

# Supplementary Materials

**Title:** A functional 4-hydroxybenzoate degradation pathway in the phytopathogen *Xanthomonas campestris* is required for full pathogenicity

**Authors:** Jia-Yuan Wang, Lian Zhou, Bo Chen, Shuang Sun, Wei Zhang, Ming Li, Hongzhi Tang, Bo-Le Jiang, Ji-Liang Tang, Ya-Wen He

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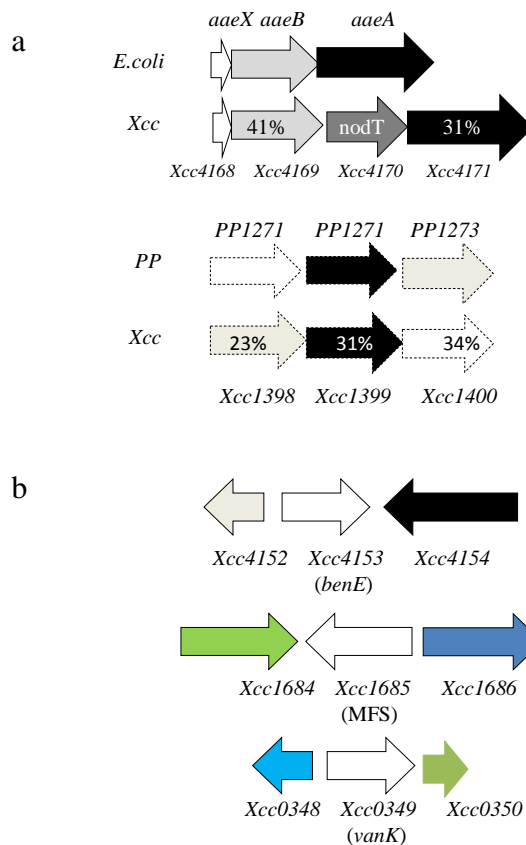
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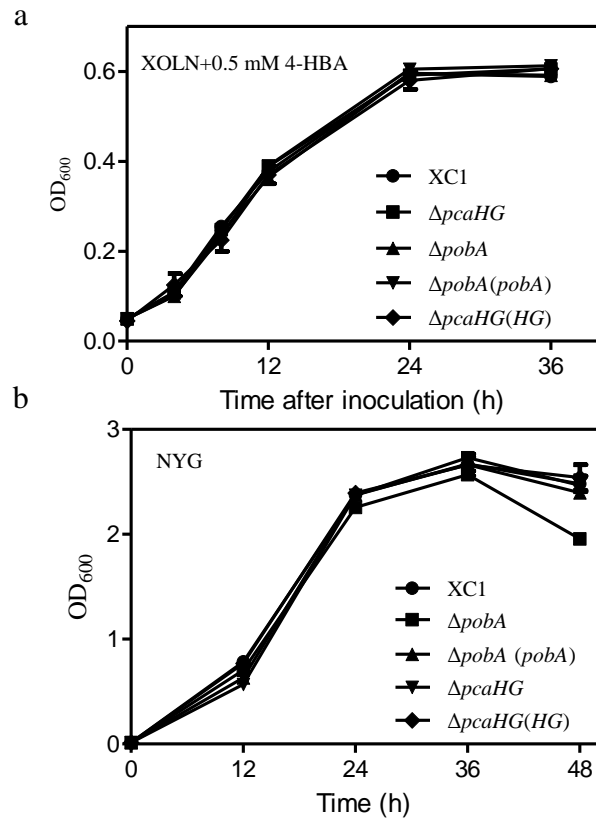
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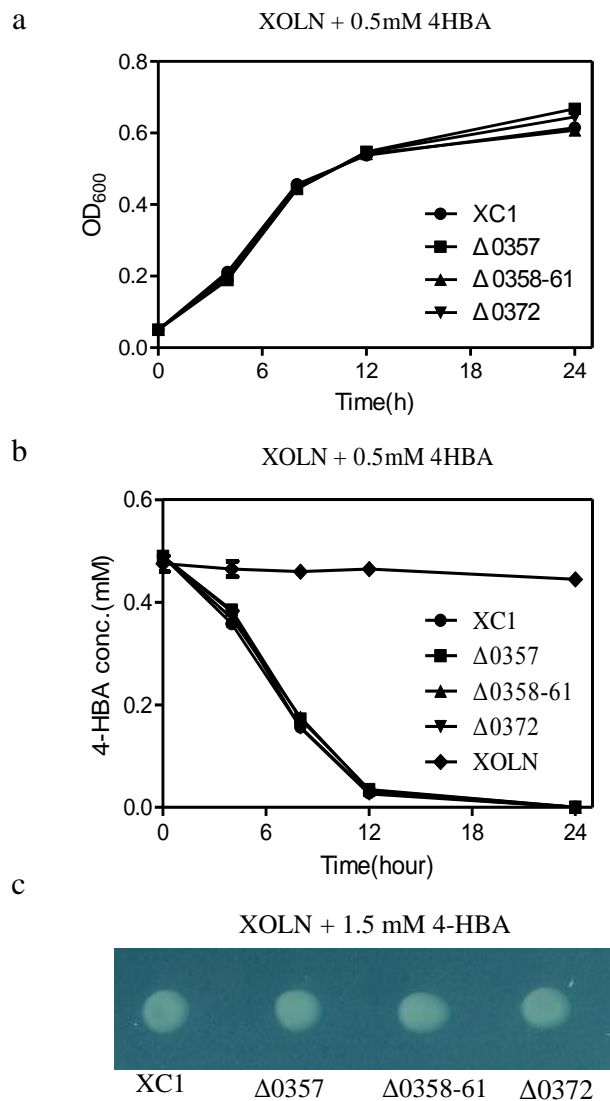
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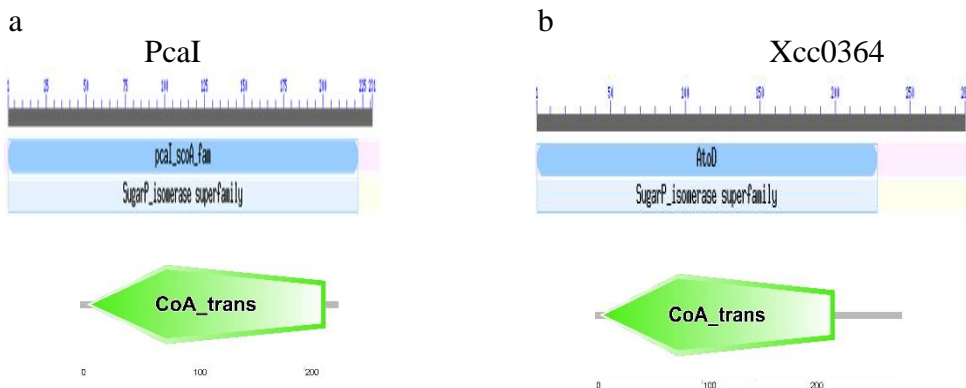
**Supplementary Fig. S1** Putative genes or gene clusters for 4-HBA transportation in *Xanthomonas campestris* pv. *campestris*. (a) Putative gene clusters for 4-HBA efflux pumping; (b) Putative genes for 4-HBA uptake. The percentage indicates amino acid identity. All sequences were downloaded from the Microbial Genome Database of NCBI.



