Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 2.1

2.1.2 Oligonucleotides

Oligonucleotides used in this study were designed either by freely available online tool Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) or Generunner software. Oligonucleotides were commercially synthesized at Eurofins MWG operons, Bangalore, India. Oligonucleotides used in this study are listed in Table 2.2.

2.1.3 Chemicals, kits and culture medium components

Agarose, phenol, dimethyl sulphoxide (DMSO), sodium acetate, sodium carbonate, sodium bicarbonate, mangenese sulphate, tris methylamine, trizma base, sodium dodecyl sulphate (SDS), formamide, ethylenediaminetetraacetic acid (EDTA), glycerol, polyethylene glycol, tributyrin, ammonium persulphate, TEMED, acrylamide, bis-acrylamide, coomassie brilliant blue (CBB), β-mercaptoethanol, chloroform, formaldehyde, nuclease free water, diethylpyrocarbonate (DEPC), isopropanol, ferrozine, glycine, sodium lauryl sarcosine, carbonylcyanidep-trifluoromethoxyphenylhydrazone (FCCP), benzyl amino purine (BAP), ferrozine, tween-20, triton-X-100, aniline blue, trisodium citrate dehydrate, remazol brilliant blue-xylan (RBB-xylan), lactic acid, nicotinic acid, hexadecyltrimethyl ammonium bromide (HDTMA), p-nitrophenol, carboxymethyl cellulose (CMC cellulose), sodium phosphate dibasic, sodium phosphate monobasic, rubidium chloride, ferrous sulphate, ferric chloride, ammonium sulphate, 2,5-diphenyloxazol (PPO), 1,4- bis (5 phenyl 1,2-oxazole) Benzene (POPOP) and 2, 2-dipyridyl were purchased from Sigma Chemicals. Sodium hypochloride, disodium hydrogen orthophosphate dehydrate, sodium chloride, sodium hydroxide, citric acid,

hydrochloric acid, sulphuric acid, methanol, acetic acid, acetone and nitric acid were purchased from Fischer Scientific. Protease inhibitor tablets were procured from Roche. Hybond-P membranes for protein transfer were purchased from Amersham Biosciences. Taq DNA polymerase and Hi-fidelity Taq DNA polymerase were purchased from Thermoscientific and Larova, respectively. SYBR-green kit for real-time PCR was procured from Qiagen and Thermoscientific. Superscript SS-III RT kit was obtained from Invitrogen. Random hexamers were obtained from Qiagen. Different restriction enzymes used for cloning and mutation generation were purchased from New England Biolabs (NEB). Plasmid DNA purification, PCR purification, gel extraction and reaction clean up kits were procured from Qiagen. Medium components for bacterial culture *viz*., sucrose, agar, Luria Bertani (LB), Nutrient Agar (NA), peptone, yeast extract, beef extract, magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and potassium sulphate (K_2SO_4) were purchased from Himedia.

Table 2.1: List of strains and plasmids used in the study

***Xanthomonas* strains**	**Description**	**Reference**
X. oryzae pv. *oryzae* (BXO43)	WT *rif*-2; Rif^r	Lab collection
rpfF	*rpfF2*::mTn*7 rif-2*; Vir– derivative of BXO43	(Chatterjee and Sonti, 2002)
rpfF/CG8	*rpfF* with the complementing plasmid; *rpfF2*::mTn7/pSC8, Tet^r	(Chatterjee and Sonti, 2002)
T2SS (Type II secretion mutant)	*xpsF*:: Tn*10 rif-2*; T2S–, derivative of BXO43	(Ray et al., 2000)
motA	*motA*:: *kan rif*-2; Km^r derivative of BXO43	This study
fliC	*fliC*:: *kan rif*-2; Km^r derivative of BXO43	This study
rpfF-motA	*rpfF2*::mTn*7 motA*:: Spc^r *rif-2*; derivative of *rpfF*	This study
rpfF-fliC	*rpfF2*::mTn*7 fliC*:: Spc^r *rif-2*; derivative of *rpfF*	This study
xpsF rpfF	*rpfF::Tn7* rif-2, km^r derivative of T2SS mutant	This study
rpfF/pSC6	*rpfF* mutant with complementing plasmid, *rpfF*2::mTn7/pSC6	This study

xpsF rpfF/pSC6	T2SS *rpfF* double mutant with complementing plasmid, *rpfF*::mTn7/pSC6; (*rpfF*$^{+}$)	This study
Xanthomonas oryzae pv. *oryzicola* (BXOR1)	Laboratory wild-type; Indian isolate	(Yashitola et al., 2000)
ΔrpfF	*rpfF::*kan*, rif-2*; derived from BXOR1	This study
Δ*rpfF* (pSC9)	Δ*rpfF* mutant harboring the complementing plasmid; *rpfF*::kan (pSC9);(*rpfF*$^{+}$)	This study
*xssA::*pk18mob	*xssA::*pK18mob *rif-2; Sid*$^{-}$ Km^{r}; derivative of BXOR1	This study
ΔrpfG	*rpfG*, rif-2; derived from BXOR1	This study
ΔrpfC	*rpfC*, rif-2; derived from BXOR1	This study
Δclp	*clp,* rif-2; derived from BXOR1	This study
BXOR1(*xsuA*::*gusA*)	*xsuA*::*gusA,*rif-2, km^{r} , amp^{r}; derived from BXOR1	This study
BXOR1(pProbe GT *xsuA*::*gfp*)	*xsuA*::*gfp*, rif-2, Gm^{r}; derived from BXOR1	This study
*rpfF** (E141A)	*rpfF*(E141A); Km^{r} amp^{r} derived from Δ*rpfF*	This study
*rpfF** (E161A)	*rpfF*(E161A); Km^{r} amp^{r} derived from Δ*rpfF*	This study
Xanthomonas campestris pv. *campestris* (*Xcc*) 8004	WT rif^{r}	(Barber et al., 1997)
Xcc8004/KLN55	*Xcc* Wild-type strain harboring the DSF responsive biosensor plasmid; P_{eng}::*gfp*	(Newman et al., 2004)
Xcc8523/KLN55	*rpfF* mutant of *Xcc* with GFP fused with DSF regulated gene	(Newman et al., 2004)
***Escherichia coli* strains**	**Description**	**Reference**
DH5α	F′/endA1 hsdR17 (rk– mk+) supE44 thi-1 recA1 gyrA relA1 f80dlacZDM15(lacZYA-argF) U169	Lab collection
S17-1	recA thi pro hsdR mutant M^{+} RP4:2-Tc::Mu-Km::Tn7 Tp^{r}	(Simon et al., 1983)
Plasmids	**Description**	**Reference**
pBlueScript KS (pBSKS)	Ap^{r}, colE1 ori, *lacZ*	Stratagene, La Jolla, CA, U.S.A.
pUC1318Ω	Ap^{r} Km^{r}, pUC1318 with a 2 kb *Hin*dIII-ended omega fragment spc^{r}	Miller et al., 2000
Pk18mob	Suicidal plasmid in *Xanthomonas,* pUC18	(Schäfer et al.,

	derivative; Mob^+ Km^r	1994)
pHM1	Broad-host range vector with pUC19 polylinker, Spc^r	(Hopkins et al., 1992)
pK18mob-sacB	Suicidal plasmid in *Xanthomonas*, *sacB*, *ori*V, Km^r	(Schäfer et al., 1994)
pVO155	pUC119 derivative carrying promoterless *gus*A ;Km^r Ap^r	(Oke and Long, 1999)
pProbeGT	Promoter probe eGFP vector; Gm^r	(Miller et al., 2000)
pSC6	pHM1 + 890 bp of the Wild-type *rpfF* allele of *Xoo* cloned in *Hin*dIII and *Eco*RI sites.	This study
pRR1	pK18mob + 321-bp internal fragment of *motA* gene from *Xoo* cloned into *Eco*RI and *Xba*I sites of pk18mob.	This study
pRR2	pK18mob + 450-bp internal fragment of *fliC* gene from *Xoo* cloned into *Eco*RI and *Xba*I sites of pK18mob.	This study
pRR3	Derivative of pRR1 containing spc^r in the *Hin*dIII site.	This study
pRR4	Derivative of pRR2 containing spc^r in the *Hin*dIII site.	This study
pRR6	300 bp fragment of 5' end of *rpfF* gene from BXOR1cloned into *Eco*RI and *Hin*dIII sites of pBSKS.	This study
pRR7	Derivative of pRR6 containing 345 bp fragment of 3' end of *rpfF* gene from BXOR1 cloned into *Hin*dIII and *Xho*I sites.	This study
pRR8	Derivative of pRR7 containing kan cassette in *Hin*dIII site.	This study
pSC9	pHM1 + 870 bp of the wild-type *rpfF* allele of *Xoc* cloned in *Hin*dIII and *Eco*RI sites.	This study
pRR9	pk18mobsacB + 270 bp fragment of 5' end of *rpfG* gene from BXOR1 cloned into *Bam*HI and *Xba*I site and 327bp fragment of 3' end cloned into *Xba*I and *Hin*dIII sites.	This study
pRR10	pk18mobsacB + 273 bp fragment of 5' end of *rpfC* gene from BXOR1 cloned into *Bam*HI and *Xba*I site and 330 bp fragment of 3' end cloned into *Xba*I and *Hin*dIII site	This study
pRR11	Pk18mobsacB + 195 bp fragment of 5' end of	This study

	clp gene from BXOR1 cloned into *Bam*HI and *Xba*I sites and 201 bp fragment of 3' end cloned into *Xba*I and *Hin*dIII sites.	
pRR12	pk18mob with 321-bp fragment of *xssA* gene from BXOR1 cloned into the *Xba*I and *Hin*dIII sites of pK18mob.	This study
pRR14	pVO155 carrying 611-bp DNA (putative promoter region, +213 to -398) upstream of *xss* operon of BXOR1 cloned in *Hin*dIII and *Bam*HI sites.	This study
pRR15	pProbeGT carrying 611-bp DNA (putative promoter region, +213 to -398) upstream of *xss* operon of BXOR1 cloned in *Hin*dIII and *Bam*HI sites.	This study
pRR16	*rpfF* full length gene with (E141A) point mutation cloned in *Eco*RI and *Xho*I sites of pBSKS.	This study
pRR17	*rpfF* full length gene with (E161A) point mutation cloned in *Eco*RI and *Xho*I sites of pBSKS.	This study

Rif^r, Ap^r, Km^r, Gm^r, Tet^r and Spc^r indicate resistant to rifampicin, ampicillin, kanamycin, gentamicin and spectinomycin, respectively.

