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Two Catechol Siderophores, Acinetobactin and Amonabactin, Are Simultaneously Produced by *Aeromonas salmonicida* subsp. *salmonicida* Sharing Part of the Biosynthetic Pathway

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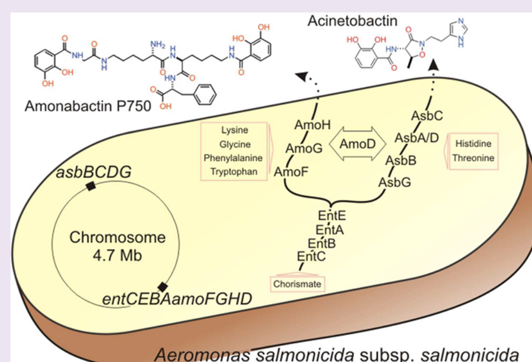
Supporting Information

ABSTRACT: The iron uptake mechanisms based on siderophore synthesis used by the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* are still not completely understood, and the precise structure of the siderophore(s) is unknown. The analysis of genome sequences revealed that this bacterium possesses two gene clusters putatively involved in the synthesis of siderophores. One cluster is a candidate to encode the synthesis of acinetobactin, the siderophore of the human pathogen *Acinetobacter baumannii*, while the second cluster shows high similarity to the genes encoding amonabactin synthesis in *Aeromonas hydrophila*. Using a combination of genomic analysis, mutagenesis, biological assays, chemical purification, and structural determination procedures, here we demonstrate that most *A. salmonicida* subsp. *salmonicida* strains produce simultaneously the two siderophores, acinetobactin and amonabactin. Interestingly, the synthesis of both siderophores relies on a single copy of the genes encoding the synthesis of the catechol moiety (2,3-dihydroxybenzoic acid) and on one encoding a phosphopantetheinyl transferase. These genes are present only in the amonabactin cluster, and a single mutation in any of them abolishes production of both siderophores. We could also demonstrate that some strains, isolated from fish raised in seawater, produce only acinetobactin since they present a deletion in the amonabactin biosynthesis gene *amoG*. Our study represents the first evidence of simultaneous production of acinetobactin and amonabactin by a bacterial pathogen and reveals the plasticity of bacterial genomes and biosynthetic pathways. The fact that the same siderophore is produced by unrelated pathogens highlights the importance of these systems and their interchangeability between different bacteria.



Iron is an essential element for the metabolism of most microorganisms, but due to its physico−chemical properties, it is hard to find in assimilable forms in most biological environments. This is also true for animal tissues where most iron is tightly bound to iron-containing proteins. Thus, bacteria have evolved a number of sophisticated mechanisms to obtain iron from the environment and from their hosts in the case of pathogens.[1] The mechanisms for acquisition of iron are generally recognized as essential factors in the survival of most bacterial pathogens within their hosts, and it is now clear that they significantly contribute to bacterial virulence, being key factors for infectious diseases development.[2,3]

One of the main strategies of microorganims to acquire iron is the synthesis and secretion of siderophores, low-molecular-weight structurally diverse Fe(III) chelators, which efficiently remove iron from iron-binding proteins or other iron-containing compounds and which then enter the cell through specific cell envelope transport proteins, including outer membrane cognate receptors.[2] Synthesis of siderophores is a complex process that usually requires a complete set of dedicated genes that encode the required biosynthetic enzymes. Many siderophores are usually synthesized by NRPS (non-ribosomal peptide synthetases), a multimodule and multi-catalytic type of enzyme that assembles the different residues that form the final compound.[4−6] From the amino acid sequence of these enzymes and through sequence comparisons with other known NRPSs, it is usually feasible, to some extent, to predict the residues that will be incorporated into the siderophore that they synthesize, hence being possible to obtain some important clues about the structure of the compound.[7−9] All catechol-type siderophores contain DHBA (2,3-dihydroxy-benzoic acid), which is formed from chorismic acid by a four-

