A siderophore biosynthesis gene cluster from the fish pathogen *Photobacterium damsela* subsp. *piscicida* is structurally and functionally related to the *Yersinia* high-pathogenicity island

Carlos R. Osorio, Sandra Juiz-Río and Manuel L. Lemos

Correspondence
Manuel L. Lemos
mlemos@usc.es

Department of Microbiology and Parasitology, Institute of Aquaculture and Faculty of Biology, University of Santiago de Compostela, Santiago de Compostela 15782, Galicia, Spain

Photobacterium damsela subsp. *piscicida*, the causative agent of fish pasteurellosis, produces a siderophore which is distinct from that produced by *P. damsela* subsp. *damsela*. Using suppression subtractive hybridization, a subsp. *piscicida*-specific DNA region of 35 kb was identified in strain DI21, and 11 genes were defined: *dahP*, *araC1*, *araC2*, *frpA*, *irp8*, *irp2*, *irp1*, *irp3*, *irp4*, *irp9* and *irp5*. The sequence of the predicted proteins encoded by these genes showed significant similarity with the proteins responsible for the synthesis and transport of the siderophore yersiniabactin, encoded within the *Yersinia* high-pathogenicity island (HPI). Southern hybridization demonstrated that this gene cluster is exclusive to some European subsp. *piscicida* isolates. Database searches revealed that a similar gene cluster is present in *Photobacterium profundum* SS9 and *Vibrio cholerae* RC385. An *irp1* gene (encoding a putative non-ribosomal peptide synthetase) insertional mutant (CS31) was impaired for growth under iron-limiting conditions and unable to produce siderophores, and showed an approximately 100-fold decrease in degree of virulence for fish. The subsp. *piscicida* DI21 strain, but not CS31, promoted the growth of a *Yersinia enterocolitica* *irp1* mutant. Furthermore, a yersiniabactin-producing *Y. enterocolitica* strain as well as purified yersiniabactin were able to cross-feed strains DI21 and CS31, suggesting that the subsp. *piscicida* siderophore might be functionally and structurally related to yersiniabactin. The differential occurrence among *P. damsela* strains, and the low sequence similarity to siderophore synthesis genes described in other members of the *Vibrionaceae*, suggest that this genetic system might have been acquired by horizontal transfer in *P. damsela* subsp. *piscicida*, and might have a common evolutionary origin with the *Yersinia* HPI.

Received 7 June 2006
Revised 26 July 2006
Accepted 3 August 2006

INTRODUCTION

Iron is an essential element for most bacteria; however, most of the iron in biological systems is chelated by high-affinity iron-binding proteins. Therefore, pathogens have developed efficient mechanisms to obtain iron from the host and to establish an infection (Ratledge & Dover, 2000). The ability to obtain iron from free haem or haem proteins from the host tissues constitutes an important source of iron for many bacterial pathogens (Genco & Dixon, 2001; Osorio & Lemos, 2002). However, one of the main strategies to obtain iron is the synthesis and secretion of Fe(III)

chelators, named siderophores, which are low molecular mass iron-chelating molecules that can remove iron from host iron-binding proteins (Crosa, 1989) and then enter the cell through outer-membrane receptor proteins. Many siderophores are small peptides synthesized by non-ribosomal peptide synthetases (NRPSs), which are multi-modular enzymes that produce peptide products of a particular sequence without an RNA template (Crosa & Walsh, 2002; Schwarzer *et al.*, 2003).

Iron acquisition has been widely recognized as a determinant of bacterial virulence (Braun, 2005). Thus, it is not surprising that several iron-transport systems have been described as part of horizontally transmitted elements, showing differential occurrence in pathogenic vs non-pathogenic strains. The siderophore aerobactin genes are found on plasmids in certain strains of *Escherichia coli* and *Salmonella* (Williams, 1979; Colonna *et al.*, 1985), but have been described to constitute a chromosomal pathogenicity

Abbreviations: A domain, adenylation domain; ArCP, aryl-carrier protein; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; HPI, high-pathogenicity island; NRPS, non-ribosomal peptide synthetase; SSH, suppression subtractive hybridization.

The GenBank/EMBL/DDBJ accession number for the sequences reported in this paper is AJ699306.

island in *Shigella flexneri* strains (Vokes *et al.*, 1999). Similarly, highly pathogenic strains of *Yersinia* (*Yersinia enterocolitica* 1B, *Yersinia pseudotuberculosis* I and *Yersinia pestis*) harbour a large chromosomal fragment termed the high-pathogenicity island (HPI), which is absent from low-pathogenic and avirulent strains (Carniel *et al.*, 1996). The *Yersinia* HPI carries a cluster of genes involved in the biosynthesis, transport and regulation of the siderophore yersiniabactin and is integrated in the chromosomal *asn-tRNA* gene (Buchrieser *et al.*, 1998). It consists of a 30.5 kb highly conserved functional core region and a 5–13 kb AT-rich variable part (Rakin *et al.*, 1999). The core region comprises genes for the biosynthesis (*irp1–irp5* and *irp9*) and uptake (*irp6–irp8* and *fyuA*) of the siderophore, as well as an AraC-like regulator (*ybtA*). An integrase gene (*intB*) is found at the upstream end of the HPI. Specific studies conducted with mutant strains have demonstrated the importance of the yersiniabactin system in the virulence of *Y. enterocolitica* (Rakin *et al.*, 1994), *Y. pestis* (Bearden *et al.*, 1997) and *Y. pseudotuberculosis* (Carniel *et al.*, 1992). Although first identified in *Yersinia* spp., the HPI has been also described in several genera of *Enterobacteriaceae* (Schubert *et al.*, 1998; Bach *et al.*, 2000; Olsson *et al.*, 2003).

Photobacterium damsela subsp. *piscicida* was initially isolated from a massive fish mortality in Chesapeake Bay, and the disease was named pasteurellosis after the classification of this agent as *Pasteurella piscicida* (Janssen & Sargalla, 1968). The pathogen was later reassigned to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida*, sharing species epithet with *P. damsela* subsp. *damsela* (formerly *Vibrio damsela*) (Gauthier *et al.*, 1995) and thus constituting a new member of the family *Vibrionaceae*. *P. damsela* subsp. *piscicida* is one of the most devastating bacterial pathogens in marine aquaculture, due to its wide geographical distribution, host range and massive mortality (Magariños *et al.*, 1996a). The virulence of this micro-organism is believed to be a multifactorial process not yet fully understood. The major virulence factor so far identified is a plasmid-encoded protein, AIP56, which induces apoptosis in fish macrophages and neutrophils (do Vale *et al.*, 2005). It is also believed that polysaccharide capsular material constitutes a main virulence factor (Magariños *et al.*, 1996b). This bacterium is able to obtain iron from haemin and haemoglobin as unique iron sources *in vitro* (Magariños *et al.*, 1994), and a genetic system for haem uptake has been recently described (Juiz-Río *et al.*, 2005a). A gene encoding the ferric uptake regulator (Fur) protein has been identified in this species, and it is believed to act as an iron-dependent transcriptional repressor regulating genes involved in iron uptake (Juiz-Río *et al.*, 2004; Osorio *et al.*, 2004). It was reported that strains of this subspecies can efficiently obtain iron from human transferrin, and this is achieved by a mechanism involving the production of siderophores (Magariños *et al.*, 1994). However, the nature of this siderophore is still unknown and the genes involved in its biosynthesis have not been characterized, although preliminary chemical assays and

bioassays indicated that it is neither a catechol nor a hydroxamate (Magariños *et al.*, 1994).

