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Irp9, Encoded by the High-Pathogenicity Island of *Yersinia enterocolitica*, Is Able To Convert Chorismate into Salicylate, the Precursor of the Siderophore Yersiniabactin

Cosima Pelludat,[1] Daniela Brem,[2] and Jürgen Heesemann[2]*

Institut für Mikrobiologie, D-BIOL, ETHZ, 8092 Zürich, Switzerland,[1] and Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, 80336 Munich, Germany[2]

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The Irp9 protein of *Yersinia enterocolitica* participates in the synthesis of salicylate, the precursor of the siderophore yersiniabactin. In *Pseudomonas* species, salicylate synthesis is mediated by two enzymes: isochorismate synthase and isochorismate pyruvate-lyase. Both enzymes are required for complementation of a Yersinia *irp9* mutant. However, *irp9* is not able to complement *Escherichia coli entC* for the production of enterobactin, which requires isochorismate as a precursor. These results suggest that Irp9 directly converts chorismate into salicylate.

The pathogenicity of *Yersinia pestis* (causing bubonic plague), *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* biogroup 1B (enteropathogens) is determined by a common virulence plasmid (pYV) and a high-pathogenicity island (HPI) which is inserted into the tRNA gene *asnT* (1, 3, 5, 20). The HPI of *Y. enterocolitica* carries two gene clusters, the cluster *irp2*, *irp1*, *irp3*, *irp4*, and *irp5* and the cluster *irp6*, *irp7*, *irp8*, and *irp9* (Fig. 1a), which are involved in the biosynthesis and transport of the siderophore yersiniabactin (Ybt), and the *fyuA* gene, which encodes the outer membrane receptor FyuA for Ybt (17, 20). Targeted disruption of *irp* genes (excluding *irp8*, whose function is unknown) results in an attenuation of virulence (1, 2, 6). Previously, it has been demonstrated that salicylate is the precursor of Ybt biosynthesis and that the *irp9* gene homolog of *Y. pestis ybtS* is required for synthesis of this precursor (11).

The predicted amino acid sequence of Irp9/YbtS demonstrates a close similarity to the sequence of anthranilate synthase component I (TrpE of *Enterobacteriaceae* and $TrpE_2$/MbtI of *Mycobacterium tuberculosis*) and a lower degree of similarity to the sequences of the isochorismate synthases (ICSs) of *Pseudomonas aeruginosa* (PchA), *Pseudomonas fluorescens* (PmsC), and *Escherichia coli* (EntC) (11) (Fig. 1b). The chorismatebinding domain is localized between amino acids 173 and 428 (according to an NCBI conserved domain search). The anthranilate synthase converts chorismate to anthranilate (the amino analog of salicylate) by using glutamine as the nitrogen source. ICSs convert chorismate into isochorismate, which is needed for the synthesis of the siderophores pseudomonine (*P. fluorescens*) (13), pyochelin (*P. aeruginosa*) (22), and enterobactin (*E. coli*) (16) (Fig. 2). The precursor of Ybt, pyochelin, and pseudomonine is salicylate, which can be generated in a second step from isochorismate by isochorismate pyruvate lyases (IPLs) (Fig. 2). *P. aeruginosa* and *P. fluorescens* carry *pchB* and *pmsB*, respectively, which have been shown to encode IPL (9, 13, 22). A similar pathway has been described previously for the salicylate biosynthesis (the precursor of mycobactin) of *M. tuberculosis* (7, 18). On the other hand, it has been suggested that $TrpE_2$/MbtI may directly convert chorismate into salicylate and thus may function like anthranilate synthase (7, 18).

Strikingly, YbtS/Irp9 does not carry related sequences to PchB or PmsB, which suggests that there is an IPL-encoding gene outside of the HPI on the chromosome. However, sequence analysis of the available genome sequence of *Y. pestis* or *Y. enterocolitica* (http://www.sanger.ac.uk/Projects/Y_pestis and http://www.sanger.ac.uk/Projects/Y_enterocolitica) failed to identify a PchB/PmsB homolog. Therefore, the question whether YbtS/Irp9 is a bifunctional enzyme with ICS and IPL activity (two-step salicylate synthesis) or converts chorismate directly into salicylate remains open.