**Supplementary Fig. S2** Growth of *Xcc* strains in (a) XOLN medium or (b) NYG medium supplemented with 0.5 mM 4-HBA.



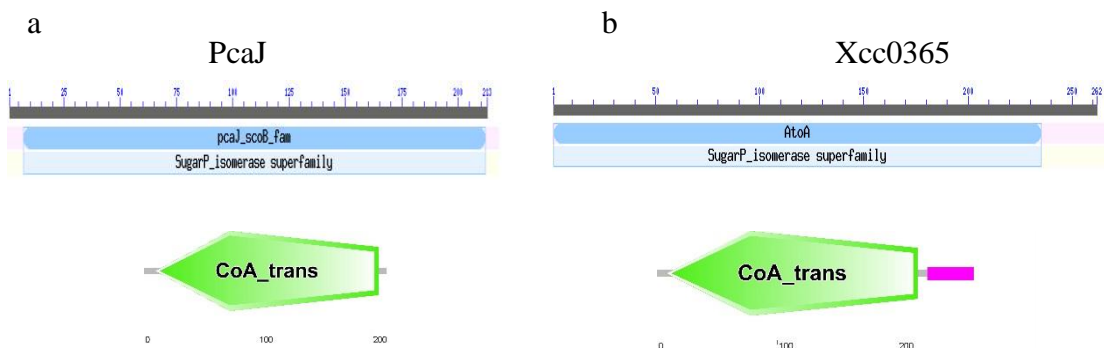
**Supplementary Fig. S3** The genes *Xcc0357*, *Xcc0358-61*, and *Xcc0372* are not required for 4-HBA degradation. (a) The growth of *Xcc* strains as determined at an optical density of 600 nm. (b) The 4-HBA levels in the cell cultures of *Xcc* strains during growth in XOLN medium. (c) Bacterial growth of *Xcc* strains on an XOLN plate supplemented with 1.5 mM 4-HBA.