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Table 1. Description of the Proteins Encoded by the Genes of the Acinetobactin Cluster (Loci Numbers Correspond to the Genome Sequence of Strain A449) in *A. salmonicida* and Their Closest Homologues

Aeromonas salmonicida subsp. *salmonicida* A449 chromosome				
locus (no. aa)	encoded protein, description	*A. hydrophila* ATCC7966[a]	*Pseudomonas entomophila*[a] pseudomonine system	*Acinetobacter baumannii*[a] acinetobactin system
ASA_4363 (701)	FhuA, TonB-dependent ferrichrome receptor	YP_858692.1 (93, 95)		
ASA_4364 (548)	FhuB, ATP-binding component	YP_858693.1 (94, 97)		
ASA_4365 (254)	FhuC, ferrichrome ABC transporter ATP-binding protein	YP_858694.1 (91, 94)		
ASA_4366 (308)	FhuD, ferrichrome-binding periplasmic protein	YP_858695.1 (80, 86)		
ASA_4367 (660)	FhuE, ABC transporter permease subunit	YP_858696.1 (88, 92)		
ASA_4368 (824)	FstA/B, TonB-dependent siderophore receptor		YP_608099.1 (46, 63)	ENW75959.1 (44, 61)
ASA_4369 (322)	AsuB, ABC transporter, periplasmic protein		YP_608100.1 (72, 82)	ENW75958.1 (54, 71)
ASA_4370 (260)	AsuE, ABC transporter, ATP-binding protein		YP_608101.1 (74, 85)	ENW75957.1 (69, 82)
ASA_4371 (316)	AsuC, ABC transporter, permease		YP_608102.1 (72, 86)	ENW75956.1 (61, 78)
ASA_4372 (307)	AsuD, ABC transporter, permease		YP_608103.1 (79, 90)	ENW75955.1 (72, 87)
ASA_4373 (589)	AsuE, ABC transporter permease/ATP-binding protein		YP_608104.1 (66, 76)	ENW75966.1 (31, 49)
ASA_4374 (607)	AsuF, ABC transporter permease/ATP-binding protein		YP_608105.1 (68, 79)	ENW75965.1 (31, 49)
ASA_4375 (165)	AsbI, RNA polymerase sigma-70 factor		YP_608106.1 (63, 77)	
ASA_4376 (709)	AsbB, nonribosomal peptide synthetase		YP_608107.1 (50, 67)	ENW75953.1 (50, 66)
ASA_4377 (440)	AsbC, L-lysine 6-monooxygenase		YP_608108.1 (71, 82)	ENW75960.1 (70, 82)
ASA_4378 (1453)	AsbA/D, nonribosomal peptide synthetase		YP_608109.1 (61, 71)	ENW75952.1 (41, 61)
				ENW75961.1 (55, 69)
ASA_4379[b] (83)	AsbF, 2,3-dihydroxybenzoate-AMP ligase		YP_608111.1 (61, 76)	ENW75963.1 (55, 82)
ASA_4380 (367)	AsbG, histidine decarboxylase		YP_608112.1 (66, 83)	ENW75964.1 (69, 84)
ASA_4381 (453)	TrmE, tRNA modification GTPase	YP_858697.1 (98, 98)		

[a]Accession number of homologues (% amino acid identity, % amino acid similarity). [b]Pseudogene.

step biosynthetic pathway requiring the presence of four genes (*entABCE* in *Escherichia coli*) encoding the corresponding enzymes. These genes are usually well conserved among many different bacteria.[4]