To address this issue, we disrupted the *irp9* gene of *Y. enterocolitica* and introduced *pmsC* and *pmsB* of *P. fluorescens* into the *irp9* mutant for restoration of Ybt biosynthesis. Moreover, for restoration of ICS activity or salicylate production, we introduced *irp9*, *pmsC*, and *pmsB* into an *E. coli entC* mutant. Our results support the assumption that Irp9/YbtS functions as salicylate synthase by converting chorismate directly into salicylate.

Inactivation of *irp9* in *Y. enterocolitica* leads to a chrome azurol S (CAS)-negative phenotype. For a first approach, we disrupted the *irp9* gene of *Y. enterocolitica* O:8 strain WA-CS. A 580-bp *Hpa*I/*Sal*I fragment from cosmid 12H2 (2, 19) (Table 1) harboring a *Sph*I site in gene *irp9* (Fig. 1a) was ligated into the *Eco*RV/*Sal*I site of plasmid pKS (Stratagene). A *Hin*dII-cut kanamycin cassette from plasmid pSB315, which lacks a transcriptional terminator (10), was inserted into the blunted *Sph*I site of *irp9*. The fragment carrying the kanamycin cassette was inserted into suicide vector pKAS32 (24) by means of the

* Corresponding author. Mailing address: Max von Pettenkofer-Institut für Hygiene und Medizinishce Mikrobiologie, Pettenkoferstr. 9a, 80336 Munich, Germany. Phone: 49 89 5160 5200. Fax: 49 89 5160 5202. E-mail: heesemann@m3401.mpk.med.uni-muenchen.de.

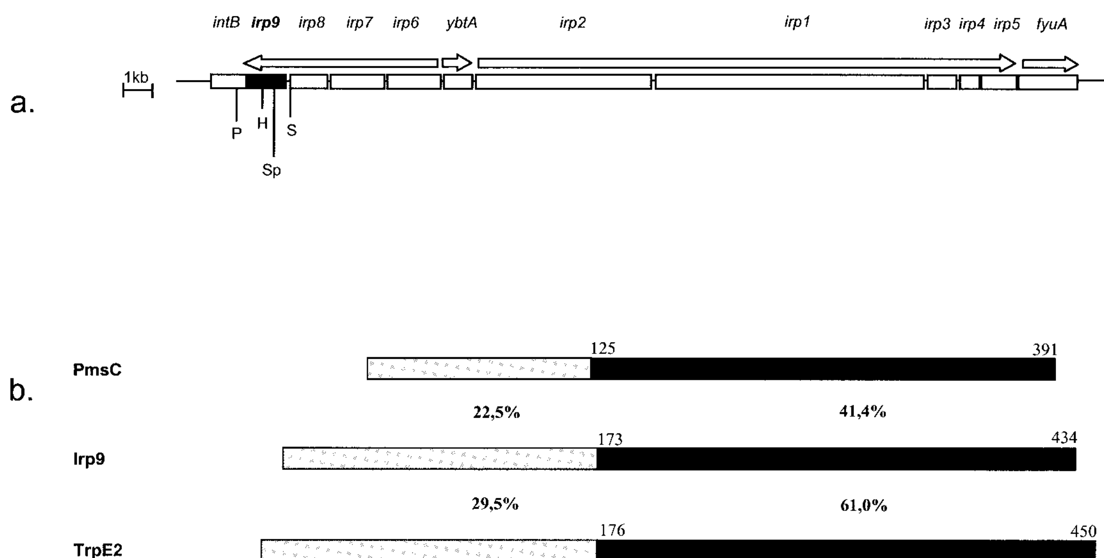

FIG. 1. (a) Structure of the HPI core in *Y. enterocolitica.* Bars indicate genes, and arrows indicate gene clusters and the direction of transcription (20). *intB*, gene for P4-like integrase (see also text); H, *Hin*cII; P, *Pst*I; Sp, *Sph*I; S, *Sal*I. (b) Alignment of chorismate binding proteins PmsC (ICS of *P. fluorescens*), Irp9 (*Y. enterocolitica*), and TrpE2/MtbI (putative anthranilate synthase of *M. tuberculosis*). Black bars indicate the regions of high homology comprising the predicted chorismate binding sites. Similarities for Irp9/PmsC and Irp9/TrpE$_2$ are indicated.