Table 2.2: List of oligonucleotides used in the study

Description	**Primer name**	**Sequence (5'-3')**
***Xanthomonas oryzae* pv. *oryzae* primers**		
MotA insertional knockout F	SCKO4F	GCGAATTCTTTCGTCGCTGTGG TCGCCGG
MotA insertional knockout R	SCKO4R	GCTCTAGATGTCCACTTCGATC TCGAGCA
fliC insertional knockout F	SCKO9F	GCGAATTCCGCTGAACGCTCAG CGT
fliC insertional knockout R	SCKO9R	GCTCTAGATGTTGATGCCGATG GTCTGGC
rpfF::Tn7 insertion mutation in *xpsF* mutant background F	SC11	GCGAATTCATGTCTGCAGTTCA ACCCTTC
rpfF::Tn7 insertion mutation in *xpsF* mutant	SC10	GCAAGCTTTCAGCCGGCGTCAA GCCCGG

background R		
rpfF full length primer F	SC7	GCAAGCTTAGGAGGACAGCTAT GTCTGCAGTTCAACCCTTC
rpfF full length primer R	SC8	GCGAATTCTCAGCCGGCGTCAA GCCCGG
***Xanthomonas oryzae pv. oryzicola* primers**		
rpfF deletion primer F1	SC14	GCGAATTCATGTCTGCTGTTCA ACCCTT
rpfF deletion primer R1	SC15	GCAAGCTTCCGGTCGCCTTCGC GAATGAGTC
rpfF deletion primer F2	SC16	GCAAGCTTATGTGCCAGCGCGT CAGTGCGC
rpfF deletion primer R2	SC17	GCCTCGAGTCAGCCGGCGTCAA GCCCGGAGC
Kanamycin cassette Primer F	SC4	GCAAGCTTCTGTCTCTTATACA CATC
Kanamycin cassette primer R	SC23	GCAAGCTTGCTTTGCCACGGAA CGGTCT
rpfF full length primer F	RpfFCola *Hind*III rbs	GCAAGCTTAGGAGGACAGCTAT GTCTGCTGTTCAACCCTT
rpfF full length primer R	RpfFCola*EcoR*IR 2	GCGAATTCTCAGCCGGCGTCAA GCCCGGAGC
xssA ko primer for insertional inactivation F	SCKOsid6F	CATCTAGAAGGACTGGCAGGTG TGCTAT
xssA ko primer for insertional inactivation R	SCKOsid6R	CTAAGCTTCTTGCAGGCTGTTG CCATC
rpfG deletion primer F1	SCR45	GTGGATCCATGCAGGATGTTTC AGGAGG
rpfG deletion primer R1	SCR46	CTTCTAGATTCCAGACCGTCCA TGCC
rpfG deletion primer F2	SCR47	GGTCTAGAATCGGCTACGAGTT GCTCAGC
rpfG deletion primer R2	SCR48	TCAAGCTTGGTGGAGAATTCCC CACAGAT
rpfC deletion primer F1	SCR49	TGGGATCCATGAAGTCTCCACT GACATG
rpfC deletion primer R1	SCR50	GATCTAGAGCAGGTGTAGTCGA GCAGCAT
rpfC deletion primer F2	SCR51	TGTCTAGAAATTTCGAAGGCGT GCTC
rpfC deletion primer R2	SCR52	GTAAGCTTGAGTGCCTCCCTTC CGGTCTG

clp deletion primer F1	SCR57	ATGGATCCATGAGCTCAGCAAACACGAC
clp deletion primer R1	SCR58	ACTCTAGAGATGCTCACCGAGCCGCTGAT
clp deletion primer F2	SCR59	TTTCTAGAGTGCGCACGCTGCACGATCTT
clp deletion primer R2	SCR60	CTAAGCTTTTAGCGCGTGCCGTACAGCA
Primer from neighbouring gene for confirmation of *clp* deletion *F*	SCR61	ATGTCCGATTCCAACGTCTC
Primer from neighbouring gene for confirmation of *clp* deletion R	SCR62	AGATCTGCCGCTCCTGAAG
Primer from neighbouring gene for confirmation of *rpfC* deletion F	SCR63	GACGAGATGAGCGTGATGAA
Primer from neighbouring gene for confirmation of *rpfC* deletion R	SCR64	ACATCACCCGCTATCAGACC
Primer from neighbouring gene for confirmation of *rpfG* deletion F	SCR65	TGCGGAATTCGTGACTCATA
Primer from neighbouring gene for confirmation of *rpfG* deletion R	SCR66	ACCACCCGGTCGAAGTGT
Promoter fusion for siderophore cluster in pVO155 and pProbeGT F	SCRsid_ pProbeGFP_F	GAAAGCTTTTTGCAGCGCCTCCTTGATCA
Promoter fusion for siderophore cluster pVO155 and pProbeGT R	SCRsid_ pProbeGFP_R	CAGGATCCCACCCGCCCAATCAGTTTCTC
Point mutation E141A in *rpfF* gene F	SCRrpfFE141A_ F	GGATTTGCAGCCGCATTGAGC
Point mutation E141A in	SCRrpfFE141A_	GCTCAATGCGGCTGCAAATCC

rpfF gene R	R	
Point mutation E161A in *rpfF* gene F	SCRrpfFE161A_F	ATGGGCTTGCCCGCAGTGCTGTTCGAC
Point mutation E161A in *rpfF* gene R	SCRrpfFE161A_R	GTCGAACAGCACTGCGGGCAAGCCCAT
Real time RT-PCR primers		
16S rRNA (endogenous control) F	16SXooRTF	GGGAATTGCAGTGGATAC
16S rRNA (endogenous control) R	16SXooRTR	CTGATGTTCCTCCCGATCTCT
XOC_3387 (*xssA*) F	SCRT99F	AATGGGATGCCGATGTAG
XOC_3387 (*xssA*) R	SCRT99R	CGATGCTGCGGTAATTGATCT
XOC_3388 (*xssB*) F	SCRT100F	ATCGTTGATGCCAGCTTG
XOC_3388 (*xssB*) R	SCRRT100R	CGGACTGGGGTCTGTCCATT
XOC_3389 F	SCRRT165F	GTGGCCTGCCTTACTACTCG
XOC_3389 R	SCRRT165R	CAGCACCACCAGATAGACCA
XOC_3386 F	SCRT98F	CGGATACCTCACCGAAAA
XOC_3386 R	SCRT98R	CACACGCAGATTGCTGAT
XOC_2243 F	SCRT164F	AGCGTAAGGAAGGACACT
XOC_2243 R	SCRT164R	GATAGAAGCCACGGCACA
XOC_0143 F	SCRRT170F	GGCTGGCTTCCCTCTCAC
XOC_0143 R	SCRRT170R	GTTCTGCGACTGGAACGAG
XOC_0619 F	SCRRT171F	GTGACTGCGCTCTATGGCATT
XOC_0619 R	SCRRT171R	TTCCTGCTGGATCTGCTG
XOC_1096 F	SCRRT172F	GATTTCCACTCGTCGCTTTC
XOC_1096 R	SCRRT172R	TGCAGGAGCAACAGTAAACG
XOC_1946 F	SCRRT173F	TGTTGAACAGAACTCCGCTCT
XOC_1946 R	SCRRT173R	AGGCACGCTTGAGATCTACC
XOC_4682 F	SCRRT174F	TGCTCAAGATCCATCAGTTCA
XOC_4682 R	SCRRT174R	CGTACCGGTATCGGCAGAC
XOC_3378 F	SCRRT175F	AGACCAGTCCACTCGCAGTT
XOC_3378 R	SCRRT175R	GGTCACGATCAGAGTGTCCA
XOC_3709 F	SCRRT176F	TATTGCGATCGCGTTGAC
XOC_3709 R	SCRRT176R	ATGCGCGAGCCAGTGATT
XOC_1736 F	SCR_*fur*_RTF	TGCTGAAGCACAATTTCGAG
XOC_1736R	SCR_*fur*_RTR	AGCGAGTGCTCTTCGAGTTC

2.1.4 Media:

2.1.4.1 Bacterial media

Peptone Sucrose (PS)

1 % Peptone

1 % Sucrose

For preparing plates, 1.2 % agar was added to the medium before autoclaving.

Minimal Medium (MM9)

Stock Solution

Minimal Salt (2X) for 250 mL

5.25 g di-Potassium hydrogen phosphate (K_2HPO_4)	125 ml
2.25 g Potassium dihydrogen phosphate (KH_2PO_4)	
0.5 g Ammonium sulphate $(NH_4)_2SO_4$	
0.25 g Tri-Sodium citrate (Na_3 citrate)	
1 M Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	- 250 µl
25 mg/mL L- Methionine	- 1 ml
25 mg/mL Nicotinic acid	- 1 ml
10 mg/mL Glutamic acid	- 25 ml
20% Glucose	-12.5 ml
3% Agar	- 100 ml

Plant mimicking medium (XOM2)

Stock preparation

100 mM L-Methionine

1 M Sodium glutamate

1 M Potassium dihydrogen phosphate (KH_2PO_4)

10 mM Manganese sulphate ($MnSO_4$)

10 mM Ferric ethylenediaminetetraacetic acid (Fe(III)EDTA)

100 mM Magnesium chloride ($MgCl_2$)

Working solution

0.18% Xylose

670 μM L-Methionine

10 mM Sodium glutamate

14.7 mM Potassium dihydrogen phosphate (KH_2PO_4)

40 μM Mangenese sulphate ($MnSO_4$)

240 μM Ferric ethylenediaminetetraacetic acid (Fe(III)EDTA)

5 mM Magnesium chloride ($MgCl_2$)

1.2% Agar

Luria Bertani (LB)

0.5% Yeast extract

1% Tryptone

1% Sodium cholride (NaCl)

Media and solutions were sterilized either by routine autoclaving at 121°C and 15 psi for 20 min or by filtration through membrane of 0.22 μM porosity.

2.1.5 Buffers and solutions

2.1.5.1 Common buffers

Phosphate-Buffered Saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

pH was adjusted to 7.3 before autoclaving.

PBS was prepared as a 10 X stock solution and used as a 1 X concentration.

Tris-HCl buffer

0.5 M Trizma Base

pH was adjusted to 7.6 using concentrated HCl.

Tris-Cl buffer was prepared as a 10X stock solution and used as 1X concentartion.

Tris-EDTA (TE) buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA

Tris Acetic acid-EDTA (TAE) buffer

40 mM Tris base

0.5 M EDTA

pH was adjusted to 8.5 with glacial acetic acid

TAE buffer was prepared as a 50 X stock solution and used at 1 X concentartion.