Aeromonas salmonicida subsp. *salmonicida* (hereafter *A. salmonicida*) is a Gram-negative γ-proteobacteria identified as the causative agent of furunculosis, a devastating disease affecting cultured and wild fish worldwide. The disease causes significant economic losses in cultivated salmonids in fresh and marine waters and also affects a variety of non-salmonid fish.[10] Although siderophore production in this bacterium was known for more than 30 years,[11−13] very little information is available about the chemistry of the siderophore(s) produced and the genetics underlying its synthesis.[14] In a previous work, we demonstrated that *A. salmonicida* produces at least one catechol siderophore related to anguibactin and/or acinetobactin and provided evidence that the siderophore might contain a histamine moiety group.[15] We demonstrate that this system was encoded on a chromosomal gene cluster (*asbGFDCBI*) with high similarity to genes related to the synthesis of the siderophore acinetobactin, from the human pathogen *Acinetobacter baumannii*.[16,17] This gene cluster was present in many strains of *A. salmonicida* isolated from different origins.[15,18] However, the completion of the genome sequence of *A. salmonicida*[19] revealed the presence of a second cluster containing genes with high similarity to those involved in the synthesis and transport of amonabactin in *A. hydrophila*[20,21] and that also contains the genes necessary for DHBA biosynthesis. These two gene clusters would also encode two different outer membrane siderophore receptors (FstB and FstC, respectively) that were identified as iron-regulated proteins in a proteomic study in *A. salmonicida*.[22]

In the present study, we report the identification of the siderophores produced by *A. salmonicida*, which resulted to be acinetobactin and amonabactin, and demonstrate that each of the two gene clusters encodes in fact the synthesis of the respective siderophore, confirming the former prediction based on the analysis of the genome of *A. salmonicida*. Furthermore, we also demonstrated that most strains tested produce both siderophores simultaneously, while certain strains produce only acinetobactin due to a mutation in one of the amonabactin biosynthetic genes.

■ RESULTS AND DISCUSSION

A search of the complete genome sequence of *A. salmonicida* subsp. *salmonicida* strain A449[19] revealed the presence of two gene clusters that show homology to pseudomonine and acinetobactin (ORFs *ASA_4368* to *ASA_4380*; Table 1) and to amonabactin (ORFs *ASA_1838* to *ASA_1851*) siderophore systems (Table 2). The two gene clusters seem to include the functions necessary for the synthesis of these siderophores and for the ferri-siderophores transport through the corresponding outer membrane receptors. Both clusters are also present in all the *A. salmonicida* subsp. *salmonicida* strains whose genome sequences are deposited in the GenBank, and with a nucleotide sequence identity higher than 99% among strains.[23]

Analysis of the Pseudomonine/Acinetobactin Cluster. The gene cluster spanning from ORF *ASA_4368* to *ASA_4380*

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Table 2. Description of the Proteins Encoded by the Genes of the Amonabactin Cluster (Loci Numbers Correspond to the Genome Sequence of Strain A449) in *A. salmonicida* and Homologies to the Corresponding Loci in *A. hydrophila*

Aeromonas salmonicida subsp. salmonicida A449		homology to amonabactin system from *A. hydrophila* (% aa identity, % aa similarity)	
locus (no. aa)	encoded protein, description		
ASA_1838 (392)	EntC, isochorismate synthase	YP_856993.1	(87, 92)
ASA_1839 (556)	EntE, siderophore synthase component E	YP_856992.1	(88, 92)
ASA_1840 (302)	EntB, isochorismatase	YP_856991.1	(93, 95)
ASA_1841 (1029)	AmoF, nonribosomal peptide synthetase	YP_856990.1	(91, 94)
ASA_1842 (259)	EntA, 2,3-dihydroxybenzoate-2,3-dehydrogenase	YP_856989.1	(94, 95)
ASA_1843 (2078)	AmoG, nonribosomal peptide synthetase	YP_856988.1	(85, 89)
ASA_1844 (536)	AmoH, nonribosomal peptide synthetase	YP_856987.1	(86, 90)
ASA_1845 (314)	periplasmic binding protein	YP_856495.1	(94, 96)
ASA_1846 (392)	AmoD, phosphopantetheinyl transferase	YP_856496.1	(71, 78)
ASA_1847 (271)	ABC transporter, ATP-binding protein	YP_856497.1	(93, 95)
ASA_1848 (351)	ABC transporter, permease	YP_856498.1	(92, 96)
ASA_1849 (338)	ABC transporter, permease	YP_856499.1	(97, 98)
ASA_1850 (657)	TonB-dependent siderophore receptor	YP_856500.1	(94, 98)
ASA_1851 (395)	major facilitator family transporter	YP_856501.1	(92, 95)