*Kpn*I and *Sac*I sites of the pKS vector. The resulting construct, pSVIrp9, was transformed into S17-1 λ *pir*$^+$ *tra*$^+$ (14, 23) and mobilized into WA-CS. Mutants were selected on agar plates containing kanamycin (40 μg/ml), streptomycin (100 μg/ml), and nalidixin (100 μg/ml), and results were confirmed by Southern blotting.

The resulting mutant strain, WA-CS*irp9*::Kan^r, was tested on CAS agar, a siderophore indicator. The CAS assay relies on the color change from a green-blue CAS-iron complex to orange desferrated CAS around siderophore-producing colonies (producing a CAS halo, or CAS positive) (12, 21). WA-CS*irp9*::Kan^r expressed no CAS halo, indicating the loss of Ybt synthesis. The CAS-positive phenotype was restored after the introduction of pTIrp9 (Table 2). The plasmid harbors the 2.8-kb *Sal*I/*Pst*I fragment of cosmid 12H2 carrying *irp9* downstream of the T7 promoter in the pT7-5 backbone (Table 1). Complementation of WA-CS*irp9*::Kan^r was possible without plasmid pGP1-2 (carrying the T7 polymerase), indicating that there was sufficient expression of the *irp9* gene for Ybt biosynthesis even in the absence of the T7 polymerase.

Salicylate feeding restores Ybt production of an *irp9* mutant. Assuming that Irp9 converts chorismate into salicylate, we examined Ybt production of WA-CS*irp9*::Kan^r after feeding the bacteria with chorismate or salicylate by a Ybt cross-inducer reporter assay and the conventional cross-feeding assay (2, 17). The cross-inducer reporter assay is based on the observation that Ybt-containing culture supernatant is able to induce the expression of the *fyuA* gene in yersinia mutants with disrupted Ybt biosynthesis genes (e.g., *irp1* or *irp9*). Reporter strains WA-CS*irp9*::Kan^{r}G and WA-CS*irp1*::Kan^{r}G carrying plasmid pCJFY5G3 (translational fusion between a *fyuA* promoter and green fluorescent protein [GFP] reporter gene *gfp*) (Table 1) show enhanced GFP fluorescence under iron limitation in response to Ybt-containing culture supernatants of

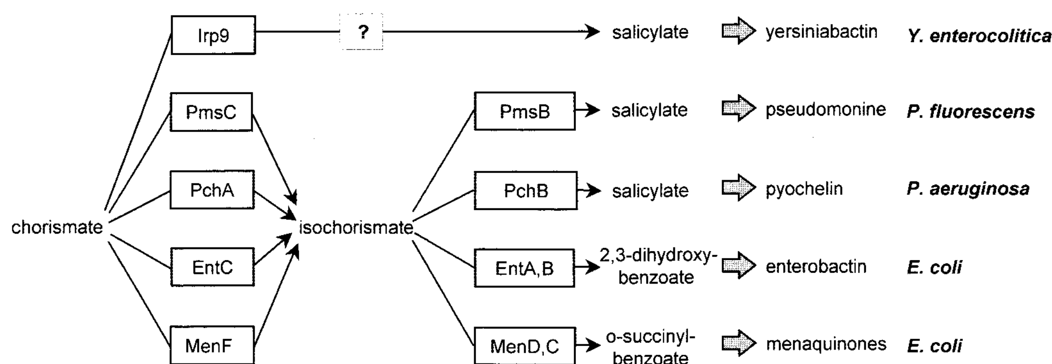

FIG. 2. Pathways of siderophore and menaquinone synthesis in *Y. enterocolitica*, *E. coli*, *P. fluorescens*, and *P. aeruginosa.* MenF (*E. coli*), EntC (*E. coli*), PmsC (*P. fluorescens*) and PchA (*P. aeruginosa*) are ICSs which convert chorismate into salicylate. In *E. coli*, isochorismate is converted into *o*-succinylbenzoate by MenD and MenC and into 2,3-dihydroxybenzoate by EntA and EntB. *Pseudomonas* converts isochorismate into salicylate by PmsB/PchB. Irp9 shows similarities to ICSs but shows a higher degree of similarity to anthranilate synthase (see Fig. 1b).