c

Xcc0364	MA-DIVSLSEAVAQLIADGDCVAMEGFTHL-IPHAAGHEVIRQRKRNLR-----
pcaI	MINKTYESIASAVEGITD <u>G</u> STIMV <u>GGFGT</u> A <u>GMP</u> SELIDGLIATGARDLTIISNNAGNGEI
	* . . :... :*:** . : : ** :* . :* *:*
	Glycine cluster
Xcc0364	-----LVRMTPDLIYDQLIGAGCASALRF <sup>51</sup> SWGGNPGVGS <sup>101</sup> LHRLRDAVE
pcaI	GLAALLMAGSVRKVVC <sup>51</sup> SFPRQSDSYVFDELYRAGKIE-----
	: * : . :*: * ** .
Xcc0364	HGWPAPLQLREHSHADMANAYVAGASGLP-FAVLRGYV-----GSDLPKVNDSIRFLQCP
pcaI	-----LEVVPQGNLAERIRAAGSGIGAFFSPTGYGTL <sup>101</sup> LAEGKETREID-----
	* . :...*: *..** : * ** *.: :..
Xcc0364	YTGQTLATSPAVNPDVTVIHAQQADRRGNVLLWGIL-GVQKEAALAARKVIVTVVEEIVDT
pcaI	--GRMYVLEMP <sup>151</sup> LHAD <sup>151</sup> FALIKAHK <sup>151</sup> GDRWGNLTYRKAARNFGPIMAMA <sup>151</sup> AKTAIAQVDQVVEL
	* : . . :. *.:*:*:*.** ** : . *:*:..* . *::*:
Xcc0364	LDAPPNACILPRWVVS <sup>201</sup> AVCVVPGGAAPS <sup>201</sup> YAHGYSARDNRFYLAWDAMARDRARFQAWIQ <sup>201</sup> A
pcaI	GELDPEHIITPGIFVQ <sup>201</sup> RVAVTGA <sup>201</sup> AASSIAKAV-----
	: * : * * .* . * * .** * * :.
Xcc0364	HILDTDDFAGFQ <sup>251</sup> RVYAGSQHAEAA
pcaI	-----

**Supplementary Fig. S4** Domain organization and multiple sequence alignment of Xcc0364 and PcaI. (a) Domain organization of PcaI in *Pseudomonas putida*. (b) Domain organization of Xcc0364 in *Xcc*. (c) Sequence alignment of PcaI and Xcc0364. Domain organization was conducted using the SMART program (<http://smart.embl-heidelberg.de>). Sequence alignment was conducted using the program Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).



c

pcaJ	MTITKKLSRTEMAQRVAADIQEGAYVNLGIGAPTLVANYL---GDKEVFLH <b>SENG</b> LLGMG
Xcc0365	--MSDYSTNEMMTVAAAARRLPDGAVCFVGIGLPSTAAANLARLTHAPEVTLIYESGPIGAR
	:. .: * : .* : :** :*** *: .** ** * *. * :*
	<b>SENG motif</b>
pcaJ	PSPAPGEEDDDLINAGKQHVTLLTGGAFFHHADS-FSMRGGHLDIAVLGAFQVSVKGDL
Xcc0365	PQVLPLSIGDGELAETAD-----TVVSTPEIFRYWLQGGRVDVGF LGAAQIDRHANL
	*. * . * : : :.. : :***::*..*** *:. :. :*
pcaJ	ANWH----TGAEGSIPAVGGAMDLATGARQVFVMMDHLLTKTGESKLVP-----
Xcc0365	NTTVIGPYAAPKTRLPGAGGAPEIATS AKQVFIIMRQSTRSFVQTLDFITTVGHLDGGDA
	. :. : :*..*** :**.*:***::* : *:: ..*
pcaJ	--ECTYPLTGIACVSRIYTDLAVLEVTPEGLKVV--EICADIDFDELQKLSGVPL-----
Xcc0365	RTRAGLPGAGP---TVVVTDLCVMEPDPVSRELTVTALHPGVTQAQVTAATGWAVRFAAT
	.. * :* : : ***.*:* * . :. : : : : * :
pcaJ	-----IK-----
Xcc0365	LTETAAPTATELHALRALQARTAAA HGTQAAGAEA
	.