contains the *asbGFDCBI* genes that we have previously described in strain RSP74.1 as genes involved in the synthesis of an uncharacterized catechol siderophore derived from histamine.[15] This cluster also contains other genes putatively involved in siderophore transport, including the outer membrane receptor FstB (Figure 1). According to *in silico* predictions using BLAST comparisons (Table 1) and the Pfam database,[24] this cluster might encode the synthesis of a siderophore closely related to pseudomonine, a phenolate siderophore produced by *Pseudomonas entomophila*.[25] Indeed, the genes of this cluster show high similarity to pseudomonine genes and an identical gene arrangement (Figure 1, Table 1). However, the *asb* cluster lacks *psmC*, *psmE*, and *psmB* homologues, three genes that encode functions essential to synthesizing activated salicylate from chorismate. In fact, a Δ*pmsCEAB* mutant of *P. entomophila* showed a complete loss of pseudomonine production.[25] In contrast, from our previous works, we know that *A. salmonicida* clearly produces DHBA,[15] an essential component of all catechol siderophores, which indicates that the siderophore produced might have a catechol functional group. It is interesting to note that although the DHBA synthesis genes are usually linked to other genes required for synthesis of catechol siderophores, the *asb* cluster of *A. salmonicida* does not contain genes encoding the synthesis of DHBA. All these observations clearly suggest that in *A. salmonicida* these genes must necessarily be located in another position in the chromosome.

The *A. salmonicida asb* cluster also shows high similarity (Figure 1, Table 1) to the cluster encoding the synthesis of acinetobactin,[17,26,27] a catechol−hydroxamate siderophore that was first described as the siderophore of the human pathogen *Acinetobacter baumannii*[16] and so far not found in any other pathogen. Pseudomonine and acinetobactin have similar structures with the only change of a functional group, salicylate in pseudomonine and DHBA in acinetobactin.[28] The *A. baumannii* acinetobactin cluster harbors the genes necessary for DHBA synthesis,[26,27] except the gene *entA*, that encodes the 2,3-dihydro-2,3-dihydroxy-benzoate dehydrogenase, which is located outside the cluster.[29]

When comparing the genomes of *A. salmonicida* and *A. hydrophila* (Figure 1), it is evident that the acinetobactin cluster represents a clear-cut insertion between the *fhuE* gene (encoding a permease for ferrichrome uptake) and *trmE* gene (encoding a putative tRNA modification GTPase), both common to all species of the genus *Aeromonas*. As mentioned above, the acinetobactin and pseudomonine gene clusters are synthenic, and their encoded proteins show high amino acid similarity values (63 to 90%). However, their G-C content differs (58% for *asb* cluster vs 64% for pseudomonine cluster), showing the *asb* gene cluster having the same G-C content as the rest of the *A. salmonicida* genome (ca. 58%). Altogether, these results suggest that, although pseudomonine and acinetobacin genes would share a common origin, the acinetobactin gene cluster was likely acquired through an ancient gene transfer event at some point during the evolution of *A. salmonicida*. The horizontal transfer of gene clusters encoding the synthesis and transport of siderophores seems to be a common feature in many bacteria.[30−33]

Analysis of the Siderophore Produced by *A. salmonicida* RSP74.1. In order to confirm the functionality of the *asb* cluster as well as the structure of the siderophore produced by *A. salmonicida* strains harboring it, we isolated, purified, and chemically characterized the siderophore present in cell free supernatants from a culture of strain RSP74.1 grown under iron-deficient conditions. We know that this strain produces only one siderophore encoded by the *asb* cluster since a mutation in the *asbD* gene (Figure 1) completely abolishes siderophore production.[15]