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype and/or phenotype	Reference or source
Y. enterocolitica		
WA-C	Derivative of *Y. enterocolitica*, BG1B, serotype O:8, WA-314, pYV plasmid cured; Nal^r	12
WA-CS	Sm resistant derivative of WA-C; Nal^r Sm^r	17
WA-CS*irp1*::Kan^r	*irp1* mutant of WA-CS, Ybt deficient strain; Sm^r Kan^r Nal^r	17
WA-CS*irp9*::Kan^r	WA-CS carrying a kanamycin cassette without transcriptional terminator in gene *irp9*; Sm^r Kan^r Nal^r	This study
WA-CSG	WA-CS carrying reporter construct pCJFY5G3; Nal^r Cm^r	This study
WA-CS*irp1*::Kan^{r}G	WA-CS*irp1*::Kan^r carrying reporter construct pCJFY5G3; Sm^r Kan^r Nal^r Cm^r	This study
WA-CS*irp9*::Kan^{r}G	WA-CS*irp9*::Kan^r carrying reporter construct pCJFY5G3; Sm^r Kan^r Nal^r Cm^r	This study
E. coli		
S17-1 λ*pir*	*pir*$^+$ *tra*$^+$	14, 23
PBB7	*entC* mutant, enterobactin deficient, CAS-negative phenotype	15
BL21(DE3)	Enterobactin positive carrying IPTG-inducible T7 polymerase gene	Stratagene
Plasmids		
pSB315	Containing kanamycin cassette without transcriptional terminator; Amp^r Kan^r	10
12H2	pLAFR2 carrying a DNA insert of WA-C comprising *irp6–9* operon, *ybtA*, and *irp2*; Tc^r	2, 19
pKAS32	Suicide vector with *rpsL* gene; Amp^r	24
pSVIrp9	pKAS32 carrying a *Sal*I/*Hpa*I DNA-fragment of the HPI with, a kanamycin cassette (without transcriptional terminator) integrated into the *Sph*I site of *irp9*; Amp^r Kan^r	This study
pGP1-2	Vector containing heat-inducible T7 polymerase; Kan^r	25
pT7-5	Cloning vector; Amp^r	25
pTIrp9	pT7-5 vector carrying *irp9* (*Sal*I/*Pst*I fragment of cosmid 12H2) upstream of T7 promoter; Amp^r	This study
pCJFY5G3	Reporter construct, pACYC184 carrying 153 bp of *fyuA* promoter region fused to *gfp*:Cm^r	2
pE3R	pGEM-3Z carrying the *pmsCEAB* operon of *P. fluorescens* WCS374; Amp^r	13
pE3	pGEM-3Z carrying the T7 promoter upstream of the *pmsCEAB* operon of *P. fluorescens* WCS374; Amp^r	13
pPmsCB	pGEM-3Z carrying *pmsC* and *pmsB* genes (*Apa*I/*Sac*II fragment of pE3R) of *P. fluorescens* WCS374; Amp^r	This study
pPmsC	pGEM-3Z carrying *pmsC* gene (*Apa*I/*Nde*I fragment of pE3R) of *P. fluorescens* WCS374; Amp^r	This study
pPmsB	pGEM-3Z carrying *pmsB* gene (*Stu*I/*Sac*II fragment of pE3R) of *P. fluorescens* WCS374; Amp^r	This study
pUC57	Cloning vector; Amp^r	MBI Fermentas
pUCEntC	pUC57 carrying *entC* of *E. coli*; Amp^r	This study

overnight cultures of tester strains. Fluorescence intensity was determined by cytofluorometry (fluorescence-activated cell sorting) on a single-cell level for 12,000 bacteria after 24 h of growth at 28°C in stoppered vessels (2). The culture supernatants of WA-CS*irp9*::Kan^r grown overnight in (i) iron-limited nutrient broth (NBD; negative control), (ii) NBD plus chorismate, (iii) NBD plus salicylate, and (iv) the supernatant of WA-CS grown in NBD (positive control) were examined for the presence of Ybt. The supernatants were collected, sterile filtered, mixed with fresh NBD medium (1:3, vol/vol), and

TABLE 2. Ybt production of WA-CS*irp9*::Kan^r carrying different plasmids as determined by CAS agar or a cross-inducer reporter assay

Strain	Plasmid	CAS phenotype	Fluorescence intensity of reporter strain WA-CS*irp1*::Kan^{r}G[a]
WA-CS		+	42 ± 14
WA-CS*irp9*::Kan^r		−	6.5 ± 0.8
	pTIrp9	+	ND[b]
	pPmsCB	+	37 ± 7
	pPmsC	−	8 ± 1
	pPmsB	−	25 ± 5

[a] Arbitrary units (au) of fluorescence intensities and standard deviations of three experiments. Note that NBD induces 5.5 ± 0.5 au (background level).
[b] ND, not determined.

inoculated with overnight cultures of the indicated reporter strains in nutrient broth (1:50, vol/vol).