This study was undertaken to obtain an insight into the genetic basis of the siderophore-mediated iron-uptake system of *P. damsela* subsp. *piscicida*, to study its role in virulence for fish and to assess if genetic differences exist among *P. damsela* subspecies and strains.

METHODS

Bacteria, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. *P. damsela* subsp. *piscicida* and *P. damsela* subsp. *damsela* strains were routinely grown at 25 °C in either Tryptic Soy Agar or Tryptic Soy Broth (Difco) supplemented with 1 % NaCl (TSA-1 and TSB-1, respectively), and in CM9 minimal medium (Mouriño *et al.*, 2004). *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) broth or LB agar, as well as in CM9 minimal medium, supplemented with antibiotics when appropriate. *Y. enterocolitica* strains were grown at 37 °C in LB, Nutrient Broth (NB) and Nutrient Agar (NBA). All strains were stored frozen at –80 °C in LB broth with 20 % glycerol. Antibiotics were used at the following final concentrations: kanamycin (Kan) at 25 µg ml^{–1}; ampicillin sodium salt (Amp) at 50 µg ml^{–1}; chloramphenicol (Cam) at 20 µg ml^{–1}; rifampicin (Rif) at 50 µg ml^{–1}. All stocks were filter-sterilized and stored at –20 °C. The iron chelator 2,2'-dipyridyl (Sigma) was prepared at 10 mM in water.

Suppression subtractive hybridization (SSH). Bacterial genome subtraction was performed using the PCR-Select Bacterial Genome Subtraction Kit (Clontech), as previously described (Juiz-Río *et al.*, 2005b). In brief, tester (DI21) and driver strain (RM71) genomic DNA (2 µg) were each digested with 10 units *RsaI* for 5 h. The tester DNA was aliquoted into two tubes, and each was ligated to a different adaptor provided with the kit. Two hybridization rounds were carried out: in the first hybridization, an excess of driver DNA was added to each adaptor-ligated tester DNA, and samples were then heat denatured and allowed to anneal. In the second hybridization round, the two samples from the first hybridization were mixed together without denaturation. The product of this last hybridization was then used as a template in a PCR reaction to amplify the tester-specific sequences, using Advantage cDNA polymerase mix (Clontech). The obtained PCR products were cloned into the pGEM-T Easy vector (Promega) and recombinant clones were screened by restriction analysis prior to DNA sequencing.

Recombinant DNA techniques, DNA sequencing and data analysis. Recombinant DNA methods were performed following standard protocols (Sambrook & Russell, 2001). Chromosomal DNA was isolated using the Easy-DNA kit (Invitrogen). Plasmid purification and elution of DNA fragments from agarose gels were performed using kits from Qiagen. Southern blot analysis was performed with Hybond-N+ membranes (Amersham Biosciences), using the ECL Direct Nucleic Acid labelling and detection system (Amersham Biosciences) and following the manufacturer's instructions. PCR reactions were carried out using the Expand Long Template kit (Roche). For inverse PCR, chromosomal DNA was digested with a single restriction enzyme, the fragments self-ligated and the ligation products subsequently used as templates in PCR reactions with suitable primers. DNA sequences were determined by the dideoxy chain-termination method using the CEQ DTCS-Quick Start Kit (Beckman Coulter) using a capillary DNA sequencer CEQ 8000 (Beckman Coulter). Comparison of the sequence data with published sequences in EMBL/GenBank was performed with the BLAST software via the internet (<http://www.ncbi.nlm.nih.gov/blast> and <http://www.ebi.ac.uk/blast/index.html>). Prediction of protein

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristic*	Source/reference
<i>P. damsela</i> subsp. <i>piscicida</i>		
DI21	Highly virulent	Seabream, Spain
DI21-Rif	DI21 derivative, spontaneous rifampicin-resistant mutant	This study
PC554.2	Highly virulent	Sole, Spain
PC435.1	Highly virulent	Sole, Spain
10831	Highly virulent	<i>Dicentrarchus labrax</i> , France
IT-1	Highly virulent	Seabream, Italy
MP-7801	Highly virulent	Yellowtail, Japan
MZS-8001	Highly virulent	Yellowtail, Japan
ATCC 17911	Moderately virulent	White perch, USA
ATCC 29690	Avirulent	Yellowtail, Japan
EPOY 8803-II	Avirulent	Red grouper, Japan
ATLIT 2	ND	<i>Morone</i> sp., Israel
666.1	ND	Seabass, Portugal
B51	ND	Seabream, Spain
CS31	DI21 with suicide plasmid pSJ52 inserted in the chromosome	This study
<i>P. damsela</i> subsp. <i>damsela</i>		
RM-71	Highly virulent	Turbot, Spain
CDC-2227-81	Highly virulent	Human, USA
RG-153	Highly virulent	Turbot, Spain
RG-214	Highly virulent	Turbot, Spain
ATCC 33539	Moderately virulent	Damselfish, USA
LD-07	Moderately virulent	Seabream, Spain
ATCC 35083	Avirulent	Brown shark, USA
158	Avirulent	European eel, Belgium
<i>Escherichia coli</i>		
DH5 α	Cloning strain	Laboratory stock
S17-1 λ pir	Tp ^r Sm ^r <i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR</i> ⁻ M ⁺ RP4-2-Tc:Mu-Km:Tn7 λ pir	Laboratory stock
<i>Yersinia enterocolitica</i>		
WA <i>fyuA2</i>	<i>fyuA</i> mutant	Brem <i>et al.</i> (2001)
WA-CS <i>irp1</i> ::Kan ^r	<i>irp1</i> mutant	Pelludat <i>et al.</i> (1998)
Plasmids		
pGEMT-Easy	PCR cloning vector, Amp ^r	Promega
pWKS30	Low-copy-number plasmid vector, Amp ^r	Wang & Kushner (1991)
pNidKan	Suicide vector, derived from pCVD442, Kan ^r	Mouriño <i>et al.</i> (2004)
pSJ52	A 4672-bp <i>EcoRI</i> - <i>XbaI</i> fragment including the 3' end of <i>irp2</i> and the 5' end of <i>irp1</i> , cloned into pNidKan	This study

*Virulence data for fish obtained from Magariños *et al.* (1992) and Osorio *et al.* (2000); ND, not determined.

domains was carried out by using the Pfam database online facilities (<http://www.sanger.ac.uk/Software/Pfam/>). Putative transcriptional terminators were predicted using RNAMotif (Macke *et al.*, 2001).

RNA isolation and RT-PCR. *P. damsela* subsp. *piscicida* DI21 was grown in TSB-1 and then subcultured on CM9 minimal medium. Total RNA was prepared from mid-exponential-phase cultures using the RNeasy Isolation Reagent (Ambion), according to the manufacturer's instructions. RNA preparations were then subjected to standard treatments with RNase-free DNase I. Reverse transcription reaction was performed by using the M-MLV reverse transcriptase (Invitrogen). For each reaction, 1 μ g total RNA was used.

Subsequent PCR amplification was carried out with *Taq* polymerase (Bioline) using suitable primers. As negative controls, DNA contamination of the RNA samples was ruled out by PCR using *Taq* DNA polymerase without reverse transcriptase. As positive controls, each primer combination was tested in PCR reactions carried out using chromosomal DNA as template.