Potassium Phosphate buffer (0.1 M)

1 M Potassium phosphate dibasic (K_2HPO_4)

1 M Potassium phosphate monobasic (KH_2PO_4)

61.5 ml of 1 M K_2HPO_4 was mixed with 38.5 ml of 1 M KH_2PO_4, pH was adjusted to 7.0 and volume was adjusted to 1 L with H_2O.

2.1.5.2 Buffers and solutions for extraction and analysis of genomic DNA and RNA

Resuspension buffer (P1)

50 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

100 µg/ml RNase

Volume was adjusted to 100 ml with sterile H_2O.

10% SDS

10 g of SDS (Sodium Dodecyl Sulfate) was dissolved in 80 ml of H_2O, and volume was adjusted to 100 ml with H_2O.

CTAB/NaCl solution

10% CTAB

0.7 M NaCl

10 g of CTAB was dissolved in 80 ml 0.7 M NaCl solution by stirring it on a hot magnetic stirrer. Volume was adjusted to 100 ml with 0.7 M NaC1 solution.

Lysozyme solution

100 mg of lysozyme was dissolved in 1 ml of H_2O (100 mg/ml).

Proteinase K solution

10 mg of proteinase K was dissolved in 1 ml of H_2O (10 mg/ml).

5 M Sodium chloride (NaCl)

292.2 g of Sodium chloride (NaC1; M.W. 58.44) was dissolved in 800 ml of H_2O.

Volume was adjusted to 1 liter with H_2O.

Sterilized by autoclaving.

3 M Sodium acetate (NaOAc) (pH 5.2 and 7.0)

24.6 g sodium acetate anhydrous (CH_3COONa; M.W. 82) was dissolved in 80 ml H_2O. pH was adjusted to 5.2 with glacial acetic acid or 7.0 with dilute acetic acid. Volume was adjusted to 100 ml with H_2O.

Sterilized by autoclaving.

Phenol:Chloroform:Isoamyl alcohol (25:24:1) solution

25 ml Tris-equilibrated phenol

24 ml Chloroform

1 ml Isoamyl alcohol

DEPC (diethyl polycarbonate) treated water

0.5% DEPC

Added in H_2O, stirred vigorusly and autoclaved prior to use.

DNA sample loading buffer

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol

DNA sample loading buffer was prepared in water.

2.1.5.3 Buffers and solutions for protein extraction, analysis by SDS-PAGE (sodium dodecyl sulphate-polyacrylamaide gel electrophoresis) and silver staining

Whole cell lysis buffer

50 mM Sodium acetate

410 mg Sodium acetate anhydrous was dissolved in 80 ml H_2O. pH was adjusted to 5.4 with glacial acetic acid and finally volume was adjusted to 100 ml with H_2O.

1 mM PMSF (phenylmethylsulfonyl fluoride) in isopropanol.

Dialysis buffer

50 mM Trizma base

pH was adjusted to 7.5 by using concentrated HCl.

Silver staining

Fixing solution

50% ethanol

10% glacial acetic acid

0.05% formaldehyde

Final volume was adjusted with sterile H_2O.

0.2% Silver nitrate solution ($AgNO_3$)

0.2 g $AgNO_3$

0.075% formaldehyde (37% stock)

Dissolved in 100 ml of H_2O. Stored at 4°C for 1 hour in brown colored bottle.

Developing solution

6% Sodium carbonate (Na_2CO_3)

0.05% Formaldehyde (37% stock)

0.02% Sodium thiosulphate

Storage buffer

50% Ethanol

SDS-PAGE

30% Acrylamide solution

29 g Acrylamide

1 g Bis-acrylamide

Acrylamide solution was prepared in H_2O.

Resolving gel mix (12%) (10 ml)

H_2O	- 3.3 ml
30% Acrylamide:Bisacrylamide mix (29:1)	- 4 ml
1.5 M Tris-HCl (pH-8.8)	- 2.5 ml
10% SDS	- 100 µl
10% Ammonium persulphate (APS)	- 100 µl
N, N, N',N',-Tetramethylethylenediamine (TEMED)	- 4 µl

Stacking gel mix (5%, 3 ml)

H_2O	- 2.1 ml
30% acrylamide:bisacrylamide mix (29:1)	- 500 µl
1.5 M Tris-HCl (pH-6.8)	- 380 µl
10% SDS	- 30 µl

10% APS - 30 µl

TEMED - 3 µl

SDS loading buffer (2X)

100 mM Tris-HCl (pH-6.8)

20% (v/v) Glycerol

4% (W/V) SDS

0.02% Bromophenol Blue

10% β-Mercaptoethanol

SDS-loading buffer was prepared as 2X stock solution in H_2O and used at 1X concentration.

SDS-PAGE running buffer

14.4 g Glycine

3.03 g Tris methylamine

1 g SDS

Dissolved in H_2O and volume was adjusted to 1L with H_2O.

Buffers for western blot analysis

Transfer buffer (1 litre)

14.4 g Glycine

3.03 g Tris methylamine

800 ml H_2O

200 ml methanol

Blocking and wash buffers (PBS-T)

5% Fat-free milk

0.05% Tween-20

Volume was adjusted to 100 ml with 1 X PBS.

2.1.5.4 Buffers for enzyme assays

Cellulase assay

50 mM Phosphate citrate buffer (pH-6.8)

0.1 M Citric acid

0.2 M dibasic Sodium phosphate

16.9 ml Citric acid (0.1 M) and 33.1 ml Sodium phosphate (0.2 M) was mixed and volume was adjusted to 100 ml with H_2O.

Lipase assay

0.1 M Tris-HCl buffer (pH-8.2)

pH was adjusted to 8.2 with HCl.

0.5 mM p-Nitrophenol standard solution

8.69 mg p-Nitrophenol was dissolved in Tris-HCl buffer (0.1 M) and volume was adjusted to 25 ml to make a final concentration of 25 mM.

1 volume of the above solution (25 mM) was diluted with 49 volume of 0.1 M Tris-HCl buffer to get a final concentration of 0.5 mM p-Nitrophenol standard solution.

p-Nitrophenyl butyrate solution (420 µM)

7.3 µl p-Nitrophenol butyrate (F.W. 209.2)

11 mg SDS

650 µL Triton-X-100

Volume was adjusted to 100 ml with H_2O. Mixture was heated to 65°C in a water bath for 15 min, mixed well, and cooled down to room temperature prior to use. It can be stored upto 3 days at 4°C.

Xylanase assay

5 mg/ml RBB-xylan

0.05 M di-Sodium hydrogen phosphate (Na_2HPO_4)

5 mg/ml RBB-Xylan was dissolved in 0.05 M Na_2HPO_4 pH-7.5

2.1.5.5 Buffers for *E. coli* electrocompetent cell preparation

Transformation buffer I (Tfb-I)

30 mM Potassium acetate

100 mM Rubidium chloride ($RbCl_2$)

10 mM Calcium chloride dihydrate ($CaCl_2.2H_2O$)

50 mM Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)

15% (v/v) Glycerol

pH was adjusted to 5.8 with 10% acetic acid and volume was adjusted to 500 ml with H_2O.

Transformation buffer II (Tfb-II)

10 mM MOPS

75 mM $CaCl_2.2H_2O$

10 mM $RbCl_2.2H_2O$

15% Glycerol

pH was adjusted to 6.5 with KOH (Potassium hydroxide) and volume was adjusted to 100 ml with H_2O.

2.1.5.6 Other solutions

CAS solution

a) 0.06 g Chrome Azurol S dye in 50 ml

b) Fe (III) solution: 10 ml

1 mM $FeCl_3$

10 mM HCl

c) 0.072 g HDTMA in 40 ml

All the above three solutions were mixed together and autoclaved prior to use.

Liquid scintillation cocktail

5 g PPO (2,5- diphenyloxazol)

0.3 g POPOP (1,4- bis (5 phenyl 1,2-oxazole) Benzene

Volume was adjusted to 1L with toluene.

MUG (4-methylumbelliferyl β-d-glucuronide) extraction buffer

1 mM MUG substrate

50 mM Sodium dihydrogen phosphate (pH-7.0)

10 mM EDTA

0.1% Triton X-100

0.1% Sodium lauryl sarcosine

10 mM β-Mercaptoethanol

Lactophenol solution (100 g)

25 g Lactic acid (20.66 ml)

25 g Phenol

50 g Glycerol (39.77 ml)

These three components were mixed together and 1 volume of lactophenol was added to 2 volumes of ethanol.

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Strains and culture conditions

Xanthomonas strains were routinely grown in rich PS medium, at 28°C with continuous shaking at 200 rpm unless otherwise stated (New Brunswick Scientific, Innova 43, U.S.A.). In general, *Xanthomonas* frozen glycerol stocks were revived on PSA medium by streaking, and allowed to grow for 3-4 days. To prepare liquid culture, a loopful of each *Xanthomonas* strain was inoculated in PS medium and grown for 24-30 h. *Xanthomonas* strains on plates were stored at 4°C for a maximum period of 1 week. For growth of *X. oryzae* pv. *oryzae* in Minimal (MM9); (Kelemu and Leach, 1990) and XOM2 media (minimal media which induces hrp genes in *X. oryzae* pv. *oryzae*), (Tsuge et al., 2002), first the strains were grown in PS medium to a cell density of 10^9 cells/ml and then centrifuged at 5000 g to concentrate the cells and washed twice with sterile water to remove media components sticking to the cells. Washed cells were inoculated in MM9 and XOM2 medium and grown for overnight.

Escherichia coli DH5α, used for cloning purposes, was revived on LB medium containing nalidixic acid and grown at 37°C with continuous shaking at 200 rpm. LB medium was supplemented with appropriate antibiotics to grow the bacterial strains carrying plasmids. For plasmid purification, bacterial strains were grown overnight in LB broth medium containing suitable antibiotics.

Antibiotics were used at a final concentration of 50 µg/ml rifampicin, kanamycin, streptomycin and trimethoprim; 100 µg/ml ampicillin; 25 µg/ml nalidixic acid; 10 µg/ml cephalexin, chloramphenicol and gentamicin; 20 µg/ml cyclohexamide and 5 µg/ml tetracyclin.