As expected, the supernatant of WA-CS*irp9*::Kan^r cultured in NBD plus salicylate was nearly as efficient as the WA-CS supernatant in inducing the *fyuA-gfp* reporter gene of the *irp9* and *irp1* mutants, indicating the presence of Ybt (Fig. 3). The supernatant collected from an *irp9* mutant grown in NBD or NBD plus chorismate showed no significant Ybt-inducing effect.

A cross-feeding assay confirmed the results of cross-induction (results not shown). Filter tips soaked with 12 μl of NBD containing 3 μM purified Ybt, 300 μM chorismate, 300 μM salicylate, or tester culture supernatants (see above) were placed on the agar layer. For the indicator strain, we used WA-CS*irp1*::Kan^r, which grows only poorly in iron-limited CDM-H (chemically defined medium with Hefe [0.25% yeast extract] and containing 40 μM EDDHA [ethylenediamine di-*o*-hydroxy-phenylacetic acid], an iron chelator) (8). The indicator strain showed no growth in the presence of salicylate or chorismate, unlike in the presence of Ybt. Tips soaked with sterile filtered supernatants of the *irp9* mutant grown in (i) NBD, (ii) NBD containing chorismate, and (iii) NBD containing salicylate revealed growth of the seeded indicator strain only around the tip that had been soaked with the supernatant of WA-CS*irp9*::Kan^r grown in NBD with the addition of salic-

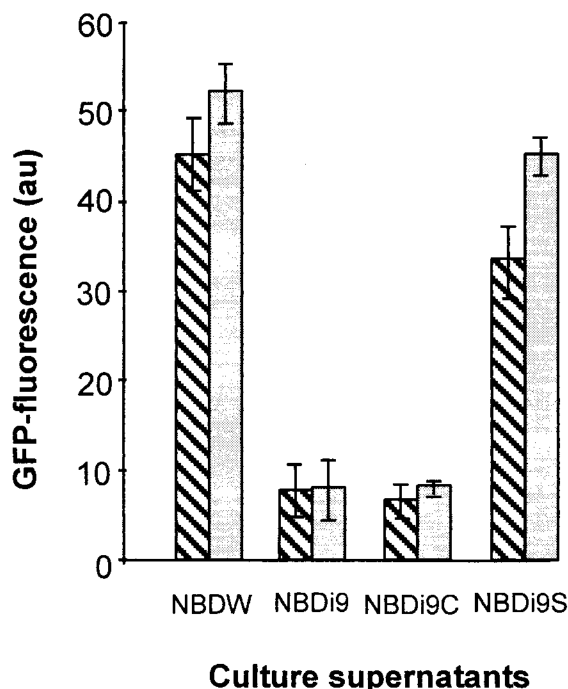

FIG. 3. Ybt cross-inducer assay performed with two reporter strains. Reporter strains (WA-CS*irp1*::Kan^{r}G [hatched bars] and WA-CS*irp9*::Kan^{r}G [shaded bars]) were grown in NBD medium containing (i) sterile filtered supernatant of WA-CS grown in NBD (NBDW), (ii) WA-CS*irp9*::Kan^r grown in NBD (NBDi9), (iii) WA-CS*irp9*::Kan^r grown in NBD plus 300 μM chorismate (NBDi9C), and (iv) WA-CS*irp9*::Kan^r grown in NBD plus 300 μM salicylate (NBDi9S). Bars represent GFP fluorescence of the strains. Standard deviations (for three experiments) are indicated by error bars. au, arbitrary units.

ylate; this result indicates that Ybt synthesis of the *irp9* mutant is salicylate dependent, as has been demonstrated with the cross-inducer assay.