Protein analysis. *P. damsela* subsp. *piscicida* DI21 and CS31 and *P. damsela* subsp. *damsela* RM71 cells were grown in CM9 supplemented with either FeCl₃ 10 μ M or 2,2'-dipyridyl 60 μ M (iron-sufficient and iron-restricted conditions, respectively). This concentration of 2,2'-dipyridyl was lowered to 30 μ M in the case of

the DI21 *irp1* mutant strain (CS31). Cells were centrifuged and total membrane proteins were obtained as previously described (Toranzo *et al.*, 1983). Protein concentration was adjusted for all the samples, and proteins were separated by SDS-PAGE. The protein bands were visualized by staining with Coomassie brilliant blue.

Construction of an *irp1*-targeted disruptant of *P. damsela* subsp. *piscicida*. An *EcoRI*–*XbaI* fragment of 4672 bp, which included ~1500 bp of the *irp2* downstream end and ~3100 bp of the *irp1* upstream end (Fig. 1a), was ligated into vector pWKS30. The DNA insert was further excised with *NotI*–*SaII* and ligated into the suicide vector pNidKan (Mouriño *et al.*, 2004), to generate pSJRS52. As a pCVD442 derivative, pNidKan contains R6K *ori*, requiring the *pir* gene product for replication. Insertion of the suicide vector into the chromosome by a single crossover results in a Kan^R phenotype. The pSJRS52 plasmid was transformed into *E. coli* S17-1 λ pir followed by mobilization into DI21-Rif. Insertional mutants were selected on agar plates containing kanamycin (50 μ g ml⁻¹) and rifampicin (50 μ g ml⁻¹), and presence of the inserted plasmid into the *irp1* gene was confirmed by Southern hybridization (data not shown).

Assay methods for siderophore production, growth under iron-limiting conditions, and virulence experiments. Production of siderophores by *P. damsela* subsp. *piscicida* DI21-Rif

and its *irp1* disruptant CS31 was tested by the universal chemical assay of Schwyn & Neilands (1987) in solid medium. The assay was performed by spotting 20 μ l supernatant of each bacterial culture, previously grown in CM9, onto chrome azurol S (CAS) agar plates. The ability of *P. damsela* subsp. *piscicida* strains to grow under conditions of iron limitation was evaluated by using CM9 minimal medium, in the presence of 60 μ M 2,2'-dipyridyl (Sigma).

The pathogenicity of *P. damsela* subsp. *piscicida* DI21-Rif and CS31 strains for turbot (*Scophthalmus maximus*) was assayed using 10 fish (mean weight 15 g) per dose, as previously described (Magariños *et al.*, 1992). Fish were inoculated with bacterial doses ranging from 10³ to 10⁷ cells. Mortalities were recorded daily for 10 days, and the LD₅₀ was calculated according to Magariños *et al.* (1992).

Cross-feeding assays. Cross-feeding assays were utilized to test the ability of *Y. enterocolitica* and *P. damsela* subsp. *piscicida* strains to induce the growth of the same strains subjected to iron starvation. Two mutants of *Y. enterocolitica* that were deficient in the biosynthesis of yersiniabactin (strain WA-CS *irp1*::Kan^r), or in the yersiniabactin receptor (strain WA *fyuA2*), respectively, were used. *P. damsela* subsp. *piscicida* CS31 was used for detection of yersiniabactin utilization. One hundred microlitres of overnight cultures of each indicator strain was added to 3 ml molten TSA-1 (*P. damsela* subsp. *piscicida* strains) or NBA (*Y. enterocolitica*

Fig. 1. (a) Physical map of the siderophore biosynthesis and transport gene cluster of *P. damsela* subsp. *piscicida* DI21, and primer design for RT-PCR operon mapping. ORFs are depicted as arrows, which indicate the direction of transcription. Predicted functions of the gene products are indicated as follows: *dahP*, *irp2*, *irp1*, *irp3*, *irp4*, *irp9* and *irp5*, biosynthetic genes; *frpA*, outer-membrane receptor; *araC1* and *araC2*, transcriptional activators; *irp8*, putative inner-membrane siderophore exporter. RT-1, RT-2 and RT-3 are the primers used in the subsequent PCR reactions. Arrow pairs located on top of A, B and C letters denote primer pairs used in the subsequent PCR reactions. Predicted Fur boxes identified in *dahP* and *araC2* putative promoters are shown. A palindromic region downstream of the stop codon of *irp5* constitutes a potential transcriptional terminator. (b) Operon mapping: results of PCR amplifications (RT) carried out with primer pairs A, B and C using as template the products of RT reactions obtained with primers RT-1, RT-2 and RT-3. M, molecular size marker (100 bp). Results indicate that a transcript originating from *dahP* putative promoter reaches the end of *irp5* gene. Negative controls (–) are PCR reactions without reverse transcriptase. Positive controls (+) are PCR reactions using chromosomal DNA as a template.

strains) and plated onto appropriate pre-poured TSA or NBA plates supplemented with 200 μM 2,2'-dipyridyl. Sterile filter-paper discs were loaded with 10 μl of cultures of different strains previously grown in TSC-1 (*P. damsela* subsp. *piscicida*) or NB (*Y. enterocolitica*) supplemented with 60 and 100 μM 2,2'-dipyridyl, respectively, to induce siderophore production. Discs loaded with 10 μl yersiniabactin (0.1 mg ml^{-1}) were also tested. Results were scored as positive or negative after 48 h incubation. Yersiniabactin was purchased from EMC microcollections GmbH.

RESULTS

Isolation of DNA fragments specific to *P. damsela* subsp. *piscicida* DI21 using SSH

P. damsela subsp. *damsela* RM71 is known to produce a hydroxamate (Fouz *et al.*, 1997). However, siderophore assays specific for catechols or hydroxamates did not detect the siderophore produced by *P. damsela* subsp. *piscicida* DI21 (Magariños *et al.*, 1994). We hypothesized that the production of different types of siderophore would be due to a differential gene content between these two strains. Therefore, we carried out SSH in order to isolate DNA fragments encountered uniquely in DI21 (tester strain), but absent from RM71 (driver strain). Some of the subtracted clones contained DNA sequences encoding transposases, tRNA hydroxylases and hypothetical proteins (to be published elsewhere). One clone, pSSH1, contained a ~ 1500 bp insert that encoded a 508 amino acid partial protein with significant similarity (63%) to the *Y. enterocolitica* high molecular mass protein 2 (HMWP2) (Table 2), a member of the family of the NRPSs involved in the synthesis of siderophore yersiniabactin. Based on this homology, this *P. damsela* subsp. *piscicida* gene was named *irp2*. Southern blot hybridization demonstrated that the DNA fragment cloned in pSSH1 was absent from the driver strain, RM71 (data not shown).

We hypothesized that the *P. damsela* subsp. *piscicida* DI21 *irp2* gene could be part of a gene cluster involved in siderophore biosynthesis. The DNA insert of clone pSSH1 was used as a probe to screen blots of DI21 chromosomal DNA digested with various enzymes, and was found to hybridize to a ~ 9 kb *Xba*I–*Xba*I DNA fragment (data not shown). This DNA fragment was further cloned into pGEM-T Easy and sequenced to completion and the complete sequence of *irp2* was established as an ORF encoding a protein of 1979 aa with a predicted molecular mass of 221 kDa. Inverse PCR was used to amplify additional DNA segments upstream and downstream of *irp2*, which were cloned and sequenced to completion. A gene cluster (*irp* cluster) spanning 35 kb and including 11 ORFs was thus identified (Fig. 1a).

