mutation plasmid
PNPAR	AATTGCCTGAAGGGCCCGTTTCACCAACTGAATATTGC	*pnpA* mutation plasmid
COMP.SKA.EX1	CTATAAAATTATTATGTTATTCTAATAATtcaatccccatcaGGGGATTGAAACTTAACTTTTAGG	Complementary *ska* extension plasmid
COMP.SKA.EX2	CCTAAAAGTTAAGTTTCAATCCCCtgatggggattgaATTATTAGAATAACATAATAATTTTATAG	Complementary *ska* extension plasmid
COMP.SKA.EX3	TTTAAGGACGTAATATTTCTC	Complementary *ska* extension plasmid
COMP.SKA.EX4	TCACTTTGAGGACTTAGGAATTG	Complementary *ska* extension plasmid
NCOMP.SKA.EX1	CTATAAAATTATTATGTTATTCTAATAATctcactcacatcaGGGGATTGAAACTTAACTTTTAGG	Non-complementary *ska* extension plasmid
NCOMP.SKA.EX2	CCTAAAAGTTAAGTTTCAATCCCCtgatgtgagtgagATTATTAGAATAACATAATAATTTTATAG	Non-complementary *ska* extension plasmid
NCOMP.SKA.EX3	TTTAAGGACGTAATATTTCTC	Non-complementary *ska* extension plasmid
NCOMP.SKA.EX4	TCACTTTGAGGACTTAGGAATTG	Non-complementary *ska* extension plasmid