To accelerate the activity assay-guided isolation of the siderophores, we employed the methodology based on HLB (hydrophilic−lipophilic balance) cartridges and MS (mass spectrometry) that we successfully applied in the isolation of piscibactin[9] and reisolation of vanchrobactin.[34] Thus, SPE (solid phase extraction) of the cell-free culture supernatants of strain RSP74.1 gave several fractions which were tested for siderophore activity and (+)−LRESIMS. The fraction eluted with acetonitrile/water (8:2) each containing 0.1% TFA (v/v) resulted to be siderophore-active and showed the presence of an intense peak at m/z 347 ($[M + H]^+$), suggesting the presence of acinetobactin (**1**; Figure 2) as it was predicted by the genomic data described above. Final purification of this fraction by HPLC allowed us to isolate a pure compound which UV and NMR spectral data (Figure 3), HRESIMS, and optical rotation matched with those reported for acinetobactin in 1994[16] (Supporting Information Figures S1−S5). In 2009, it was suggested that the reported structure was an unstable intermediate, named preacinetobactin, and the correct structure was proposed as **1**.[28] The structure of acinetobactin as **1**, including its absolute configuration, was confirmed by chemical synthesis.[35] In addition, we found that the pure compound

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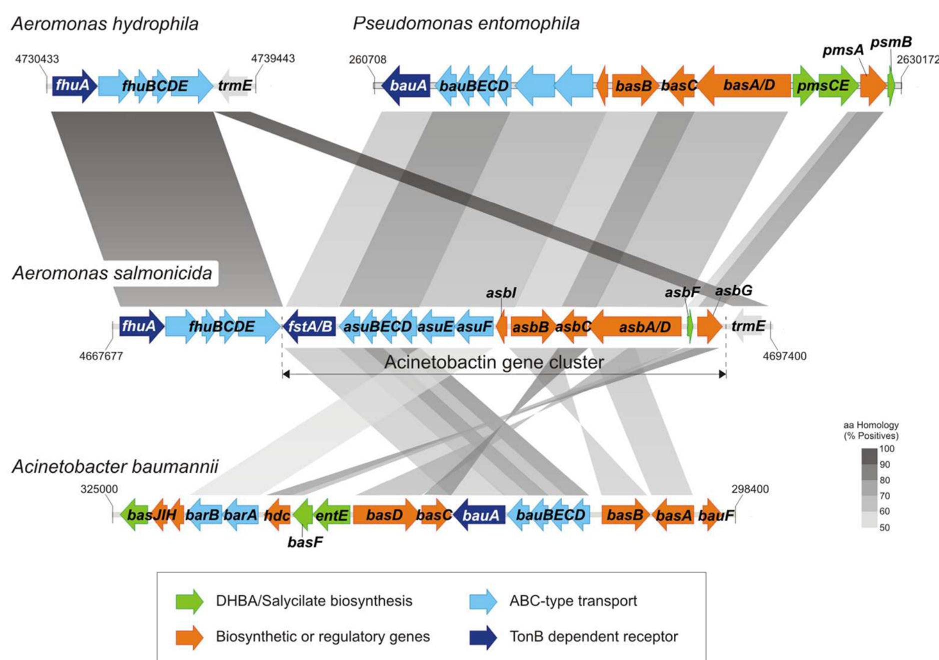

Figure 1. Genetic map of the acinetobactin gene cluster and surrounding genes in *A. salmonicida*, and its comparison to that of *Acinetobacter baumannii* and with the pseudomonine gene cluster from *Pseudomonas entomophila*. Gray blocks link orthologous genes in each cluster. The percentage of amino acid sequence similarity in each protein is represented by different gray tones. The acinetobactin cluster of *A. salmonicida* is inserted between genes *fhuE* and *trmE* shared with *A. hydrophila*. DNA sequences used were those deposited in GenBank: *Aeromonas hydrophila* ATCC 7966 (accession no. NC_008570.1), *Aeromonas salmonicida* A449 (accession no. CP000644), *Pseudomonas entomophila* L48 (accession no. NC_008027), and *Acinetobacter baumannii* ATCC 19606 (accession no. AB101202).