Predicted protein sequences

Homologues of the proteins encoded by the *irp* cluster predicted ORFs are summarized in Table 2. The first ORF, *dahP*, encodes a putative 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase. It showed the highest similarity with a DAHP synthase of *Vibrio vulnificus*.

Homologues of this enzyme are responsible for the condensation of the pentose phosphate pathway intermediate D-erythrose 4-phosphate and the glycolytic pathway intermediate phosphoenolpyruvate to DAHP, a step in the biosynthesis of chorismate. The second and third genes of this cluster were termed *araC1* and *araC2*, respectively. The closest relatives of AraC1 and AraC2 proteins were putative AraC-family transcriptional regulators of *Photobacterium profundum* and *Vibrio cholerae* RC385. Homologues also included putative transcriptional regulators of other bacterial species, but the homology was restricted to the C-terminus. A protein domain prediction by using the Pfam database identified a helix–turn–helix motif within approximately the last 100 residues of the C-terminus of the deduced AraC1 and AraC2 proteins (data not shown), which is a conserved domain typical of this family of regulators. Interestingly, AraC2 showed similarity to YbtA, a transcriptional activator that controls expression of genes within the *Yersinia* HPI (Fetherston *et al.*, 1996; Anisimov *et al.*, 2005).

The fourth ORF of the cluster was termed *frpA*, encoding a predicted 660 amino acid protein that showed significant similarity with the *Y. pestis* and *Pseudomonas fluorescens* yersiniabactin and quinolobactin outer-membrane receptors, respectively. The fifth ORF, *irp8*, encodes a protein that showed homology to putative proteins whose function has not been ascertained in any species. A search conducted using the COG database (<http://www.ncbi.nlm.nih.gov/COG>) placed this protein within the group of permeases of the major facilitator superfamily (MFS). Similarity was found between this protein and YbtX, a highly hydrophobic cytoplasmic membrane protein encoded in the *Yersinia* HPI (Fetherston *et al.*, 1999). Its subcellular location and the similarity to MFS proteins suggest that Irp8 could be involved in siderophore export.

The sixth ORF of the cluster corresponds to the *irp2* gene, isolated by subtractive hybridization between strains DI21 and RM71 (see above). The seventh ORF was termed *irp1*; its start codon was located 50 bp downstream of the stop codon of *irp2*. Irp1 is a protein of 3996 aa, with a predicted molecular mass of 442 kDa. The first N-terminal 430 aa of Irp1 show homology to typical PKS (polyketide synthase) domains, and the identity percentage in this region was 62% to *Y. enterocolitica* HMWP1.

The eighth ORF was termed *irp3*; it encodes a protein with homology to thiazolinyldeductase components of the siderophore yersiniabactin biosynthesis pathway (YbtU component) (Miller *et al.*, 2002). The protein encoded by the ninth ORF, Irp4, was found to be 43% and 41% identical to *Y. pestis* YbtT and *Y. enterocolitica* Irp4, respectively, described as thioesterase-like proteins involved in yersiniabactin biosynthesis (Geoffroy *et al.*, 2000; Pelludat *et al.*, 1998), and 36% identical to a thioesterase-like protein located in the anguibactin biosynthetic gene cluster of *Vibrio anguillarum* (Farrell *et al.*, 1990).

Table 2. Proteins with homology to products of *P. damsela* subsp. *piscicida* DI21 genes of the siderophore biosynthesis cluster described in this study (asterisks denote that homology is restricted to the last 100 residues of the protein)

<i>P. damsela</i> protein (no. of aa)	EMBL/GenBank no.	Identity (%)	Similarity (%)
DAHP synthase (354)			
<i>V. vulnificus</i> DAHP synthase	Q8D5S3	54	68
<i>Escherichia coli</i> phospho-2-dehydro-3-deoxyheptonate aldolase	Q8FIY4	51	67
<i>V. cholerae</i> El Tor N16961 phospho-2-dehydro-3-deoxyheptonate aldolase	Q9KRX6	50	64
<i>Y. pseudotuberculosis</i> phospho-2-dehydro-3-deoxyheptonate aldolase	Q66A12	53	68
AraC1 (327)			
<i>P. profundum</i> hypothetical transcriptional regulator, AraC family	Q6LRJ3	59	75
<i>V. cholerae</i> RC385 AraC-type protein	ZP_00753343	58	77
<i>Ralstonia solanacearum</i> probable transcription regulator	Q8XYE5	35*	56*
<i>Ps. syringae</i> transcriptional regulator, AraC family	Q882M0	35*	61*
AraC2 (323)			
<i>P. profundum</i> hypothetical transcriptional regulator, AraC family	Q6LRJ4	63	77
<i>V. cholerae</i> RC385 AraC-type DNA-binding domain-containing protein	ZP_00753342	60	77
<i>Bacteroides fragilis</i> putative AraC family transcriptional regulatory protein	Q5LHP7	41*	61*
<i>Y. enterocolitica</i> AraC family transcriptional regulator YbtA	Q9X9I4	34*	54*
FrpA (660)			
<i>P. profundum</i> hypothetical outer membrane receptor	Q6LRJ5	65	81
<i>V. cholerae</i> RC385 outer membrane receptor protein	ZP_00753341	65	81
<i>Ph. luminescens</i> putative ferrisiderophore receptor FyuA	Q7N4L5	40	60
<i>Y. enterocolitica</i> yersiniabactin receptor	P46360	26	46
<i>Ps. fluorescens</i> quinolobactin receptor	Q84HG2	25	42
Irp8 (412)			
<i>P. profundum</i> hypothetical protein	Q6LRJ6	57	75
<i>V. cholerae</i> RC385 putative permease of the major facilitator superfamily	ZP_00753340	57	74
<i>Ph. luminescens</i> hypothetical protein	Q7N4L4	41	61
<i>E. coli</i> putative cytoplasmic transmembrane protein	Q8FGF7	35	52
<i>Y. enterocolitica</i> Irp8 protein	Q9X9I7	32	49
Irp2 (1979)			
<i>P. profundum</i> hypothetical NRPS	Q6LRJ7	55	70
<i>V. cholerae</i> RC385 NRPS	ZP_00753339	56	70
<i>Ph. luminescens</i> HMWP2-like protein	Q7N4L1	47	63
<i>Ps. syringae</i> yersiniabactin NRPS	Q48HQ4	47	61
<i>Y. pestis</i> yersiniabactin biosynthetic protein HMWP2	Q9Z399	45	61
<i>Y. enterocolitica</i> yersiniabactin biosynthetic protein HMWP2	P48633	45	61
Irp1 (3996)			
<i>P. profundum</i> putative beta-ketoacyl synthase	Q6LRJ8	50	67
<i>V. cholerae</i> RC385 polyketide synthase	ZP_00753338	52	66
<i>Ph. luminescens</i> HMWP1-like protein	Q7N4L0	45	60
<i>Ps. syringae</i> yersiniabactin polyketide/NRPS	Q882M6	51	64
<i>Y. pestis</i> yersiniabactin biosynthetic protein HMWP1	Q3V8D5	50	63
<i>Y. enterocolitica</i> HMWP1	O54511	49	62
Irp3 (361)			
<i>V. cholerae</i> RC385, oxidoreductase	ZP_00753422	61	74
<i>P. profundum</i> putative reductase component	Q6LRK0	59	76
<i>Ps. syringae</i> yersiniabactin synthetase, thiazolanyl reductase component	Q882M7	44	62
<i>Y. pestis</i> thiazolanyl-S-HMWP1 reductase (YbtU)	Q8D0C4	42	59
<i>Y. enterocolitica</i> yersiniabactin biosynthetic protein Irp3	O54512	42	59
Irp4 (233)			
<i>P. profundum</i> hypothetical thioesterase	Q6LRK1	60	77
<i>V. cholerae</i> RC385 predicted thioesterase	ZP_00753421	53	62
<i>Ph. luminescens</i> hypothetical protein similar to <i>Y. enterocolitica</i> Irp4	Q7N4K8	50	62
<i>Y. pestis</i> yersiniabactin biosynthetic protein YbtT	Q7BS79	43	56
<i>Y. enterocolitica</i> yersiniabactin biosynthetic protein Irp4	O54513	41	57

Table 2. cont.