2.2.1.2 Growth analysis and determination of generation time

For growth analysis of *Xanthomonas* strains, a loopful of bacterial colony was inoculated in appropriate broth medium and grown for 14-16 h. 0.2% of overnight grown culture was used to inoculate the test medium (for iron limitation, PS with 50 or 100 µM of 2,2'-dipyridyl, and for iron supplementation, different concentrations of either $FeCl_3$ or $FeSO_4$ was added). Cultures were transferred to a shaker incubator set at 28°C and 200 rpm. Absorbance of cultures was measured using Ultraspec 2100 pro UV/visible spectrophotometer (Amersham Biosciences) at 600 nm at regular time-intervals till 48 h. Absorbance values were plotted with respect to time and generation time was determined from the logarithmic (log) phase of bacterial growth using the following formula.

$$\text{Generation time (G)} = (T_2\text{-}T_1) \times \frac{\log 2}{\log (N_f/N_i)}$$

G = Generation time (h)

T_1 = Initial time point taken for analysis

T_2 = Final time point taken for analysis

N_f = Absorbance at time T_2 (Final OD)

N_i = Absorbance at time T_1 (Initial OD)

2.2.1.3 Serial dilution plating and spotting assay

Xanthomonas strains were grown in PS medium for 14-16 h at 28°C with continuous shaking at 200 rpm. 1 ml of bacterial cultures were ten-fold serially diluted in water and 100 µl volume of each dilution was plated on PS agar plates to get the colony forming units (CFUs). Similarly, 5 µl volume of each dilution was spotted on PS agar plates containing different concentration of streptonigrin and different detergents for intracellular iron and membrane sensitivity assay, respectively. Plates were incubated at 28°C and images were captured after 2-8 days of incubation depending upon medium

used.

2.2.1.4 Preparation of *E.coli* ultracompetent cells

A single colony of *E.coli* DH5α strain was inoculated in 5 ml LB medium and incubated at 37°C for overnight. 1% of overnight grown culture was inoculated in 500 ml fresh LB medium and incubated at 37°C for 2-3 h till the OD_{600} reached to 0.4-0.5. Culture was chilled on ice for 5 min followed by centrifugation at 3000 g for 15 min at 4°C. Harvested cells were washed gently with 200 ml ice-cold TFb-I buffer. Cells were collected by centrifugation at 3000 g for 5 min at 4°C and gently resuspended in 20 ml ice-cold TFb-II buffer. Bacterial cell suspension was kept on ice for 15 min and was aliquoted in 100 µl volumes in chilled sterile microcentrifuge tubes. Cells were immediately snap-frozen in liquid nitrogen and stored at -80°C.

2.2.1.5 *E.coli* transformation

E.coli DH5α strain was transformed with plasmids carrying appropriate inserts to generate clones, and *Xanthomonas* deletion strains. Ultracompetent cells stored at -80°C were thawed on ice for 5-10 min. 5 µl ligated plasmid was added to 100 µl ultracompetent cells and incubated on ice for 30 min. Next, competent cells were subjected to heat shock at 42°C for 90 seconds. Cells were immediately transferred on ice for 2-3 min. Next, 1 ml LB medium was added and cells were allowed to recover for 1 h on a shaker incubator set at 37°C. After the recovery, cells were centrifuged at 3000 g for 3 min. Medium supernatant was discarded and cells were resuspended in 100 µl fresh sterile medium. Cells were plated on LB agar containing appropriate antibiotics. Plates were incubated at 37°C for 12-16 h.

2.2.1.6 *Xanthomonas* transformation

For electrocompetent cell preparation, single colony of desired *Xanthomonas* strain was inoculated in 5 ml PS medium and grown overnight at 28°C with constant

shaking at 200 rpm. 1% of overnight grown culture was inoculated in 100 ml fresh PS medium and grown to obtain log-phase culture. Log phase *Xanthomonas* culture was kept on ice for 10-15 min, aliquoted in 50 ml pre-chilled centrifuge tubes and centrifuged at 4000-5000 g at 4°C for 10 min. Supernatant was discarded and pellet from each tube was gently resuspended in 10-20 ml sterile chilled water. Next, cells were harvested by centrifugation at 4000 g at 4°C for 10 min and supernatant was discarded. Harvested cells were washed twice and finally resuspended in adequate amount of prechilled sterile water. 100 µl of cell suspension was aliquoted in sterile 1.5 ml microcentrifuge tubes and kept on ice.

For transformation, *Xanthomonas* electrocompetent cells and appropriate amount of plasmid DNA was mixed, and kept on ice in laminar hood. This mixture was added to 1 mm electroporation cuvettes (Biorad) and tapped gently to allow the cells to settle properly in order to avoid air bubbles. Competent cells were electroporated (1800 V, 25 µF, 200 Ω, 1mm cuvette) followed by immediate addition of fresh PS broth in the cuvette, mixed properly and taken in the microcentrifuge tubes. Microcentrifuge tubes containing transformed cells were incubated at 28°C for 2 hours with continuous shaking for recovery. After recovery, cells were plated on specific medium with appropriate antibiotics and incubated in 28°C plate incubator.

2.2.1.7 *Xanthomonas* conjugation

Since compatible conjugation does not exist between *Xanthomonas* and *E.coli* DH5α strain. Therefore, upon getting the appropriate clones in DH5α, conjugation was performed with S17-1 (recipient strain) and PRK600 (helper strain). All the three strains (DH5α with clone, S17-1 and PRK600 strain of *E.coli*) were grown overnight at 37°C with constant shaking at 200 rpm in 3 ml LB broth. Cells from 1 ml overnight grown cultures were harvested by centrifugation followed by three washes with sterile water

and finally resuspended in 100 µl sterile water. Bacterial cell suspension was aliquoted in 20 µl volume. The above procedure was followed for all the three strains and cell suspension of three different strains were mixed together in 1:1:1 ratio. For conjugation to occur, 20 µl of the above mixture was spotted on the LB agar plate and incubated at 37°C for 12-16 h. Next, the conjugation drops were streaked on LB agar plate containing appropriate antibiotics to select the S17-1 recipient containing recombinant plasmid.

S17-1 was directly conjugated with *Xanthomonas* strain. S17-1 strain containing recombinant plasmid (3 ml) and recipient *Xanthomonas* strain (100 ml) was grown overnight with appropriate antibiotics. Cells were harvested and washed thrice as mentioned earlier. *Xanthomonas* strain was finally dissolved in 600-700 µl sterile water and S17-1 strain was dissolved in 3 ml sterile water. 50 µl *Xanthomonas* cell suspension and 10 µl S17-1 cell suspension were mixed together and 20 µl was spotted on PS agar plate. After 40 h of incubation at 28°C, each conjugation drop was dissolved in 400 µl water separately and plated on PS agar medium with rifampicin (counter-selectable marker) and plasmid specific antibiotics for specific selection of *Xanthomonas* colony with recombinant plasmid.

2.2.1.8 *Xanthomonas* and *E.coli* colony PCR

A microtipful cells of bacterial strain from appropriate medium was resuspended in 20 µl sterile water and incubated at 98°C for 10 min for cell lysis. 2 µl of heat-lysed cell suspension was used as template in 25 µl PCR reaction.

2.2.2 Molecular biology methods

All standard molecular biology and genetics were performed as described previously (Sambrook et al. 1989).

2.2.2.1 Plasmid DNA purification

E.coli strains carrying plasmids were inoculated and grown overnight at 37°C and

200 rpm in LB broth supplemented with appropriate antibiotics (plasmid antibiotic marker). Cells were harvested by centrifugation at 12,000 g for 5 min. Plasmids were extracted using Qiagen plasmid miniprep or midiprep kit following the manufacturer's instructions. Concentration of the extracted plasmid DNAs was measured using spectrophotometer at 280 nm and stored at -20°C.

2.2.2.2 Genomic DNA isolation

Xanthomonas strains were grown overnight in 3 ml PS medium. Cells were harvested at 12,000 g for 5 min, resuspended in RNase added P1 buffer and were transferred to 2 ml microcentrifuge tube. Cells were lysed by adding 40 µl lysozyme followed by adding 80 µl 10% SDS and incubated at 50°C for 10 min. Further, proteins were removed by treating the cell suspension with 16 µl proteinase K and incubated at 37°C for overnight. Next day, 200 µl CTAB/NaCl was added and cell suspension was heated at 65°C for 10 min. Next, 1 ml chloroform-isoamyl alcohol was added to the cell suspension and tubes were vortexed for 2-3 min. After centrifugation at maximum speed for 10 min at room temperature, aqueous phase was carefully transferred to a fresh microcentrifuge tube. To further remove cell debris, previous step was repeated with 1 ml of phenol-chloroform-isoamyl alcohol and aqueous phase containing DNA was taken out carefully. Genomic DNA from the aqueous phase was precipitated by adding 700 µl isopropanol and 170 µl sodium acetate (3M, pH-7). Next, DNA pellet was washed with 70% ethanol and dried at room temperature for 20 min. Genomic DNA pellet was dissolved in 50 µl nuclease free water and stored at -20°C. Quality of extracted genomic DNA was checked on 0.7% agarose gel by electrophoresis.

2.2.2.3 RNA extraction

For RNA experiments, all solutions were prepared in RNase free diethylpyrocarbonate (DEPC) treated water. Microcentrifuge and tips used for RNA

work were autoclaved twice and dried at 80°C for overnight before use. RNA was isolated from *Xanthomonas* culture using Trizol method. *Xanthomonas* cells were harvested at 12,000 g for 5 min at 4°C, resuspended in approximately 1 ml Trizol (Invitrogen), mixed properly and incubated at room temperature (RT) for 5 min. 200 µl chloroform was added to the tube, shaken for 15 seconds and incubated at RT for 2-15 seconds. Next, tubes were centrifuged at 13,000 g for 15 min at 4°C. Aqueous phase was transferred to new 1.5 ml microcentrifuge tube and RNA was precipitated by adding 500 µl isopropanol and incubated for 5-10 min at RT. Precipitated RNA was collected by centrifugation at 10,000 g for 10 min at 4°C. RNA pellet was washed with 70% ethanol and resuspended in 20 µl nuclease-free water. RNA concentration was determined by measuring absorbance at 260 nm. Quality of RNA was examined by gel electrophoresis on 0.8% agarose gel with TAE buffer prepared in DEPC treated water.

2.2.2.4 Synthesis of complementary DNA (cDNA)

Complementary-DNA synthesis was performed using reverse transcriptase enzyme (Invitrogen) and random hexamers (Qiagen). For this, 1 µg good quality RNA was treated with 1 µl (1 unit) DNase I (Invitrogen) for 20 min to remove DNA contamination. Next, Superscript III Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA according to the manufacturer's instructions. cDNA synthesized was further confirmed by using it as a template for amplification in PCR. cDNA was stored at -20°C till further use.