**Supplementary Fig. S5** Domain organization and multiple sequence alignment of Xcc0365 and PcaJ. (a) Domain organization of PcaJ in *Pseudomonas putida*. (b) Domain organization of Xcc0365 in *Xcc*. (c) Sequence alignment of PcaJ and Xcc0365. Domain organization was conducted using the SMART program (<http://smart.embl-heidelberg.de>). Sequence alignment was conducted using the program Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

## XCC0364 homologues in Bacteria

a

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Xcc0364  MADIVSLSEAVAQLIADGDCVAMEGFTHLIPHAAGHEVIRQQRKRNRLVRMTPDLIYDQL
SMB20587 MARIMSLAEAVEENVRDGDTVAMEGFTHLIPYAAGHEVIRQGRKDLFLVRMTPDILYDQL
      ** *: **:*** : : *** *****:***** : : * *****: : ****
Xcc0364  IGAGCASALRFSWGNGPGVSLHRLRDAVEHGWPAPLQLREHSHADMANAYVAGASGLPF
SMB20587 IGVGAARGMKFSWGGNPGVSLHRFRDAVENQWPRPLEIEEHSHAAMANAYEAGAGNLPF
      ** . * . * . : : *****:***** . ** * : : . ***** ***** * . * . *
Xcc0364  AVLRGYVGSDDLPKVNDSIRFLQCPYTGQTLATSPAVNPDVTVIHAQQADRRGNVLLWGIL
SMB20587 AAFRGYIGADLPKVNLNIRSVTFCPTGEVLAAPAIRPDVTIIHAQRADRRGNVLIIEGIV
      * . : * * : * : * * * * . * * : * * : * * : * * : * * * * : * * : * * :
Xcc0364  GVQKEAALAARKVIVTVVEEIVDTLDAP-PNACILPRWVVSAVCVVPGGAAPSYAHGYSAR
SMB20587 GVQKEAVLAARRSIVTVVEEIVDELSPPSPNSVVLPGWAVTAVAHVPGGAFPSYAHGYPR
      * * * * * . * * * * : * * * * * * . * * * * : * * * . * : * * . * * * * * *
Xcc0364  DNRFYLAWDAMARDRARFQAWIQAHILDTD--DFAGFQRVYAGSQHAEEAA
SMB20587 SNAFYIRWDEIARDREFTTAWIRENVLAAKPEDFARHAAKTAAKV----A
      . * * * : * * : * * * * * * * : . : * . . * * * . * . . *
    