<i>P. damsela</i> protein (no. of aa)	EMBL/GenBank no.	Identity (%)	Similarity (%)
<i>V. anguillarum</i> thioesterase for anguibactin biosynthesis Irp9 (480)	Q79JZ0	36	53
<i>P. profundum</i> hypothetical anthranilate synthase	Q6LRK2	64	76
<i>V. cholerae</i> RC385 anthranilate/ <i>p</i> -aminobenzoate synthase component	ZP_00753420	61	72
<i>Ph. luminescens</i> hypothetical protein	Q7N4K7	45	62
<i>Desulfotobacterium hafniense</i> anthranilate synthase	Q429X2	39	57
<i>Y. pestis</i> YbtS protein	Q9Z396	39	53
<i>Y. enterocolitica</i> salicylate synthetase Irp9 Irp5 (532)	Q9X9I8	39	53
<i>V. cholerae</i> RC385 peptide arylation enzyme	ZP_00753419	62	79
<i>P. profundum</i> putative peptide arylation enzyme	Q6LRK3	60	78
<i>V. vulnificus</i> vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase	Q7MCT0	60	74
<i>Y. pestis</i> salicyl-AMP ligase YbtE	Q56950	49	64
<i>V. cholerae</i> vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase, VibE	O07899	47	62
<i>Y. enterocolitica</i> salicyl-AMP ligase Irp5	O54514	46	62

The tenth ORF encodes a protein which we termed Irp9. It is similar to *Y. enterocolitica* Irp9, an enzyme that directly converts chorismate into salicylate, which is further used as a precursor in the synthesis of yersiniabactin (Pelludat *et al.*, 2003). Irp5, encoded by the eleventh ORF, was found to be homologous to a series of aryl-activating enzymes, involved in the adenylation and activation of aryl groups and amino acids. It is 47 % identical to *Y. pestis* YbtE, a salicyl-AMP ligase which activates salicylic acid and transfers the activated molecule to the first ArCP (aryl-carrier protein) domain of the non-ribosomal peptide synthetase HMWP2 (Gehring *et al.*, 1998). Interestingly, Irp5 is 44 % identical to *V. cholerae* VibE, the vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase, the enzyme that adenylates and activates 2,3-dihydroxybenzoate, a precursor of vibriobactin (Wyckoff *et al.*, 1997).

Transcriptional organization of the cluster

The 11 genes of the cluster described in this study have the same transcriptional polarity. Downstream of *irp5* is an ORF with opposite transcriptional polarity to the 11-gene cluster described and its predicted protein product shows similarity to putative inner-membrane ATPases (data not shown). Upstream of *dahP* we sequenced 3 additional kb, but no ORFs were found.

Sequence analysis identified a conserved putative Fur-binding site (Fur box), upstream of *dahP* gene, which shares 17 out of 19 identical nucleotides with the *E. coli* Fur-box consensus sequence. Similarly, a potential Fur box with identity in 16 of 19 positions with the consensus was found upstream of *araC2* (Fig. 1a). This would suggest the existence of at least two iron-regulated promoters within this cluster. To study the transcriptional organization of the cluster, we conducted a series of reverse-transcriptase reactions with primers targeted to the downstream ends

of *irp5* (RT-1), *araC2* (RT-2) and *araC1* (RT-3), and subsequent PCR reactions were performed with three different primer combinations, A, B and C, that amplified internal fragments of *irp1* (653 bp), *araC2* (407 bp) and *dahP* (470 bp), respectively (Fig. 1a). All the reactions yielded a product of the expected size, and negative and positive controls (see Methods) corroborated the accuracy of the RT-PCR results (Fig. 1b). These results demonstrate that the 11 genes of the cluster described in this study can be co-transcribed from the promoter upstream of *dahP*, although the existence of additional promoters, particularly upstream of *araC2*, cannot be ruled out. When we applied the RNAMotif program, a unique transcriptional stop was predicted, starting at 18 bp downstream of the *irp5* stop codon (Fig. 1a). No additional transcriptional terminators were predicted within this gene cluster.

Presence of iron-regulated high molecular mass proteins in *P. damsela* subsp. *piscicida* DI21

The presence of Fur-binding sequences similar to the *E. coli* consensus upstream of *dahP* and *araC2* would suggest that iron concentration plays a role in regulating the expression of genes within the *irp* cluster. *P. damsela* subsp. *piscicida* DI21 cells were grown in iron-rich and iron-limited media, and the profiles of total membrane proteins were compared. As a result, two high molecular mass protein bands were observed when the cells were cultured in iron-restricted media, whereas these bands were not observed under conditions of iron sufficiency (Fig. 2). The molecular masses of these two proteins likely correspond to those predicted for the Irp1 (442 kDa) and Irp2 (221 kDa) as described above, encoded by the *irp* cluster. These two protein bands were absent under both iron-limiting and iron-sufficient conditions in the *P. damsela* subsp. *damsela* strain RM71, which was used as the driver strain in the SSH experiment described above. Although the subcellular

Fig. 2. Total membrane proteins purified from *P. damsela* subsp. *piscicida* DI21, *P. damsela* subsp. *piscicida* CS31 mutant and *P. damsela* subsp. *damsela* RM71 under conditions of iron excess (Fe+) and iron limitation (Fe-). M1 and M2 are molecular mass markers, and numbers at side denote kDa. Arrows denote bands corresponding to the iron-regulated Irp1 and Irp2 proteins.

location of Irp1 and Irp2 homologues is not well known, there is some evidence that they may contain hydrophobic domains that may be membrane associated (Guilvout *et al.*,

1993) and this could explain the purification of these proteins in the membrane fraction.

Irp1, Irp2 and Irp5 are putative members of the NRPS family

Most of the bacterial siderophores described to date are synthesized by NRPSs (Crosa & Walsh, 2002), which are modularly organized multi-enzyme complexes that represent at the same time template and biosynthetic machinery (Schwarzer *et al.*, 2003). Using the Pfam database, we found that the predicted domain organizations of the *P. damsela* subsp. *piscicida* Irp5, Irp2 and Irp1 proteins are highly similar to those predicted for the *Y. enterocolitica* Irp5, HMWP2 and HMWP1 proteins. However, subsp. *piscicida* Irp2 lacks an ArCP domain, which is otherwise encountered at the N-terminus of *Y. enterocolitica* HMWP2. Interestingly, Irp1 is larger than HMWP1 and contains different domains (Fig. 3).