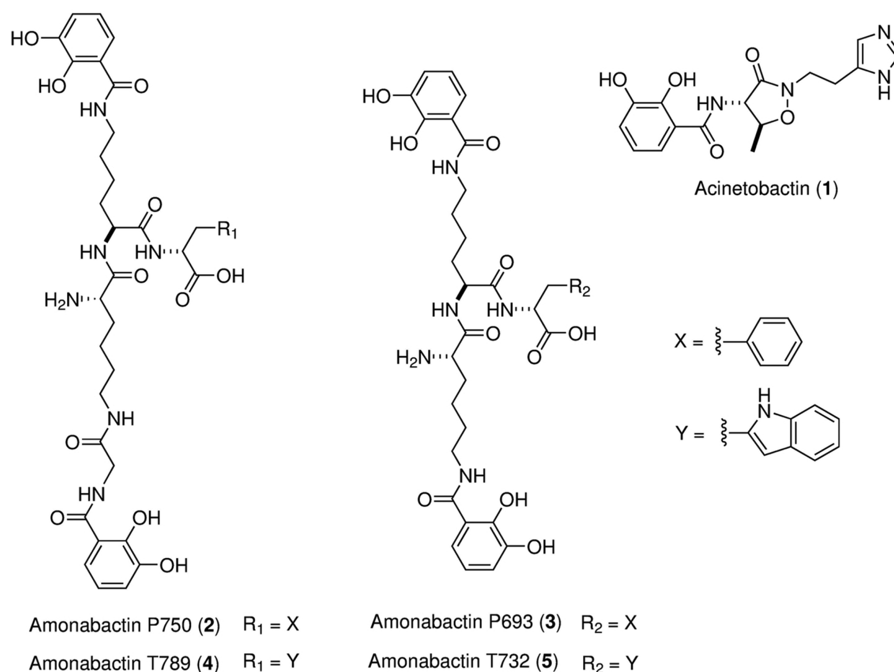

Figure 2. Structures of the siderophores identified in this study: acinetobactin (**1**), amonabactin P750 (**2**), amonabactin P693 (**3**), amonabactin T789 (**4**), and amonabactin T732 (**5**).

isolated could be used as a siderophore by *A. salmonicida* strain RSP74.1Δ*asbD* in plate bioassays (data not shown). Furthermore, acinetobactin was not detected in the supernatants of RSP74.1Δ*asbD* mutant. Thus, all these results clearly demonstrate that *A. salmonicida* produces at least acinetobactin as a siderophore when growing in iron-limiting conditions. To the best of our knowledge, this is the first report of acinetobactin acting as a siderophore in bacteria outside the *Acinetobacter* genus.

Analysis of the Gene Cluster Involved in Amonabactin Biosynthesis. The analysis of the *A. salmonicida* A449 genome revealed a second cluster (ORFs ASA_1838 to ASA_1851) that contains genes with high similarity to those involved in the synthesis and transport of amonabactin, a