```

b

Species	Homologues of Xcc0364	Amino acid identity (%)	NCBI access No.
<i>Xac</i>	XAC0364	92	AAM35256.1
<i>Xcr</i>	XCR_4151	99	AEL09008.1
<i>Xff</i>	XFF4834R_chr03 480	92	CDF60017.1
<i>Xoc</i>	XOC_4548	91	AEQ98605.1
<i>Lysobacter capsici</i>	AZ78_14030	79	EYR67734.1
<i>Pseudomonas aeruginosa</i>	PA0226	73	AAG03615.1
<i>Pseudomonas knackmussii</i>	PKB_2952	72	CDF84299.1
<i>Sinorhizobium meliloti</i>	SMB20587	67	CAC49899.1
<i>Mesorhizobium loti</i>	MAFF_RS17350	58	BAB50895.1

**Supplementary Fig. S6** Homologs of Xcc0364 in other bacterial species. (a) Multiple sequence alignment analysis of Xcc0364 and SMB20588. (b) Putative homologs of Xcc0364 in other bacterial species.

a

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Xcc0365  -----MSDYSTNEMMTVAAARRLPDGA VCFVGI GLPSTAANLARLTHAPEVTLIYESG
SMB20588 MLEGKTKMTHFTPTMMTVAAARALSND DVC FVGI GAPS AACNVARLTNAPDITLIYESG
          *:..: .***** * : ***** *:*.*:****.**:*****
Xcc0365  PIGARPQVLP LPSIGDGELAE TADTVVSTPEIFRYWLQGG RVDVGF LGAAQIDRHANLNTT
SMB20588 TVGTPDVLPLS IGDGELCDTALFTVSVPEMFRYWLQGG RITTGFLGGAQIDRFANLNTT
          :*:*:*****.**:** . **.*:*****: .****.*****.*****
Xcc0365  VIGPYAAPKTR LPGA GGAPEIATS AKQVFIIMRQSTRSFVQ TLD FITTVGHLDGGDARTR
SMB20588 VVGPYDHPKVR LPGA GGAPEIASNCGRIFITMALT KRGFVERLPFV TSMGHGEGGNHRER
          *:*** *.****.*****:.. **:** * :.*.**: * *:*:*** **: * *
Xcc0365  AGLPGAGPTVV VTDLCVME PDPVSRELTVTALHPGV TQAQVTAATGWAVRFAATLTETAA
SMB20588 LGLTTS GPTRVITDLC ILEPDPETKELTVVSMHPG VARDQIAGNCGWPIKFAETVIETPA
          ** : *** *:****.:**** :*****.:*****: *:. . ** ::** * : ** *
Xcc0365  PTATELHALRAL QARTAAAHGTQAAGAEA
SMB20588 PTETELVVL RDINARTKKAHKAAGKEAA-
          ** *** .** ::** ** : . *

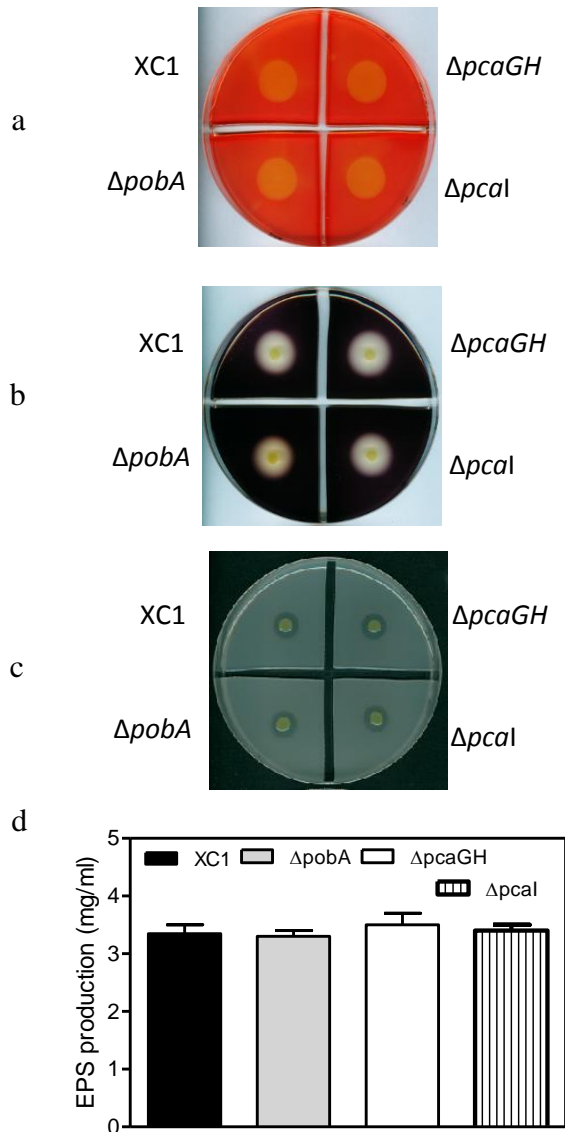
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b

Species	Homologues of Xcc0365	Amino acid identity (%)	NCBI access No.
<i>X. axonopodis</i> pv. <i>citri</i>	XAC0365	89	AAM35257.1
<i>Xcr</i>	XCR_4150	100	AEL09007.1
<i>Xff</i>	XFF4834R_chr03490	89	CDF60018.1
<i>Xoc</i>	XOC_4547	90	AEQ98604.1
<i>Lysobacter capsici</i>	AZ78_14025	76	EYR67733.1
<i>Pseudomonas aeruginosa</i>	PA0227	72	AAG03616.1
<i>Pseudomonas knackmussii</i>	PKB_2951	74	CDF84298.1
<i>Sinorhizobium meliloti</i>	SMB20588	60	CAC49900.1
<i>Mesorhizobium loti</i>	MAFF_RS17345	63	BAB50894.1

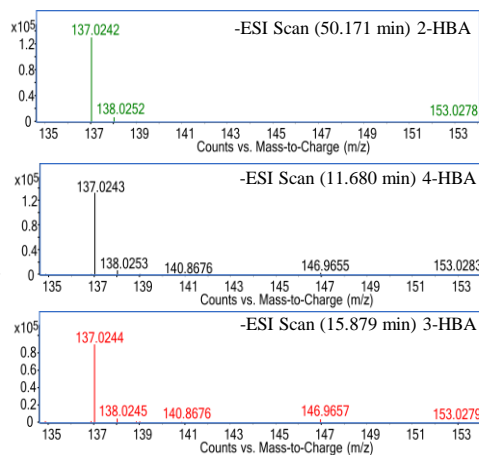
**Supplementary Fig. S7** Homologs of Xcc0365 in other bacterial species. (a) Multiple sequence alignment analysis of Xcc0365 and SMB20588. (b) Putative homologs of Xcc0365 in other bacterial species.



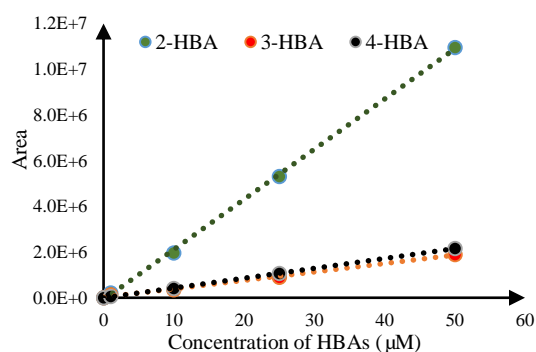