The adenylation (A) domains are responsible for substrate recognition and activation as acyl-adenylate derivatives. It has been reported that the substrate specificity of the A domains is mostly determined by ten residues of the substrate-binding pocket and these residues can be considered as the codons of non-ribosomal peptide synthesis (Stachelhaus *et al.*, 1999; Schwarzer *et al.*, 2003). Thus, it is possible to predict the substrates that will be recognized and adenylated by the three A domains predicted in this study: one in Irp5, and one in each of Irp2 and

Fig. 3. Schematic representation of the functional domains predicted for the *P. damsela* subsp. *piscicida* Irp5, Irp2 and Irp1 and the *Y. enterocolitica* Irp5, HMWP2 and HMWP1 proteins using the Pfam database. Abbreviations denoting domain nomenclature are as follows: A, adenylation domain; ACP, acyl-carrier protein domain; ArCP, aryl-carrier protein domain; AT, acyl transferase domain; C, condensation domain; Cy, condensation-cyclization domain; Dh, dehydrogenase domain; KS, ketoacyl synthase domain; MT, methyltransferase domain; PCP, peptidyl-carrier protein domain; TE, thioesterase domain. Superscripts denote the substrate specificity predicted for the A domains: Sal, salicylate; Cys, cysteine. Boxes in white denote the *P. damsela* subsp. *piscicida* A domains for which the substrate specificity was predicted following the non-ribosomal code.

Table 3. Selectivity-conferring amino acids in the adenylation domains of *P. damsela* subsp. *piscicida* Irp1, Irp2 and Irp5 NRPSs

Prediction of the substrate specificity according to the non-ribosomal code, and comparative alignment with the respective positions of the substrate-binding pocket described in A domains of *Y. enterocolitica* HMWP2 and YbtE, and in *V. anguillarum* AngR. Positions refer to the *B. brevis* GrsA (gramicidin S synthetase). Cys, cysteine; Sal, salicylate.

A domain	Substrate specificity	Residue positions according to GrsA Phe*									
		235	236	239	278	299	301	322	330	331	517
Irp2	Cys	D	L	Y	N	L	S	M	I	W	K
Irp1	Cys	D	L	F	N	M	S	L	I	W	K
HMWP2	Cys	D	L	Y	N	N	S	M	I	W	K
AngR	Cys	D	L	Y	N	M	S	M	I	W	K
Consensus		D	L	Y	N	L	S	L	I	W	K
Irp5	Sal	N	F	C	A	Q	G	V	I	C	K
YbtE	Sal	N	F	C	A	Q	G	V	L	F	K
Consensus		N	F	C	A	Q	G	V	I	Y	K

*Stachelhaus *et al.* (1999).

Irp1. We carried out a sequence alignment of these three predicted A domains with that of the gramicidin S synthetase (GrsA) from *Bacillus brevis* and determined the ten conserved residues of the substrate-binding pocket. As shown in Table 3, the Irp5 A domain is predicted to select salicylate, thus having the same substrate specificity as *Y. enterocolitica* Irp5. On the other hand, the A domains of Irp2 and Irp1 are predicted to select and activate cysteine, which is the same substrate as the homologous A domain present in *Y. enterocolitica* HMWP2.

Fig. 4. Growth of DI21-Rif parental strain (▲), and CS31 mutant (□) in CM9 minimal medium supplemented with 10 μ M FeSO₄ (iron-sufficient conditions; continuous lines), and in CM9 in the presence of the iron chelator 2,2'-dipyridyl at a concentration of 60 μ M (iron-limited conditions; discontinuous lines).

***irp1* insertional inactivation renders *P. damsela* subsp. *piscicida* unable to produce siderophores and to grow under iron-limiting conditions, and decreases the virulence for fish**

In order to demonstrate that the *irp* gene cluster identified in this study is involved in siderophore biosynthesis in *P. damsela* subsp. *piscicida* DI21, an operon disruption was constructed by inserting a suicide plasmid in the *irp1* gene. The *irp1* insertional mutant, which was termed CS31, was tested on CAS agar plates and found to be CAS negative, indicating loss of siderophore production. When we selected for a second cross that eliminated the suicide plasmid and restored the wild-type gene, the strain recovered the ability to produce siderophores, demonstrating that loss of siderophore production was due to *irp1* insertional mutation (data not shown).

Parental and mutant strains showed a similar growth in CM9 minimal medium supplemented with 10 μ M FeSO₄ (iron-sufficient conditions) (Fig. 4). The same strains were grown in CM9 in the presence of the iron chelator 2,2'-dipyridyl at a concentration of 60 μ M. Under these iron-limiting conditions, the *irp* insertional mutant CS31 was impaired for growth with respect to the parental strain (Fig. 4). In addition, when the protein profiles of CS31 and the parental strain, under both iron-sufficient and iron-limiting conditions, were compared, the mutant lacked the high molecular mass iron-regulated protein believed to correspond to Irp1 (Fig. 2).

To analyse the role of siderophore-mediated iron uptake in the virulence of *P. damsela* subsp. *piscicida* for fish, we carried out experimental inoculations with the parental strain DI21-Rif and the CS31 mutant in turbot fingerlings. The LD₅₀ obtained with the CS31 mutant (2.6×10^7

Table 4. Results of the cross-feeding assays performed with different *P. damsela* subsp. *piscicida* and *Y. enterocolitica* strains and with purified yersiniabactin

Tester	Ability to be cross-fed			
	<i>P. damsela</i> subsp. <i>piscicida</i>		<i>Y. enterocolitica</i>	
	DI21	CS31	WA <i>fyuA2</i>	WA-CS <i>irp1::Kan^r</i>
Yersiniabactin	+	+	–	+
DI21	+	+	–	+
CS31	–	–	–	–
WA <i>fyuA2</i>	+	+	–	+
WA-CS <i>irp1::Kan^r</i>	–	–	–	–

bacterial cells per fish) was ~100-fold higher than with the parental strain (8×10^5), demonstrating that production of siderophores mediated by the *irp* cluster contributes significantly to the virulence of *P. damsela* subsp. *piscicida* for fish.

Cross-feeding assays

Based on protein-domain sequence predictions, the siderophore synthesized by *P. damsela* subsp. *piscicida* might be similar to yersiniabactin. To assess this possibility, *P. damsela* subsp. *piscicida* DI21 and CS31, and *Y. enterocolitica* *fyuA* and *irp1* mutants, were cultured under iron-limiting conditions and the ability of the different strains as well as of purified yersiniabactin to induce the growth of the indicator strains was evaluated. The cross-feeding assays showed that *Y. enterocolitica* WA *fyuA2*, which produces yersiniabactin but lacks the yersiniabactin FyuA receptor, is able to cross-feed both *P. damsela* subsp. *piscicida* DI21 and CS31, whereas *Y. enterocolitica* WA-CS *irp1::Kan^r*, which has the yersiniabactin FyuA receptor but is unable to synthesize yersiniabactin, is not (Table 4). The assays using purified yersiniabactin corroborated that both the parental *P. damsela* subsp. *piscicida* and the CS31 mutant are able to use this siderophore (Table 4).

We also tested if the *P. damsela* subsp. *piscicida* DI21 siderophore can stimulate the growth of *Y. enterocolitica* strains subjected to iron starvation. The results showed that *P. damsela* subsp. *piscicida* DI21 can promote the growth of *Y. enterocolitica* *irp1* but not that of *Y. enterocolitica* *fyuA*, demonstrating that the siderophore produced by *P. damsela* subsp. *piscicida* can efficiently be used by *Y. enterocolitica* and this growth promotion is dependent on the presence of the functional outer-membrane yersiniabactin receptor FyuA. On the other hand, *P. damsela* subsp. *piscicida* CS31 could not cross-feed any of the indicator strains (Table 4). This indicates that induction of growth in *Yersinia* strains by *P. damsela* subsp. *piscicida* is actually due to the production of a siderophore molecule whose synthesis is dependent on the *irp* gene cluster. Altogether, these results suggest that the siderophore produced by subsp. *piscicida* could be structurally and functionally related to yersiniabactin.