2.2.2.5 Quantitative real-time PCR

Primers for real-time PCR analysis were designed using Primer3 plus software and are listed in Table 2.2. For RNA isolation, *X. oryzae* pv. *oryzae* wild-type, *rpfF* mutant, *rpfF*/CG8 complemented strains were grown in PS medium at 28°C for 28 h at 200 rpm. Similarly, for RNA isolation from *X. oryzae* pv. *oryzicola*, the Wild-type

BXOR1, Δ*rpfF* and Δ*rpfF* (pSC9) strains were grown to OD_{600} of 1 in rich media (PS), PS + 50 μM 2,2'-dipyridyl (DP) and PS + DP + 30 μM $FeSO_4$. RNA was isolated by Trizol (Invitrogen) method as described above. Optimal primer and cDNA concentrations were standardized, and qRT-PCR was performed using ABI 7500 Fast Real-Time PCR system (Applied Biosystems). In brief, 1 μl cDNA, 0.25 picomoles of gene specific primers and 10 μl 2X SYBR GREEN qPCR Mastermix (Qiagen) were mixed in the wells of 96-well PCR plate (Axygen). Final reaction volume was adjusted to 20 μl with nuclease-free water. Transcript levels were quantified with an end-point value known as C_t (cycle thresold) value. Expression of 16S rRNA was used as an internal control. The C_t values defines the number of PCR cycles required for the fluorescent signal of SYBR green dye to cross beyond the background level. Fold-change in transcript expression was determined using following formula.

Fold change in expression = $2^{-\Delta\Delta Ct}$

$\Delta\Delta C_t = \Delta C_t$ treated- ΔC_t untreated

ΔC_t treated = C_t value for the gene of interest under treated condition - C_t value for the internal control gene (16S rRNA) under treated condition

Δ C_t untreated = C_t value for the gene of interest under untreated condition - C_t value for the internal control (16S rRNA) gene under untreated condition

2.2.2.6 Microarray analysis

Xanthomonas oryzae pv. *oryzae* strains grown in PS medium to an OD_{600} of 1, were collected, washed once with 150 mM sodium chloride (NaCl) solution to remove excess EPS. RNA isolation was performed using Trizol method described above. After isopropanol precipitation, RNA was frozen at -80°C. Quality of RNA was examined by determining the RNA integrity number (RIN) before microarray analysis. Microarray experiments were performed at Genotypic Technology Pvt. Ltd., Bangalore. Briefly, a

8x15k (AMADID: 25096) custom Agilent platform comprised of coding sequences for the three strains of *Xanthomonas*- *X. oryzae* pv. *oryzae* (KACC10331), *X. oryzae* pv. *oryzicola* (BLS256) and *X. axonopodis* pv. *citri* 306 gathered from National Center for Biotechnology Information (NCBI). A total of 8113 probes were designed wherein 2120 probes corresponding to genes of interest replicated three times on Agilent platform. Feature extraction software GeneSpring GX version 10.5.1 of Agilent and GeneSpring GX percentile shift normalization was used for data analysis. Genes that were significantly up or down regulated by more than 1.5 fold and less than 0.5 fold were identified. Hierarchical clustering was performed for the differentially regulated genes and classified based on functional category. Data are the average of two hybridizations from biological replicates of each sample and raw data sets for this study are available at the Gene Expression Omnibus database (Accession number – GSE217809).

Likewise, Microarray analysis for *Xanthomonas oryzae* pv. *oryzicola* was performed by isolating RNA from the strains grown under low-iron condition. The labeled cRNA samples were hybridized on to a Genotypic Technology Private Limited designed 8x15k (AMADID: 41087) Agilent platform. Data extraction from Images was done using Feature Extraction software v 10.7 of Agilent. Data normalization was done in GeneSpring GX using 75th percentile shift and normalization to specific samples. Differentially regulated genes were clustered hierarchically to identify significant gene expression patterns. Genes were classified based on functional category. Hierarchical clustering of DSF regulated genes in *X. oryzae* pv. *oryzicola* grown under low-iron conditions is based on similar expression profiles in Δ*rpfF* mutant vs either the Wild-type BXOR1 strain or Δ*rpfF* (pSC9). Clustering analysis was performed using GeneSpring GX Software using Average Linkage rule with pearson uncentered distance metric. $\log_2$ –fold change differences between the Δ*rpfF* mutant with either the Wild-type BXOR1

strain or the Δ*rpfF* mutant harboring the Wild-type allele in plasmid (pSC9). Genes that were significantly up regulated by 0.6 or more or down regulated by -0.6 or less fold (log_2 –fold change) were identified. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) under the GEO series accession number GSE53255.

2.2.2.7 Gel extraction of DNA

QIAGEN QIAquick Gel extraction kit containing required buffers, spin columns and collection tubes was used to extract and purify DNA from agarose gels. Digested DNA samples and PCR products were resolved on 1% agarose gel and gel piece containing desired fragment was cut on an UV-transilluminator. DNA fragment was purified following manufacturer's instructions.

2.2.2.8 DNA precipitation

For DNA precipitation after digestion, 500 µl nuclease free water was added to the digested DNA fragment. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture and centrifuged at 13,000 g for 10 min at RT. Upper aqueous phase containing DNA fragment was transferred to fresh microcentrifuge tube and DNA was precipitated by adding 0.7 volume of iso-propanol and $1/10^{th}$ volume of sodium acetate. Precipitated DNA was washed with 70% ethanol, pellet was air dried for 20-30 min at RT and dissolved in nuclease-free water.

2.2.2.9 Ligation

After restriction enzyme digestion, digested products were resolved on agarose gels, and desired DNA fragments were extracted from the gel. Otherwise digested DNA fragments were precipitated by Phenol-choloroform-isoamyl alcohol method. Concentration of gel extracted or precipitated fragments were determined using spectrophotometer and ligation reactions were set up using a molar ratio of vector to

insert of 1:3 for sticky end ligations. Ligation mix was incubated either at 22°C for 30 min or 16°C for 14-16 h. After incubation, T_4 DNA ligase was inactivated at 65°C for 20 min.

2.2.2.10 Construction of mutants in *X. oryzae* pv. *oryzae*

To obtain the insertional nonpolar mutant in the *motA* (encodes flagellar motor stator protein) and *fliC* (flagellin) genes, a 321 bp internal fragment of the *motA* gene and a 450 bp internal fragment of *fliC* containing the *Eco*RI and *Xba*I site were amplified using respective primer listed in Table 2.2. These fragments were cloned in pk18mob suicide vector, in which the *lacZ* promoter drives the expression of downstream gene (Schäfer et al., 1994; Windgassen et al., 2000), to obtain pRR1 and pRR2, respectively (Table 2.2). The resulting plasmid (pRR1& pRR2) was introduced into *Xoo* BXO43 strain by electroporation. Single Km^r recombinants were selected on PSA plate containing kanamycin. Insertion of the pK18mob vector in *motA* and *fliC* gene was confirmed with PCR and sequencing. To further confirm the mutation in the flagellar genes, we did swimming motility assay on 0.1% peptone-sucrose agar (PSA). Swimming plate assay indicated that both *motA* and *fliC* mutant of *Xoo* was deficient in motility.

Further, to obtain *motA* and *fliC* insertional knock out mutants in *rpfF* background, we cloned spectinomycin cassette obtained from pUC1318Ω plasmid, into the *Hind*III site of pRR1 and pRR2 plasmid to obtain pRR3 and pRR4. The resulting plasmid (pRR3 & pRR4) were transformed in *rpfF*. Single $spec^r$ recombinants were selected on PSA plate containing kanamycin and spectinomycin. Insertion of the vector was further confirmed by PCR and sequencing.

T2SS *rpfF* double mutant was constructed by transforming the plasmid with *rpfF::Tn7* Kanamycin cassette in the T2SS (*xpsF*) mutant background, and Km^r recombinants were selected on PSA plates containing kanamycin antibiotic and

confirmed through PCR (by using primers SC11 and SC10) and squencing. Double mutant was complemented for DSF production by cloning whole *rpfF* gene of *Xoo*, cloned in *Hind*III and *Eco*RI sites of pHM1 (a broad host range vector for *Xanthomonas*) to get pSC6 plasmid. The resultant pSC6 plasmid was introduced into double mutant by electroporation.

2.2.2.11 Construction of mutants in *X. oryzae* pv. *oryzicola* and rescue of the mutation

Two fragments, each approximately 300 bp in length corresponding to 5'and 3' end of the *rpfF* gene were amplified using genomic DNA of *Xoc* wild-type strain BXOR1, and cloned in pBSKS vector to obtain pRR7 (Table S1 and S5). pRR8 was obtained after ligation of Km^r cassette (EZ::Tn5TM<Kan-2>; Madison, WI) in the *Hind*III site of pRR7. The resulting plasmid (pRR8) was introduced into *Xoc* BXOR1 strain by electroporation. Double recombinants (Km^r and Ap^s) were screened on PSA plates containing appropriate antibiotics. Deletion of *rpfF* (76 amino acids) in the Δ*rpfF* mutant strain was confirmed by PCR and sequencing. For complementation analysis, full length *rpfF* gene was amplified from genomic DNA of *Xoc* Wild-type BXOR1 strain with *Hind*III and *Eco*RI restriction sites and cloned into stable broad host range vector pHM1 (Hopkins et al., 1992) downstream to *lacZ* promoter to obtain pSC9. The pSC9 plasmid harboring the wild-type *rpfF* allele was introduced into Δ*rpfF* mutant strain by electroporation.

To obtain the insertional nonpolar mutant in the *xssA* (xanthomonas siderophore synthesis A), a 321 bp internal fragment of the *xssA* gene containing the *Xba*I and *Hind*III sites was cloned in pK18mob suicide vector, in which the *lacZ* promoter drives the expression of downstream gene (Schäfer et al., 1994; Windgassen et al., 2000) to obtain pRR12. The resulting plasmid (pRR12) was introduced into *Xoc* BXOR1 strain by

electroporation. Single Km^r recombinants were selected on PSA plate containing kanamycin. Insertion of the pK18mob vector in *xssA* gene was confirmed with PCR and sequencing. To further confirm the mutation in the siderophore biosynthetic gene, we did siderophore production assay on Peptone-sucrose agar (PSA)-chrome azurol sulfonate (CAS) (Schwyn and Neilands, 1987). PSA-CAS plate assay indicated that the *xssA* mutnat of *Xoc* was deficient in production of secreted siderophore.