**Supplementary Fig. S8** Production of extracellular enzymes and polysaccharide (EPS) in *Xcc* strains. (a) Cellulase activity. About 1  $\mu$ L of the cell culture ( $OD_{600} \approx 1.0$ ) was spotted onto NYG containing 0.5% CMC. (b) Amylase activity. *Xcc* strains were grown on an NYG plate supplemented with 0.1% soluble starch. (c) Protease activity. The strains were grown on an NYG plate supplemented with 1.0% skim milk. (d) EPS production in NYG medium.

a



b



**Supplementary Fig. S9 LC-MS analysis of radish hydrolysates.** (a) Molecular weights of the putative 2-HBA, 3-HBA and 4-HBA detected in radish hydrolysates. (b) The established standard curves for determining the concentration of 2-HBA, 3-HBA and 4-HBA.

**Supplementary Table S1.** Bacterial strains used in this study

Strains	Relevant characteristics	Reference/source
<b><i>Xcc</i> XC1</b>	Wild type, Rif <sup>R</sup>	Laboratory collection
Δ0355	<i>xcc0355</i> in-frame deletion mutant of XC1	This study
Δ0356	<i>xcc0356</i> in-frame deletion mutant of XC1	This study
Δ0357	<i>xcc0357</i> in-frame deletion mutant of XC1	This study
Δ0358-61	<i>xcc0358</i> , <i>xcc0359</i> , <i>xcc0360</i> , and <i>xcc0361</i> in-frame deletion mutant of XC1	This study
Δ0364	<i>xcc0364</i> in-frame deletion mutant of XC1	This study
Δ0365	<i>xcc0365</i> in-frame deletion mutant of XC1	This study
Δ0364-65	<i>xcc0364</i> and <i>xcc0365</i> in-frame deletion mutant of XC1	This study
Δ0367-68	<i>xcc0367</i> and <i>xcc0368</i> in-frame deletion mutant of XC1	This study
Δ0370	<i>xcc0370</i> in-frame deletion mutant of XC1	This study
Δ0372	<i>xcc0372</i> in-frame deletion mutant of XC1	This study
Δ0373	<i>xcc0373</i> in-frame deletion mutant of XC1	This study
Δ0356C	Δ0356 mutant containing pBBR_0356 which contains full length <i>xcc0356</i>	This study
Δ0364C	Δ0364 mutant containing pBBR_0364 which contains full length <i>xcc0364</i>	This study
Δ0365C	Δ0365 mutant containing pBBR_0365 which contains full length <i>xcc0365</i>	This study
Δ0364( <i>pcaI</i> )	Δ0364 mutant containing pBBR_0364 which contains full length <i>pcaI</i>	This study
Δ0365( <i>pcaJ</i> )	Δ0365 mutant containing pBBR_0365 which contains full length <i>pcaJ</i>	This study
Δ0367-68C	Δ0367-68 mutant containing pBBR_0367-68 which contains full length <i>xcc0367</i> and <i>xcc0368</i>	This study
<b><i>P.putida</i></b>		
KT2440	Wild type	Prof. Ping Xu lab collection
<b><i>E.coli</i></b>		