Distribution of *irp* cluster genes in *P. damsela* subsp. *piscicida* and subsp. *damsela* isolates

The presence of genes of the *irp* cluster described in this study in various *P. damsela* isolates of the two subspecies and with diverse isolation origins was tested by Southern hybridization. Chromosomal DNA samples were digested with *Hind*III, and Southern blotting was individually performed with probes internal to the *dahP*, *araC1*, *frpA*, *irp2*, *irp1*, *irp9* and *irp5* genes. For all the hybridization probes, the results were equivalent: all these genes were found to be absent in all the subsp. *damsela* strains and were present uniquely in subsp. *piscicida* strains DI21, B51, 666.1, PC554.2, PC435.1 and ATLIT 2 (Fig. 5). Interestingly, the two subsp. *piscicida* avirulent strains (EPOY 8803-II and ATCC 29690) tested negative for all these genes (Fig. 5). All the positive strains are recognized as virulent for fish (Table 1) and were isolated in Spain and Portugal, with the exception of ATLIT 2, which was isolated in Israel. When the primer pairs used to amplify the DNA probes were also used in a PCR-based screening with all the strains, the data for the presence of *irp* cluster genes were equivalent to those obtained by Southern blotting (data not shown).

Similarity to gene clusters of other species: the *Yersinia* HPI

We searched DNA sequence databases of both complete and incomplete bacterial genome projects to find homologues of the *P. damsela* subsp. *piscicida* siderophore biosynthesis gene cluster. The highest similarity in sequence and gene arrangement of the *P. damsela* cluster corresponds to a region of the *P. profundum* SS9 genome (Vezzi *et al.*, 2005). This DNA region contains homologues of all the *P. damsela* subsp. *piscicida* genes, with the exception of *dahP* (Fig. 6). Although the *P. profundum* PBPR1666 gene, which is annotated as a putative AMP-binding enzyme, does not have a gene counterpart in subsp. *piscicida*, it encodes a protein that is highly homologous to the C-terminus of *P. damsela* subsp. *piscicida* Irp1, suggesting that the subsp. *piscicida* Irp1 protein is actually the counterpart of the *P. profundum* proteins Irp1 and PBPR1666. None of the published *Vibrio* genomes was shown to contain a gene cluster homologous to that described in this study (Heidelberg *et al.*, 2000; Chen *et al.*, 2003; Makino *et al.*,

Fig. 5. Results of Southern hybridization screening for the presence of *irp1* gene, using *Hind*III-digested chromosomal DNA from a collection of *P. damsela* strains. Similar results were obtained with *dahP*, *araC1*, *frpA*, *irp2*, *irp9* and *irp5* probes (results not shown). Lanes 1–12, *P. damsela* subsp. *piscicida* strains DI21, B51, ATCC 17911, ATCC 29690, 666.1, 10831, MP-7801, EPOY 8803-II, MZS-8001, PC554.2, PC435.1 and ATLIT 2. Lanes 13–19, *P. damsela* subsp. *damsela* strains RM-71, ATCC 33539, ATCC 35083, CDC 2227-81, RG-214, LD-07 and 158.

2003; Ruby *et al.*, 2005). However, we identified genes similar to those of the *P. damsela* subsp. *piscicida* *irp* cluster in *V. cholerae* RC385 (GenBank ref. AAKH00000000), a strain whose genome project is being completed at TIGR (The Institute for Genomic Research, <http://www.tigr.org/>). Interestingly, these genes are distributed into two gene clusters separated by approximately 75 kb (Fig. 6).

The 11-gene cluster described in this work shows similarity with the HPI of *Y. enterocolitica*. The HPI contains homologues of all the *P. damsela* subsp. *piscicida* genes described here, with the exception of *dahP* and *araC1*. However, the arrangement of genes differs between the two species

(Fig. 6), and whereas the *Yersinia* HPI contains genes that are divergently transcribed, the *P. damsela* subsp. *piscicida* genes are all transcribed from the same DNA strand.

DISCUSSION

Previous studies reported that *P. damsela* subsp. *piscicida* strains produce a siderophore which is different from that synthesized by isolates of *P. damsela* subsp. *damsela* (Magariños *et al.*, 1994; Fouz *et al.*, 1997). The genetic content can vary considerably between strains of the same species, and this variation is particularly frequent in genes

Fig. 6. Comparative analysis of gene arrangement in the *P. damsela* subsp. *piscicida* DI21 siderophore biosynthesis and transport gene cluster, and related clusters in *Yersinia enterocolitica* HPI, *Photobacterium profundum* and *Vibrio cholerae* RC385. For clarity and for comparative purposes, the same gene nomenclature of *P. damsela* subsp. *piscicida* genes was applied to the *P. profundum* and *V. cholerae* RC385 homologues. The small grey squares denote potential Fur-binding sites (Fur boxes).

that encode virulence factors. In this scenario, SSH has been successfully applied to the identification of genomic differences between bacterial strains (Winstanley, 2002), and it has been recently applied to the assessment of genetic diversity among *P. damsela* subsp. *piscicida* isolates (Juiz-Río *et al.*, 2005b). In the present study, by using SSH, we have identified a subsp. *piscicida*-specific gene cluster spanning ~35 kb, involved in siderophore biosynthesis. This cluster includes 11 putative ORFs that can be co-transcribed from a promoter upstream of *dahP*. However, we understand that this does not exclude the possibility that additional promoter sequences may exist upstream of other genes of this cluster.

The Fur protein is the major repressor of iron-uptake systems in Gram-negative bacteria, repressing transcription of the genes and thus limiting the entry of excess iron into the cell (Escobar *et al.*, 1999). Two high molecular mass protein bands, which are predicted to correspond to Irp1 and Irp2 encoded within the *irp* cluster, are induced under conditions of iron limitation, which could be explained by the presence of Fur-binding sequences predicted upstream of *dahP* and *araC1*. In accordance with this, previous studies demonstrated that *P. damsela* subsp. *piscicida* DI21 harbours a *fur* homologue that can act as an iron-dependent transcriptional repressor (Juiz-Río *et al.*, 2004; Osorio *et al.*, 2004).

Genes involved in siderophore biosynthesis can also be subject to transcriptional activation. The AraC1 and AraC2 proteins described in this study are putative members of the AraC family of transcriptional activators, which are classified based on a conserved 99 aa stretch usually found within the C-terminus. This conserved region is predicted to encode two helix–turn–helix motifs that function in DNA binding (Gallegos *et al.*, 1997). The homology of AraC1 and AraC2 with other proteins in databases was shown to be restricted to the C-terminus, indicating that the main variability resides within the N-terminus. Interestingly, there is increasing evidence that the N-terminus of AraC-family activators regulating siderophore synthesis and transport genes contains cofactor-binding regions, and this co-factor has proved to be the cognate ferri-siderophore complex (Tanabe *et al.*, 2005; Michel *et al.*, 2005). It is also interesting that AraC2 shows similarity to YbtA, a protein that controls expression of yersiniabactin synthesis genes within the *Yersinia* spp. HPI in a yersiniabactin-dependent manner (Fetherston *et al.*, 1996; Anisimov *et al.*, 2005). It is noteworthy that *Yersinia irp1* mutants do not produce HMWP1 and HMWP2 (Pelludat *et al.*, 1998), since yersiniabactin is required for upregulation of *irp* genes. In contrast, we have demonstrated here that a *P. damsela* subsp. *piscicida irp1* mutant still produces *irp2* (Fig. 2). These results suggest that differences in gene regulation exist between *Yersinia* and *P. damsela* subsp. *piscicida* gene clusters, and either AraC1, AraC2 or both could play a regulatory role distinct from YbtA. In this context, we could not find predicted YbtA-binding sites in the putative promoter regions of *dahP* and *AraC2* (data not shown).