In summary, these results clearly demonstrate that *irp9* inactivation may be complemented by the addition of salicylate in the culture medium, which confirms that Irp9 is involved in salicylate synthesis.

Both *pmsC* and *pmsB* are required for complementation of WA-CS*irp9*::Kan^r. Irp9 has sequences homologous to the sequences of ICSs (Fig. 1b), but neither *irp9* nor any other gene of the HPI encodes a protein with significant homology to PchB or PmsB, the IPLs of the *Pseudomonas* species. This raises the question whether Irp9 functions as ICS in cooperation with an IPL-encoding gene unrelated to *pchB* or *pmsB* elsewhere on the chromosome. As a first step, we subcloned the genes *pmsC* and *pmsB* as *Apa*I/*Sac*II fragments of pE3R (carrying the *pmsCEAB* operon of *P. fluorescens*) (Fig. 4). Blunt ends were generated with T4 DNA polymerase (MBI Fermentas) and religated. The resulting plasmid, pPmsCB, restored the CAS-positive phenotype of WA-CS*irp9*::Kan^r (Table 2). In contrast, WA-CS*irp9*::Kan^r carrying only *pmsC* (pPmsC, cut with *Apa*I/*Nde*I) or *pmsB* (pPmsB, cut with *Stu*I/*Sac*II) did not produce a halo on CAS agar. As CAS agar might not be sensitive enough for detection of low Ybt concentrations, we analyzed the NBD supernatants of WA-CS*irp9*::Kan^r carrying plasmid pPmsCB, pPmsB, or pPmsC for the presence of Ybt by using the WA-CS*irp1*:Kan^{r}G reporter strain (Ybt cross-inducer assay). As expected, a strong induction of the *fyuA-gfp* reporter gene was detected after the addition of the supernatant from WA-CS*irp9*::Kan^r(pPmsCB) cultivated in NBD medium (which was comparable to the supernatant of the parental strain WA-CS) (Table 2). However, a significant induction of *fyuA-gfp* was also detected after the addition of the culture supernatant from WA-CS*irp9*::Kan^r(pPmsB), although plasmid pPmsB expressing an IPL cannot restore the CAS-positive phenotype of the *irp9* mutant. These results indicate that both the *pmsC* and *pmsB* genes are necessary for the restoration of the CAS-positive phenotype of the *irp9* mutant but that *pmsB* is sufficient to partially restore Ybt production under liquid culture conditions (in stoppered vessels).

These apparently contrary results suggest that growth in liquid culture results in a level of isochorismate production sufficient for conversion by PmsB to salicylate and subsequently to Ybt. Is there a plausible explanation for this? First, we have to consider that yersiniae grow aerobically on CAS agar but microaerobically in the stoppered liquid culture vessels used in the cross-inducer assay. Second, for *E. coli* it is known that about 2% of the isochorismate produced by MenF (an isochorismate synthase of the menaquinone pathway, expressed predominantly under anaerobic conditions) (Fig. 2)

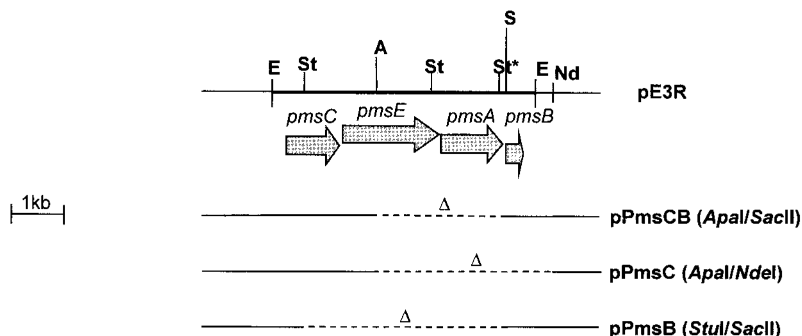

FIG. 4. Plasmid pE3R carrying the *pmsCEAB* operon of *P. fluorescens*. Plasmid pE3R was cut with different restriction enzymes and religated. E, *Eco*RI; A, *Apa*I; S, *Sac*II; St, *Stu*I; St*, methylated *Stu*I cutting site. Arrows indicate genes and the direction of transcription. Dashed lines denote generated deletions. The resulting plasmids were designated pPmsCB (cut with *Apa*I/*Sac*II, harboring *pmsCB*), pPmsC (cut with *Apa*I/*Nde*I, harboring *pmsC*), and pPmsB (cut with *Stu*I/*Sac*II, harboring *pmsB*).