DH5α	F <sup>+</sup> φ80 <i>lacZ</i> ΔM15 <i>endA</i> 1 <i>hsdR</i> 17 (r <sub>k</sub> <sup>-</sup> m <sub>k</sub> <sup>-</sup> ) <i>supE</i> 44 <i>thi</i> <sup>-1</sup> <i>gyrA</i> 96 Δ( <i>lacZYA-argF</i> )	Laboratory collection
S17-1	<i>recA pro</i> (RP4-2Tet::Mu Kan::Tn7)	Simon et al., 1983

Simon, R., Prierer, U., and Puhler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotech 1:784-791

**Supplementary Table S2.** Plasmids used in this study

Plasmids	Relevant characteristics	Refernce/source
pK18mobSacB	Suicide plasmid in XCC, Km <sup>R</sup>	Schafer et al., 1994
pRK2013	ColE1 replicon TraRK+ Mob+; Helper plasmid for mobilisation of non-self-transmissible plasmids, Km <sup>R</sup>	Figurski and Helinski, 1979
pBBR1MCS2	Plasmid for gene complementation, Km <sup>R</sup>	Kovach et al., 1995
pK18-0355	Sucide construct for in-frame deletion of <i>xcc0355</i>	This study
pK18-0356	Sucide construct for in-frame deletion of <i>xcc0356</i>	This study
pK18-0357	Sucide construct for in-frame deletion of <i>xcc0357</i>	This study
pK18-0358-61	Sucide construct for in-frame deletion of <i>xcc0358</i> , <i>xcc0359</i> , <i>xcc0360</i> , and <i>xcc0361</i>	This study
pK18-0364	Sucide construct for in-frame deletion of <i>xcc0364</i>	This study
pK18-0365	Sucide construct for in-frame deletion of <i>xcc0365</i>	This study
pK18-0364-65	Sucide construct for in-frame deletion of <i>xcc0364</i> and <i>xcc0365</i>	This study
pK18-0367-68	Sucide construct for in-frame deletion of <i>xcc0367</i> and <i>xcc0368</i>	This study
pK18-0370	Sucide construct for in-frame deletion of <i>xcc0370</i>	This study
pK18-0372	Sucide construct for in-frame deletion of <i>xcc0372</i>	This study
pK18-0373	Sucide construct for in-frame deletion of <i>xcc0373</i>	This study
pBBR0356	Complementary vector containing whole length of <i>xcc0356</i>	This study
pBBR0364	Complementary vector containing whole length of <i>xcc0364</i>	This study
pBBR0365	Complementary vector containing whole length of <i>xcc0365</i>	This study
pBBR0367-68	Complementary vector containing whole length of <i>xcc0367</i> and <i>xcc0368</i>	This study
pBBR-pcaI	Complementary vector containing whole length of <i>pcaI</i>	This study
pBBR-pcaJ	Complementary vector containing whole length of <i>pcaJ</i>	This study

Figurski, D.H., and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proceedings of the National Academy of Sciences of the United States of America 76:1648-1652.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and Peterson, K.M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175-176.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene 145:69-73.