FrpA is the putative outer-membrane siderophore receptor of *P. damsela* subsp. *piscicida* DI21. It shows high similarity to the yersiniabactin receptor FyuA and to the quinolobactin receptor of *Ps. fluorescens*, but not to any of the siderophore receptors described in vibrios, suggesting that *P. damsela* subsp. *piscicida* might utilize a siderophore that is not related to any of the catecholate- or hydroxamate-type siderophores described to date in other vibrios.

The insertional inactivation of the *irp* cluster led to an impairment of growth under conditions of iron limitation, suggesting that this cluster encodes a siderophore-based mechanism that is crucial for iron uptake. The absence of reaction in the CAS agar plates of the CS31 mutant indicates that this is the only high-affinity iron-scavenging system present in *P. damsela* subsp. *piscicida* DI21. The *irp1* mutant displayed reduced virulence in fish, demonstrating that the presence of this siderophore-mediated iron-acquisition mechanism contributes significantly to the virulence of *P. damsela* subsp. *piscicida*. The high LD₅₀ exhibited by the mutant strain (2.6×10^7) could even be due to LPS toxicity, which would mean that the siderophore mutant could be avirulent. Mutants in the *irp1* gene of extraintestinal pathogenic *E. coli* carrying HPI were also less virulent than the parental strain (Schubert *et al.*, 2002). The connection between siderophore-mediated iron-acquisition mechanisms and bacterial virulence is well established (Faraldo-Gómez & Sanson, 2003), and recent studies have demonstrated the importance of siderophore production in the virulence of fish pathogens (Stork *et al.*, 2004; Fernández *et al.*, 2004).

Protein sequence comparisons suggested that the siderophore produced by *P. damsela* subsp. *piscicida* is synthesized by an NRPS-mediated mechanism similar to that described for yersiniabactin. Based on the *in silico* analysis, the as yet uncharacterized siderophore produced by *P. damsela* subsp. *piscicida* DI21 might contain at least one salicylate and two cysteine residues in its structure. The *dahP*-encoded DAHP synthase is probably involved in the synthesis of chorismate. This is an intermediate in the biosynthesis of aromatic compounds, some of which are precursors of aromatic carboxy and amino acids which are part of the structure of siderophores. In this context, a *dahP* homologue has been described as part of the gene cluster involved in siderophore anguibactin biosynthesis in *V. anguillarum* (Alice *et al.*, 2005). Irp9 is the candidate enzyme to be involved in the synthesis of salicylate, based on its high similarity to the *Y. enterocolitica* Irp9. Irp3 and Irp4 are counterparts of the *Yersinia* spp. Irp3 (or YbtU) and Irp4 (or YbtT). Therefore, the *P. damsela* subsp. *piscicida* gene cluster described in this study contains all the gene counterparts of the yersiniabactin biosynthetic complex (Crosa & Walsh, 2002). All these data suggest that the DI21 siderophore might be structurally related to yersiniabactin (Drechsel *et al.*, 1995), and this hypothesis is supported by the results obtained in the cross-feeding assays. However, the differences in the domain composition between the

Yersinia and DI21 Irp1 and Irp2 homologues suggest that the two siderophores could have some differences in their structures.

The gene cluster described in this study was found to be absent in subsp. *damsela* strains, but was present in subsp. *piscicida* strains isolated from Spain and Portugal and one isolate from Israel. The two subsp. *piscicida* avirulent strains as well as all the isolates from Japan lacked this system, although they have been reported to produce siderophores (Magariños *et al.*, 1994). This suggests that these strains harbour an as yet uncharacterized genetic system for siderophore production. In a previous study (Magariños *et al.*, 2000), it was proposed that two distinct clonal lineages exist within *P. damsela* subsp. *piscicida*, represented by the European isolates on the one hand, and the Japanese isolates on the other hand. Our data support this hypothesis and indicate that DNA fragments from the operon described here could be used as genetic markers for the epidemiological typing of strains of this fish pathogen.

As *P. damsela* subsp. *piscicida* is a member of the *Vibrionaceae*, it is expected that the genes responsible for siderophore biosynthesis in this species would show high similarity to homologous genes in other vibrios. However, most of the predicted protein sequences encoded by this gene cluster did not show significant similarity to described siderophore synthesis and transport gene clusters of the *Vibrionaceae*. Homologous gene clusters exist in *P. profundum* SS9 and *V. cholerae* RC385, although their role in siderophore biosynthesis remains untested. It is noteworthy that the *P. profundum* *irp* homologues are located downstream of an *asn*-tRNA gene (Fig. 6), which suggests that this gene cluster may have been integrated in the chromosome in a similar way as described for the *Yersinia* HPI. Although we did not find evidence of *asn*-tRNA genes upstream of the *P. damsela* subsp. *piscicida* *irp* cluster, this does not exclude the possibility that this cluster is part of a laterally transmitted integrating element, since genetic rearrangements of the flanking sequences could have occurred after integration, or a mechanism of integration independent of the presence of tRNA genes could have taken place. In this context, no tRNA genes were found in the *V. cholerae* RC385 homologous cluster.

Among the gene clusters showing homology to the *irp* cluster of *P. damsela* subsp. *piscicida*, the *Yersinia* HPI is the most widely studied. The HPI is widespread among both pathogenic and non-pathogenic taxa of *Enterobacteriaceae* (Schubert *et al.*, 1998; Bach *et al.*, 2000; Olsson *et al.*, 2003) and its ability to be horizontally transmitted has recently been demonstrated (Lesic & Carniel, 2005). The similarity between HPI genes and those described here in the *irp* cluster suggests that the *P. damsela* subsp. *piscicida* siderophore biosynthesis genes might have a common evolutionary origin with the *Yersinia* HPI. Recently, gene clusters similar to the *Yersinia* HPI have also been described in yersiniabactin-producing strains of *Pseudomonas syringae* and *Photobacterium luminescens* (Bultreys *et al.*, 2006),

providing more clues about the evolution and dispersion of yersiniabactin-related genes.

The differential occurrence of this gene cluster among *P. damsela* strains, together with its low similarity to siderophore synthesis genes of other vibrios, suggest that the *P. damsela* subsp. *piscicida* siderophore biosynthesis gene cluster could have been acquired by horizontal transfer. Future studies will help to ascertain whether the *irp* cluster is part of a genomic island and if this DNA region can be mobilized in a similar way as that described for the HPI.

ACKNOWLEDGEMENTS

We thank J. Heesemann and A. Rakin for providing *Y. enterocolitica* strains, and K. E. Klose for helpful suggestions and useful comments. This work was supported by grants AGL2003-00086 and AGL2006-00697 from the Ministry of Education and Science of Spain (grants cofunded by the FEDER Programme from the European Union) and grant PGIDIT04PXIC23501PN from Xunta de Galicia to M. L. L.; S. J. R. also thanks the Spanish Ministry of Education and Science for a Predoctoral Fellowship.

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