Deletion of the chromosomal *rpfG, rpfC* and *clp* gene of the *X. oryzae* pv. *oryzicola* was accomplished by allelic exchange, following homologous recombination, utilizing the suicide vector pK18mobsacB harboring 5' region and 3' regions of the gene of interest (Katzen et al., 1999). 5' and 3' regions of *rpfG* and *rpfC* and *clp* gene were first amplified from the BXOR1 by PCR using primers indicated in Table no. 2.2 and products were ligated together. After restriction digestion of ligated PCR products and the pK18mobsacB vector with appropriate restriction enzymes, they were ligated to get the plasmids pRR9, pRR10 and pRR11, respectively. These plasmids were then transformed into *E. coli* DH5α cells. The transformed *E. coli* cells were selected on the LB agar plates containing nalidixic acid and kanamycin. The positive colonies carrying vector with correct inserts were further selected by colony PCR. These donor cells carrying pRR9, pRR10 and pRR11 containing 5' and 3' regions of the gene of interest were then transformed into electrocompetent BXOR1 wild-type cells. First crossover (single crossover) was achieved by culturing the cell mixture on Nutrient agar (NA) containing rifampicin and kanamycin, after transformation. The second crossover was allowed by passaging the cells with single crossover in nutrient broth medium and then selecting on PSA plates containing rifampicin and 5% sucrose. BXOR1 with deletion of the *rpfG, rpfC* and *clp* genes by double crossover was identified by PCR using primers

(SCR65/ SCR66, SCR63/ SCR64 and SCR61/SCR62, respectively) designed from the neighbourhood region of the deleted gene.

Replacement of Δ*rpfF* deletion mutant with the point mutant allele (E141A and E161A: Glutamate to Alanine) (*rpfF**) was carried out by transforming *Xoc* Δ*rpfF* mutant with pbsks suicide vector harbouring full length *rpfF** allele. The DNA fragment carrying the *rpfF** allele was constructed by overlap PCR as described previously (Ionescu et al., 2013) using two 21 and 28 bp complementary primers for E141A-F/R and E161A-F/R, respectively; harbouring GAA to GCA substitution (Table 2.2). The mutated *rpfF** allele was amplified by using the end primers only (SC14 and SC17) and cloned into pbsks vector with *Hind*III and *Xho*I restriction sites. The resulting suicide vector (pRR16 and pRR17) was transformed into Δ*rpfF* mutant and single recombinants were selected on PSA medium with kanamycin and ampicillin. Colonies were screened for integration of *rpfF** (E141A or E161A) allele through homologous recombination with the flanking region of deleted *rpfF* allele.

2.2.2.12 Construction of *xsuA*::*gusA* and *xsuA*::*gfp* strains in *X. oryzae* pv. *oryzicola* background

Glucuronidase (GUS) reporter gene fusion and GFP reporter fusion was created by using the suicide plasmid pVO155 having a promoterless *gusA* gene (Oke and Long, 1999), and pProbeGT having a promoterless GFP (Miller et al., 2000). To construct the *xsuA::gusA* and *xsuA::gfp* transcriptional fusion, a 611-bp DNA fragment containing the putative promoter of the *xss* operon (+213 to −398) was amplified by using the SCRsid_ pProbeGFP_F and SCRsid_ pProbeGFP_R primers (Table 2.2). This promoter fragment was subsequently digested with *Hind*III and *BamH*I, and directionally cloned upstream of the promoterless *gusA* and *gfp* gene in pVO155 and pProbeGT plasmids to create the *xsuA::gusA* and *xsuA*::pProbeGT(*gfp*) reporter constructs pRR14 and pRR15,

respectively. The resulting constructs pRR14 and pRR15 were transferred in *E.coli* DH5α. Through triparental mating using pRK600 helper plasmid the construct were transferred in *E.coli* S17-1. After confirming pRR14 and pRR15 constructs by sequencing, the constructs were then introduced into BXOR1 strain through biparental mating using *E. coli* S17-1. *X. oryzae* pv. *oryzicola* GUS and GFP reporter strains were selected on PS medium plates containing suitable antibiotics. Since pVO155 cannot replicate in *X. oryzae* pv. oryzae, ampicillin and kanamycin-resistant colonies were obtained upon chromosomal integration of the plasmid using the cloned DNA sequence as a region of homology. pProbeGT can replicate independently in *Xanthomonas* and report for the gene expression.

2.2.3 Other methods

2.2.3.1 Extracellular enzyme assays

For extracellular enzyme assays, *X. oryzae* pv. *oryzae* strains were grown in PS, MM9 and XOM2 media to an OD of 0.6, and centrifuged at 12,000 g for 10 min to collect the supernatant. The supernatant was taken as an extracellular fraction and cell pellet was plated by dilution plating to get the CFUs per milliliter of culture. Extracellular cellulase activity was measured using phenol-sulphuric acid (H_2SO_4) method, which measures pentoses and hexoses (concentration of glucose released) upon cellulase activity (DuBois et al., 1956). Briefly, a specific amount of supernatant was taken and volume was adjusted to 300 µl by adding 50 mM acetate buffer (pH-5.4). To this, 1% carboxy methyl cellulose (CMC) substrate solution was added and mixed well. This mixture was incubated at 28°C for 30 min, and the reaction was stopped by adding 1 ml ice-cold ethanol. Solution was mixed well, kept on ice for 5 min and centrifuged at 12,000 g for 5 min. Supernatant was recovered and 5% phenol was added to it, mixed well followed by adding 1 ml H_2SO_4. The tube was incubated at RT for 20 min for color

development. Absorbance was measured at 490 nm, and concentration of glucose production was calculated against glucose standard. Cellulase activity is expressed as micromoles of reducing sugar (glucose) released per minute per 10^9 cells. For plate assay, cell-free culture supernatant of *X. oryzae* pv. *oryzae* strains were inoculated in wells of 0.2% CMC agarose plates. In addition, cellulase assay was also performed by spotting the colony on 0.2% CMC PSA plates. Plates were incubated for 8 to 24 h and stained with congo red to observe the halo formation as described previously (Wood and Bhat, 1988). Extracellular xylanase activity in different *X. oryzae* pv. *oryzae* strains was measured using 0.2% 4-O-methyl-D-glucurono-D-xylanremazol Brilliant Blue R (RBB-Xylan) (Sigma-Aldrich) as substrate (Biely et al., 1988) on 1% agarose plates. Xylanase activity is indicated by production of halo around the bacterial colony (Ray et al., 2000). Similarly, for lipase activity p-nitrophenyl butyrate was used as substrate. Lipase activity was calculated by measuring the level of p-nitrophenol released upon hydrolysis of p-nitrophenyl butyrate at 410 nm (Acharya and Rao, 2002). Lipase activity was expressed as micromoles of p-nitrophenol released per min per 10^9 cells. For plate assay, colonies were spotted on 1% PSA plates containing 0.5% Tributyrin in 100 mM Tris (pH 8) and 25 mM $CaCl_2$ and halo formation was observed for lipase activity.

2.2.3.2 Protein extraction and immunoblotting

For protein extraction, *Xanthomonas oryzae* pv. *oryzae* strains with eGFP plasmid were grown for 24-30 h in PS medium to an OD of 0.8 as described above and centrifuged at 12,000 g for 10 min. The supernatant was taken as extracellular fraction and protein was extracted as described previously (Ray et al., 2000). Extracellular proteins were precipitated from this fraction by constantly adding 50% (wt/vol) ammonium sulphate at 4°C. After precipitation, the solution was kept on ice for 15-20 min and centrifuged at 12,000 g for 30 min at 4°C. The pellet was dissolved in suitable

volume of 50 mM acetate buffer (pH-5.4), and dialyzed overnight with 10 mM Tris buffer, pH 7.5. Pellet was used for dilution plating for calculating CFUs. For whole cell protein isolation, bacterial pellet was dissolved in 50 mM sodium acetate buffer (pH-5.4) and sonicated for 30 min (1 min on and off, Amplitude 32) by adding phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM in ice-cold solution. Both extracellular proteins and whole cell lysate fractions were aliquoted in 1.5 ml microcentrifuge tube, and protein quantification was performed using a Pierce BCA protein assay kit (Thermo Scientific) as per manufacturer's instructions using bovine serum albumin as standard and stored at -80°C for further use. Cell normalized extracellular and whole cell lysate proteins fractions from different strains were resolved on 12% SDS-PAGE gel at 90 V till the dye front reached the bottom. One gel was processed for silver staining (Sambrook et al., 1989), and other for western-blot analysis by using anti-GFP antibody. For western blot analysis, resolved proteins were transferred to Hybond-ECL membrane (Amersham biosciences) at 35 V for overnight in the cold room. Transfer of the proteins were visually confirmed by examining marker's lane and membranes were incubated in small box for 2-3 h in 5% fat free milk prepared in 1X PBST for blocking. Blocking solutions were discarded, and primary antibody, appropriately diluted in 5% fat free milk prepared in 1X PBST, was added to the box containing membrane. After 2-3 h incubation in primary antibody, membranes were washed thrice with 1X PBST for 10 min. Membranes were incubated for 2 h in appropriate secondary antibody (anti-Rabbit antibody) diluted in 5% fat free milk prepared in 1X PBST. Blots were either developed by chemiluminescence based ECL-plus western detection system or alkaline phosphatase method. For HRP based chemiluminescence method, detection was performed using the ECL plus kit (Amersham biosciences) and incubated for 3 min. Blot was exposed to the film and developed in

developer solution for appropriate time and immediately kept in fixer solution to see the protein band. For alkaline phosphatase method, blot was incubated with 5 ml of BCIP/NBT solution (Amresco) under dark condition. After incubation, blot was washed with water to see the blue-violet color protein band.

2.2.3.3 Motility assay

For determining the motility of *Xanthomonas oryzae* pv. *oryzae* strains, swim plate assay was performed as described previously (Robleto et al., 2003; Tremaroli et al., 2010) with slight modifications. Briefly, swim plates were prepared with PSA medium containing 0.1% agar. For motility assay, cells were grown at a density of 10^9 cells, which corresponds to an OD of 0.6. Cells were concentrated by centrifugation at 3000 g for 5 min, washed and resuspended in 1/10 volume of sterile water. 5 µl cell suspension was inoculated at the center of the swim plates and incubated for 36-48 h at 28°C. To get the quantitative measurement of the motility of each strain, diameter of the motility zone was determined at appropriate time point.