**Supplementary Table S3.** Primers used in this study

Application	Primers	Sequence(5'-3')
In-frame deletion		
Δ0355	0355-F1	cccagccaccgaaacggatacacc
	0355-R1	cgctacagccgcagatacattacgc
	0355-F2	tctgcggtgtagcgggtggctgccgacctggatttttc
	0355-R2	caccacagacggcctgttctatgc
Δ0356	0356-F1	ggaattctcggcaaggctggcaaagacaaga
	0356-R1	aacctgggtgcgcatacggagt
	0356-F2	atgcgcaccaggttgcggcggtgtgcagcagtattc
	0356-R2	cgggatcccagcaacaggcgcgggagaac
Δ0357	0357-F1	gcccagccgcagccgttactacct
	0357-R1	cgctcgccgtagacgctttg
	0357-F2	tctagcggcgagcggaccgcgtgatccgtccaaga
	0357-R2	ccgggcatggcgaagaacacctat
Δ0358-61	0361-F1	cgacgcggcgaatgtgggtt
	0361-R1	cgcccatacgccaggttctcc
	0358-F2	ctggcgtatcgggcgcggcaccagcaaggaacct
	0358-R2	ccgcaacccaaccaccagaaa
Δ0364	0364-F1	ggaattccggcctgcgcgtttagacc
	0364-R1	ggcgtgcgggatcaggtgggtaa
	0364-F2	ctgatcccgcacgccgcggaggcggcatgagcgacta
	0364-R2	cgggatcctggactgccgcatgatgatgaagc
Δ0365	0365-F1	ggaattccccggacctgatttacgaccaac
	0365-R1	gcccgatgccgacgaaacacacc
	0365-F2	ttcgtcggcatcggcggagccgaggcatgagcgagg
	0365-R2	cgggatcctccaggcgcggattgatgaagc
Δ0364-65	0364-F1	ggaattccggcctgcgcgtttagacc
	0364-R1	ggcgtgcgggatcaggtgggtaa
	0365-F3	ctgatcccgcacgccgagccgaggcatgagcgagg
	0365-R2	cgggatcctccaggcgcggattgatgaagc
Δ0367-68	0367-F1	cgcgcgggtgctggggtttg
	0367-R1	gcccgatagccgagcaggacat
	0368-F2	ctcggctatcggcgcctcgtgttcacgcgggctgt
	0368-R2	gtggcactggcggcaatgacc
Δ0370	0370-F1	gcggcgtatggtgctgctga
	0370-R1	gcatgtgtcggcaactggagaaa
	0370-F2	ttgccacacatgcacacgctgctgctgggtttctc
	0370-R2	tcgggtcgtatgtctttgttga

Δ0372	0372-F1	gcctgcccgccacactcg
	0372-R1	cggcagccgcgaccacaccac
	0372-F2	tggtcgcggctgccgctgctgctgcgcggtgtggatg
	0372-R2	cctggcggccctgggttatgtg
Δ0373	0373-F1	ggaattcacgccggcgtccaccagttg
	0373-R1	gggacgtttgcggcgggaagag
	0373-F2	cgccgcaaacgtcccctgcgtggcgaggtgattgc
	0373-R2	cgggatcctgttgcggcgtggtgctgtatg
Complementation		
0356	0356-F	cccaagctttcggcaaggctggcaaagacaaga
	0356-R	cgggatcccgcctcggcgtagacgctttgac
0364	0364-F	cccaagcttcggccctgcgcgtttagacc
	0364-R	gctctagagccgatgccgacgaaacacacc
0365	0365-F	cccaagcttatccgttctgcagtgccctac
	0365-R	gctctagactggacagtgcgccgccgtaa
0367-68	0367-68-F	cccaagcttcgcgcgggtgctggggtttg
	0367-68-R	gctctagaccctggcgacctgtgcaacaactac
pcaI	3951-F	ggaattcactgcatcgaaccactggaaageg
	3951-R	cgggatcccgggtgcgggagagcttttgggtga
pcaJ	3952-F	ggaattccaacctggccgagcgtatccg
	3952-R	cgggatcctcgaagcggctgtaaaggtagtctcaag
qRT-PCR		
0364	RT0364-F	cttttctggggcggcaatcct
	RT0364-R	ccatgtccgcgtggctgtgttc
0365	RT0365-1	caccccgagatctttcgtattgg
	RT0365-2	cagatgaccgacggtggtgatgaa
atpD	atpD-F	gcgatggtgtacggccagatgaa
	atpD-R	cggccagcgttagcggtagatgt