TABLE 3. Salicylate synthesis and enterobactin production (CAS agar) of strain *E. coli* BL21(DE3) and *E. coli entC* mutant PBB7(pGP1-2) carrying different plasmids

Strain	Plasmid	Salicylate concn (mean ± SD) (μM)[a]	CAS phenotype
BL21 (DE3)	pT7-5	0	+
	pPmsB	114 ± 24	ND[b]
	pTIrp9	439 ± 81	ND
PBB7(pGP1–2)	pT7-5	0	−
	pE3	34 ± 7	ND
	pPmsC	ND	+
	pUCEntC	ND	+
	pTIrp9	57 ± 13	−

[a] Results are means for three experiments.
[b] ND, not determined.

can channel into the enterobactin pathway (4, 26). As *Y. enterocolitica* harbors a gene encoding a protein with 66% similarity to MenF of *E. coli* (http://www.sanger.ac.uk/Projects/Y_enterocolitica), it is very likely that a sufficient level of isochorismate is produced by WA-CS*irp9*::Kan^r(pPmsB) in liquid culture and is then converted to salicylate by PmsB and subsequently to Ybt.

***irp9* leads to salicylate synthesis in an *E. coli entC* mutant.** To differentiate between one-step and two-step conversion of chorismate into salicylate by Irp9, we introduced *pmsC*, pmsCEAB of *P. fluorescens*, irp9 of *Y. enterocolitica*, and, as a control, *entC of E. coli* into the *E. coli entC* mutant PBB7. The *entC* gene and its promoter region were amplified by using primers EntC70 (AATCCGTCCCCTCGCCTTTG) and EntC1637 (TGCGTCAGAATGTCGGTCAG). The PCR product was subcloned into vector pUC57 (MBI Fermentas), yielding pUCEntC (Table 1). As a first step, the resulting transformants were tested for enterobactin synthesis on CAS agar (Table 3). As expected, introduction of *entC* (pUCEntC) restored siderophore production (CAS positive) in PBB7. Transfer of pPmsC led also to a CAS-positive phenotype of *E. coli entC*, indicating that *entC* and *pmsC* are interchangeable. In contrast, transfer of pTIrp9 did not restore siderophore production in PBB7 even though pGP1-2 (carrying the T7 polymerase) was present and induced (30 min at 42°C). Obviously, Irp9 does not produce isochorismate as an available intermediate for enterobactin biosynthesis in the *E. coli entC* mutant.

To verify Irp9-mediated salicylate synthesis in *E. coli* strains, the plasmids pT7-5 (negative control), pE3 or pPmsB (positive control), and pTIrp9 were transferred into PBB7 (pGP1-2) and into the enterobactin-positive *E. coli* strain BL21(DE3), as indicated in Table 3. After induction at an optical density of 0.5 [PBB7 (pGP1-2) at 42°C for 30 min and BL21(DE3) in 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside)], strains were grown for 15 h at 37°C. Salicylate was detected as described by Mercado-Blanco et al. (13). The purple iron-salicylate complex in the aqueous phase was quantified photometrically at 527 nm. PBB7 and BL21(DE3) produced detectable amounts of salicylate when harboring pTIrp9 (Table 3). Similar results were obtained with PBB7 carrying pE3 and BL21(DE3) carrying pPmsB. Thus, *E. coli* becomes a salicylate producer after receiving the corresponding *Yersinia* or *Pseudomonas* genes.

In conclusion, these results demonstrate that *Yersinia* Irp9 functions as a salicylate synthase by the conversion of chorismate into salicylate (probably in one step), in contrast to *Pseudomonas* species, which require the two enzymes ICS and IPL.

Considering the high degree of similarity between Irp9 and $TrpE_2$/MbtI and that both corresponding genes are located within the gene cluster of yersiniabactin and mycobactin biosynthesis, respectively, we suggest that Irp9 and $TrpE_2$/MbtI function similarly to anthranilate synthase component I by channelling a hydroxyl residue to the active site for salicylate production instead of an amino residue for anthranilate production.

Daniela Brem and Cosima Pelludat contributed equally to this work.

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