2.2.3.4 Isolation and detection of DSF

For DSF extraction, *X. oryzae* pv. *oryzicola* strains were grown in PS media to an OD_{600} of 1.2 as described earlier. Supernatant was collected by pelleting down the cells at 7000 g for 10 min. Next, water-saturated ethyl acetate was added to the cell-free culture supernatant in a ratio of 2:1, and mixed properly for 5-10 min. The mixture was centrifuged at 5000 g to separate the DSF containing organic phase. The ethyl acetate layer (organic phase) was evaporated at 37°C, remaining residue was dissolved in methanol, and assayed for DSF by using *Xcc* biosensor strain *8523/KLN55* (Newman et al., 2004). Biosensor strain is a DSF minus strain comprised of DSF responsive endoglucanase promoter fused to promoterless *gfp* and expressed through plasmid (P_{eng}::*gfp*). To check the DSF production by a particular strain, 0.2% inoculum of the

biosensor strain *8523/KLN55* was inoculated in fresh medium, and grown with the ethyl acetate extract isolated from the test strain as described earlier. After 30 h of growth, cells were pelleted by centrifugation, washed once with sterile water and resuspended in sterile miliQ water for measuring the GFP fluorescence intensity at excitation and emission wavelength of 472 and 512 nm, respectively. 1 DSF unit is equivalent to increase in fluorescence by 1 arbitary unit in DSF biosensor strain.

2.2.3.5 Virulence assay on rice plant

To study the virulence of *Xanthomonas oryzae* pv. *oryzicola* strains on rice plant two different inoculation methods, syringe infiltration and wound inoculation methods, were implimented. For infiltration method, bacterial suspension comprising of 1×10^8 cells/ml were infiltrated with needleless syringe into leaves of 4 to 6 week-old rice cultivar of susceptible Taichung Native-1 (TN-1) (Hopkins et al., 1992; Wang et al., 2007). Wound inoculation method was carried out by dropping an aliquot of 20 µl bacterial suspension comprised of 1×10^8 cells/ml onto fully expanded leaf of 6-8 week green-house grown Taichung Native-1 cultivar of rice, and pricking with sterile needle for facilitating the entry of *Xoc* inside the leaves throgh wound. For inititation of disease symptom, the inoculated plants were incubated in greenhouse with minimum and maximum temperatures of approximately 25 to 30 °C, respectively, and a relative humidity of approximately 60%. Water soaking symptom and lesion development was measured 4 to 10 days after inoculation. Likewise, for infiltration by wound inoculation method, lesion length was measured 14 days after inoculation. In both the cases, no lesions were observed in control experiments in which the leaves were inoculated with sterile water.

2.2.3.6 *In planta* bacterial growth assay

In planta growth assay for different strains of *Xanthomonas oryzae* pv. *oryzicola* was performed by counting CFUs. For getting the CFUs, 1 cm^2 leaf area surrounding the site of inoculation was cut and surface sterilized by dipping the leaf in 1% (vol/vol) sodium hypochlorite for 2 min followed by three washes with sterile water. To get the CFUs, sterilized leaves were crushed using mortar and pestle, and diluted appropriately for plating on PSA plate containing suitable antibiotics for differentially marked strains.

2.2.3.7 Static biofilm and attachment assay

For biofilm and attachment assays, *Xanthomonas oryzae* cells were grown in PS media with appropriate antibiotics at 28°C with constant shaking at 200 rpm. 0.2% of the overnight grown culture was inoculated into the fresh PS media and grown till the OD reached 0.6-0.7 at 600 nm. 4 ml culture was inoculated into 12 well polystyrene culture plates, and incubated for 24 h and 48 h at 28°C without shaking. After 24 h, cultures were discarded, and wells were washed with 4 ml of water to remove loosely attached cells. The adherence was examined by staining the cells with 1% crystal violet solution for 30 min at room temperature. After incubation, excess crystal violet stain was removed by washing the wells with 3 ml water. Images were captured for visualizing the stained biofilm on polystyrene plate. Finally, crystal violet stained biofilm was dissolved in 80% ethanol, and quantified by taking OD at 560 nm. Similar procedures were repeated for the polystyrene plate with culture incubated for 48 h. For attachment, cells were grown similarly in 12 well polystyrene culture plates for 24 h, rinsed once with sterile water to remove loosely attached cells then attached cells were collected by vigorous washing with sterile water. Attached cells were diluted, and plated to get the CFUs.

2.2.3.8 EPS isolation and estimation

For EPS isolation, *X. oryzae* pv. *oryzicola* strains were plated on PS agar plate and incubated at 28°C. Bacterial lawn was dissolved in 15 ml 1X PBS and 100 µl formamide, and centrifuged at 12,000 g for 6-8 min at RT. Before centrifugation, 1 ml cell suspension was diluted, and plated to get the CFUs. For EPS precipitation, 250 ml chilled acetone was added to the supernatant, and kept at 4°C for overnight (Dharmapuri and Sonti, 1999). EPS was pelleted down at 7000 g for 10 min at 4°C, washed with 10 ml acetone, and kept for drying. After drying, it was dissolved in appropriate volume of water, and quantitated by colorimetric method for estimation of pentoses and hexoses by phenol-sulphuric acid method (Dharmapuri and Sonti, 1999).

2.2.3.9 Streptonigrin sensitivtity assay

For streptonigrin sensitivity assay, different strains of *Xanthomonas oryzae* pv. *oryzicola* were grown overnight with appropriate antibiotics as described earlier. 0.2% of primary inoculum was added into fresh PS medium and grown for 24 h till the OD_{600} reached 0.6. Serial dilution of bacterial cultures were performed as mentioned earlier, and 5 µl diluted cultures were spotted on PSA plates containing different concentration of streptonigrin (0.05 µg/ml, 0.1 µg/ml and 0.15 µg/ml). Plates were incubated at 28ºC for 72 h and plate images were captured and analyzed for comparative growth inhibition in different strains caused by streptonigrin.

Further, streptonigrin sensitivity assay in liquid broth was performed by growing different strains as described previously (Wilson et al., 1998). Briefly, *Xanthomonas oryzae* pv. *oryzicola* strains were grown to an OD of 1 in PS medium with appropriate antibiotics. Cells were pelleted down, and resuspended in fresh PS medium at an OD_{600} of 0.6. Next, 100 µl culture was inoculated in 4 ml PS medium with or without streptonigrin. Streptonigrin was added to a final concentration of 0.1µg/ml into 12 well

culture dishes and dishes were incubated at 28ºC. OD_{600} was measured after 16 and 42 h of incubation, and percentage inhibition of growth was determined with respect to the growth in the corresponding control cultures containing PS media without streptonigrin.

2.2.3.10 Estimation of intracellular iron content

Intracellular iron content in different *Xanthomonas oryzae* pv. *oryzicola* strains was measured by using atomic absorption spectroscopy as described previously with few modifications (Velayudhan et al., 2000). For estimation of intracellular iron, different strains of *Xanthomonas oryzae* pv. *oryzicola* were grown overnight in 3 ml PS media with appropriate antibiotics for differentially marked strains. 0.2% of the overnight grown culture was inoculated in 250 ml PS medium alone or PS plus 2, 2'-dipyridyl for iron stravation, and grown to an OD_{600} of 1.2. Cells were then pelleted down by centrifuging at 7000 g for 10 min, and washed twice with phosphate buffer saline (PBS). After washing, cells were lyophilized, and their dry weights were determined. Lyophilized cells were then dissolved in 30% nitric acid at 80ºC for 12 h and diluted 10-fold with miliQ water. Iron content was determined by atomic absorption spectroscopy using ICP-OES (JY 2000 sequential Inductively Coupled Plasma Optical Emisson spectrometer, Jobin Yvon, Horiba, France). Iron level was quantified against aqueous standard of iron traceable to NIST (National institute of standards and technology, India).

2.2.3.11 ^{55}Fe uptake assay

In vitro transport assay was performed by using radiolabelled iron to measure the capacity of *Xanthomonas oryzae* pv. *oryzicola* strains to transport ^{55}Fe(II) and ^{55}Fe(III) forms of iron as described previously with slight modifications (Ardon et al., 1997; Velayudhan et al., 2000). For iron uptake asssay, *Xoc* wild-type BXOR1 strain, Δ*rpfF* mutant and the complemented strain harboring full length *rpfF* gene were grown overnight in PS medium. 0.2% of the overnight grown culture was inoculated in fresh PS

media containing 50 µM 2'2'-dipyridyl and grown for 24 h at 28°C with continuous shaking at 200 rpm. Cells were harvested by centrifugation at 7000 g for 10 min at 4 °C, washed twice with 50 mM phosphate buffer (pH-7.4), and finally resuspended in phosphate buffer. The bacterial suspension was then diluted with chelex-100 treated PS to get a final OD_{600} of 1.0 and incubated at 28°C for 5 min. Iron transport assay was initated by adding $^{55}FeCl_3$ (American radiolabeled chemicals, Inc., St. Louis, USA, specific activity 10.18 mci/mg) to a final concentration of 0.4 µM into the bacterial suspension. The radiolabelled stock solution was diluted with water and 1M sodium ascorbate for $^{55}Fe^{3+}$ uptake and $^{55}Fe^{2+}$ uptake studies, respectively. For uptake of $FeCl_3$ bound vibrioferrin, both vibrioferrin (7.6 mM stock) and $^{55}FeCl_3$ were incubated in 1:1 ratio by diluting it appropriately with water and uptake was initiated with a final concentartion of 0.4 µM. To stop the uptake, 200 µl of bacterial cell suspension was layered and immediately centifuged (13000 g; 1 min) through 300 µl of di-butyl phthalate and di-octyl phthalate (1:1) mixture. The upper aqueous layer and organic solvent was aspirated, and pellet was resuspended in 100 µl Triton-X-100. The suspension was added to 5 ml scintillation cocktail, and radioactivity count was determined in the ^{3}H channel of scintillation counter (Perkin Elmer, Liquid Scintillation analyzer, Tri- Carb 2910 TR, USA). As control, both Fe^{2+} and Fe^{3+} uptake assays were performed in presence of proton motive force uncoupler carbonylcyanidep-trifluoromethoxyphenylhydrazone (FCCP; 50 µM), to distinguish between non-specific uptake of readiolabelled Fe by the bacterial cells. However, no significant increase in the incorporation of Fe^{2+} and Fe^{3+} was observed in presence of FCCP, which indicated that iron uptake by these strains is energy-dependent process.

2.2.3.12 Assay for ferric reductase activity

Ferric-iron-reduction activity of *Xanthomonas oryzae* pv. *oryzicola* was measured using ferrozine, a chromogenic ferrous iron chelator, as described previously (Velayudhan et al., 2000; Worst et al., 1998). For estimating the ferric reductase activity, *Xanthomonas oryzae* pv. *oryzicola* strains were grown in 20 ml PS medium carrying appropriate antibiotics for 24 h to OD_{600} of 1. Cell free PS media was incubated under similar condition to be used as control. Chromogenic ferrous iron chelator, ferrozine was added to a final concentration of 1 mM, and $FeCl_3$ was added as ferric iron source to a final concentration of 50 µM, and incubated at 28ºC. At regular time intervals, 1 ml aliquotes were taken from the test culture and control, centrifuged to remove the cells, and absorbance of the magenta coloured Fe^{2+}-ferrozine complex in the cell free culture supernantant was measured at 535 nm by using control supernatant as reference. The Fe^{2+} reduction activity was quantified as micromoles of Fe^{2+}- Ferrozine complex formed.

2.2.3.13 Exogenous iron supplementation and bacterial growth assay in rice leaves

Exogenous iron supplementation was performed as described previously (Chatterjee and Sonti, 2002). Briefly, leaves of 40-day-old greenhouse-grown rice plants of the susceptible rice cultivar Taichung Native-1 (TN-1) were cut with scissors 2 cm above the junction of the leaf blade and leaf sheath. These cut leaves (25 leaves per flasks) were dipped in 250 ml conical flasks containing 200 ml 1µg/ml of Benzyl amino purine (BAP) in double distilled water. BAP (a cytokine hormone) maintain the detached rice leaves in fresh condition for longer period. For iron supplementation, $FeCl_3$ was added to a final concentration of 50 µM (stock-10 mM). Prior to inoculation with different strains of *Xanthomonas oryzae* pv. *oryzicola*, the leaves were maintained overnight on a laboartory bench top. Strains were inoculated into the leaves by needle pricking method by dropping 20 µl of bacterial suspension (approx. 1×10^8 bacterial

CFUs/ml) onto fully expanded leaf, and pricking with sterile needle to facilitate the entry of bacteria inside the leaves through wound. To detrmine the growth of bacteria inside leaves, 1 cm^2 leaf area surrounding the inoculation site was cut at regular time intervals, surface sterilized by dipping in 2% (vol/vol) sodium hypochlorite for 2 min, and washed twice in sterile water. For getting the CFUs, leaves were crushed using mortar and pestle, serially diluted, and plated on PSA medium containing appropriate antibiotics.

2.2.3.14 Oxalic acid estimation from culture supernatant of different strains of *X. oryzae* pv. *oryzicola* by HPLC and GCMS analysis

Xanthomonas oryzae pv. *oryzicola* strains were grown overnight in PS medium supplemented with appropriate antibiotics. 0.2 % of the overnight grown culture was reinoculated in 250 ml of fresh PS medium supplemented with 50 µM 2,2'-dipyridyl, and allowed to grow till OD_{600} reached 1. Cultures were centrifuged to obtain cell free culture supernatant, concentrated on vaccum evaporator, and freeze dried at regular time intervals to remove the water completely. Oxalic acid was estimated from the dried supernatant by using Agilent 1100 series HPLC system as described previously with slight modifications (Ding et al., 2006). In brief, dried supernatant fractions of different cultures were dissolved in mobile phase of pH 2.7, and allowed to stand for 3 h for the precipitation of humic substances. These samples were filtered through membrane filter (porosity, 0.45 µm), and 20 µl volume of the filtrate was injected into the Agilent C_{18} (4.6 mm× 250 mm× 5 µm) column. The mobile phase used was 10 mM KH_2PO_4-CH_3OH (95:5, pH 2.7), and the samples were separated by isocratic elution at 0.8 mL/min at 26°C temperature. Standard oxalic acid was detected in similar way in mobile phase (pH 2.7 at 210 nm) with retention time (RT) of 6.7 min. Likewise, oxalic acid in the test samples were also detected at 210 nm with RT 6.7 min. The oxalic acid

concentarion in the samples were determined based on their peak area against standard oxalic acid plot.

For GC-MS analysis, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization was performed with the dried HPLC fraction of samples as well as standards as described previously (Šťávová et al., 2011). Briefly, 200 µl BSTFA, and 100 µl hexane were added to the sample, and incubated at 50 °C for 70 min. GC analyses were performed using a Shimazdu GP 2010 plus instrument equipped with an autosampler, and a split injector. Separations were accomplished using a 30-m long DB-5 capillary column, 0.25 mm internal diameter (I.D.) at a constant helium flow rate of 1.5 mL/min. Samples (10 µL) were injected with a split ratio of 10 into the column at 100 °C. The final column temperature program started at 100 °C and attained final temperature 280°C with a gradient increase of 5 °C/min. The MS data (total ion chromatogram,TIC) was acquired in the full scan mode (m/z of 50–500) at a scan rate of 1000 amu using the electron ionization (EI) with an electron energy of 70 eV. The acquired spectrum was searched against standard NIST-05 library.

2.2.3.15 HPLC based siderophore estimation from culture supernatant of different strains of X. *oryzae* pv. *oryzicola*

Different *Xanthomonas oryzae* pv. *oryzicola* strains were grown overnight in PS medium at 28 °C and 200 rpm. 0.2% of the overnight grown culture was inoculated in the the fresh PS medium supplemented with 50 µM 2, 2'-dipyridyl, and grown till OD_{600} reached to 1. Cultures were centrifuged at 12,000 g for 50 min to get the cell free culture supernantant, which was collected into acid treated bottles. Excess exoplysaccharide was removed by centrifugation for longer time. Siderophore was initially isolated by column chromatography as described previously (Wright, 2010). Briefly, 220 g of XAD-16 resin was soaked overnight and packed into the column (2.4×30 cm), column was washed with

2 bed volumes of methanol, and equilibrated with 5 bed volumes of distilled water. In order to reduce the water solubility of siderophore in the supernatant, it was acidified to pH 2 using concentarted HCl. This acidified supernatant was passsed through the column, and finally eluted with 160 ml methanol by collecting approximately 60 fractions (2 ml each) of the flow through. Siderophore assay was done on CAS plate with each collected fraction. Fraction that gave orangish-yellow halo for the siderophore on CAS plate, was combined together, dried in rotary evaporator and finally reconstituted in 1 ml methanol for further quantification using HPLC as described previously (Amin et al., 2009).

For HPLC analysis, siderophore samples were filtered through filter membrane (porosity, 0.45 µ). Next, 10 µl sample was injected into Agilent C18 (4.6mm×250mm×5µm) column (gradient:(A=H_2O/0.1%TFA), (B= CH_3CN/0.1%TFA) 0-30% B in 10 min, 30-45% B in 15 min,45-0%B in 20 min at a flow rate of 1 ml/min). Similarly, standard vibrioferrin (siderophore produced by *Xanthomonas*) was also estimated through HPLC for comparison. Fe(III) bound vibrioferrin complex was prepared by incubating $FeCl_3.6H_2O$ and apo-vibrioferrin for overnight. This complex was detected at 300 nm (RT 10.998 min), whereas apo-vibrioferrin was detected at 220 nm at RT 10.988 min. The siderophore concentration in the samples were determined by peak area and calculated against the standard curves obtained from standard vibrioferrin. The siderophore from the test samples were detected at 300 nm, which confirms that majority of the vibrioferrin isolated from the culture was present in bound form.

2.2.3.16 Reporter assays with β-Glucuronidase (GUS) and green fluorescent protein (GFP)

For reporter assay, GUS and GFP marked *Xanthomonas oryzae* pv. *oryzicola* strains and control strains were grown overnight in PS medium. 0.2 % of overnight

grown culture was inoculated in fresh PS medium with or without 50 µM 2, 2'-dipyridyl and grown at 28°C. At regular time intervals, 1 ml culture was removed to determine OD at 600 nm. Furthermore, for GUS assay, 1 ml culture was centrifuged to obtain the pellet, which was washed once in sterile miliQ water, and resuspended in 250 µl volume of 1 mM MUG (4-methylumbelliferyl β-D-glucuronide) extraction buffer (50 mM sodium dihydrogen phosphate [pH 7.0], 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β-mercaptoethanol), and incubated at 37°C (Jefferson et al., 1987). After appropriate time intervals, 75 µl aliquotes were taken from each reaction mixture, and reaction was terminated by adding 675 µl Na_2CO_3 (0.2 M). Fluorescence was measured against 4-methyl-umbelliferone as the standard at excitation/emission wavelength of 365/455 nm, respectively. Likewise, GFP activity was measured in Varioscan flash (Thermoscientific) at exitation/emission wavelength of 472/512 nm, respectively by taking 200 µl of culture directly.

2.2.3.17 *In planta* GUS expression assay for siderophore cluster

In planta siderophore gene expression was studied by measuring β-glucuronidase activity. GUS marked BXOR1 strain and wild-type BXOR1 (control) were inoculated in the leaves of 14 day old susceptible Taichung Native 1 (TN-1) variety of rice. After 10 days of inoculation, leaves were crushed and dissolved in 1 ml of MUG extraction buffer without adding MUG substrate (4-methylumbelliferyl β-D-glucuronide). Subsequently, 250 µl extraction buffer containing MUG was added, and incubated at 37°C for appropriate time (Jefferson et al., 1987). Next, 75-µl aliquots were taken from each reaction mixture, and the reaction was terminated by the addition of 675 µl Na_2CO_3 (0.2 M). Fluorescence was measured against 4-methyl-umbelliferone (MU; Sigma) as standard at excitation/emission wavelength of 365/455 nm, respectively in Varioscan

flash (Thermoscientific). β-Glucuronidase activity for GUS was expressed as nanomoles of MU produced/minute/10^8 cells.

2.2.3.18 *In planta* hypersensitive response (HR) and callose deposition assay

Four week old tomato S-22 cultivar (acts as non-host for *Xanthomonas oryzae* pv. *oryzicola*) were syringe-infiltrated with a suspension of *Xoc* strains and water control. Plants were incubated in green house for 24 h with minimum and maxium temperature of 26 and 28°C, respectively and relative humidity of 65%. Callose deposition assay was performed as a marker for hypersensitvity response in non host plant as described previously (Hauck et al., 2003). Leaf picture was captured at this stage to observe the HR browning of leaf. For assaying callose deposition by aniline blue staining, infilterated leaves were removed from plant, dipped in lactophenol solution and incubated at 65°C in water bath until the cholorohyll is completely removed. Leaves were rehydrated by washing with 50% ethanol, and finally rinsed with water. For aniline blue staining, leaves were incubated in 0.01% aniline blue solution, prepared in 100 mM K_2HPO_4 (pH 9.5), for 15-20 min in dark. Subsequently, leaves were washed with water and observed for callose deposition in epifluorescence microscope (Stereo, Lumar V7, Zeiss) under UV illumination.