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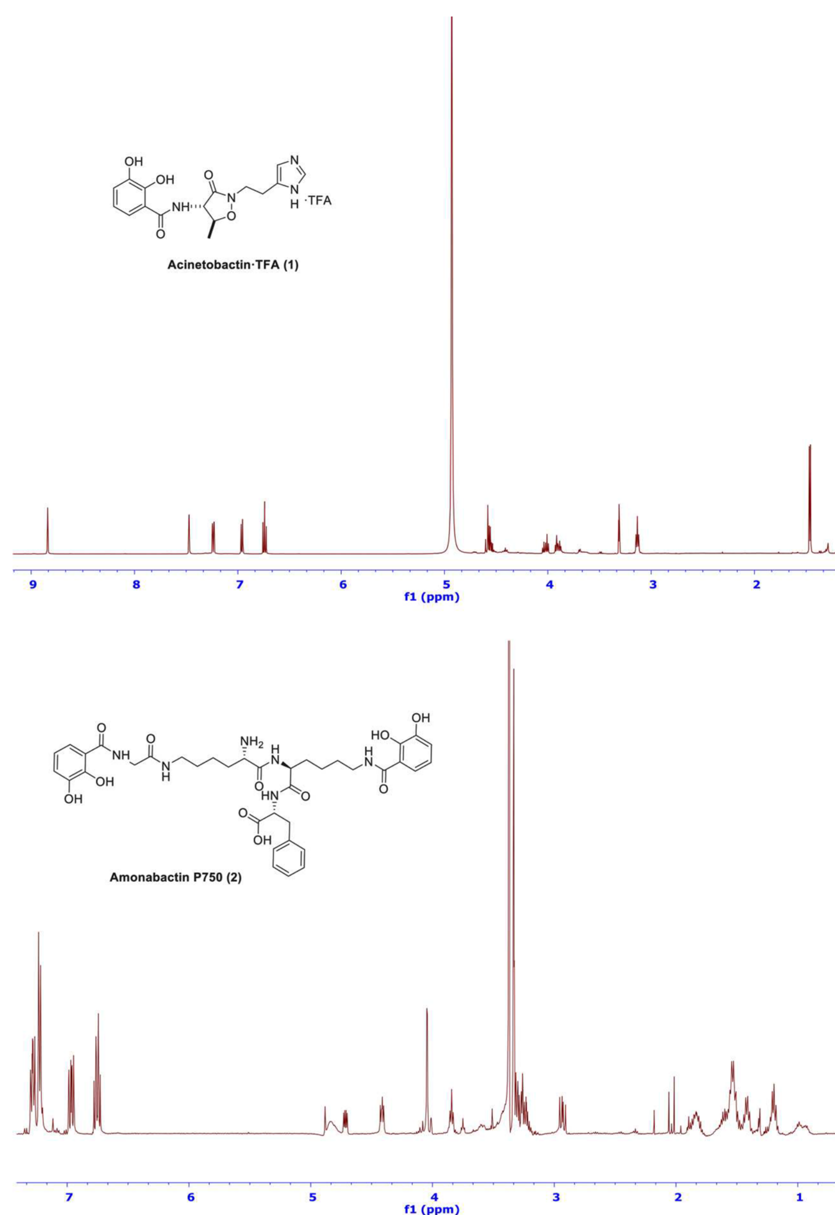

Figure 3. ^{1}H NMR spectra of acinetobactin (1) and amonabactin P750 (2).

catechol isolated from *A. hydrophila*,[20,21] and that would include the genes necessary for DHBA biosynthesis (*entCEBA*).[36] It also includes the genes necessary for siderophore transport (Figure 4). Closed homologues of these genes are extensively present in all *Aeromonas* species for which genomic data are available. We renamed the genes putatively involved in siderophore assembly as *amoF*, *amoG*, *amoH* (encoding nonribosomal peptide synthetases), and *amoD*, that would encode a phospho-pantetheinyl transferase. The percentage of similarity of the *A. salmonicida* proteins with the *A. hydrophila* homologues is above 90% in most of the cases (Table 2). The only noticeable difference between the two species is that in *A. hydrophila* the genes encoding the transport proteins, including the outer membrane receptor, are located in a different locus in the chromosome (Figure 4).

Since, as shown above, strain RSP74.1 produces only acinetobactin, we selected another highly virulent strain of *A. salmonicida* (VT45.1) to study the amonabactin cluster functions. Using the allelic exchange procedure, we constructed several mutants in strain VT45.1 and test the resulting phenotype for growth in iron-deficient conditions, as well as DHBA and siderophore production. We first mutated the *asbD* gene of the acinetobactin cluster in this strain, abolishing acinetobactin synthesis. This mutant showed a phenotype almost identical to the wild-type strain, as it was capable of growth under iron-limiting conditions, produced about the same amount of catechols, and displayed almost the same siderophore activity in the CAS test (Figure 5). This suggests that another siderophore is being produced. When we constructed a single deletion mutant of the *amoG* gene of the amonabactin cluster, we again found that the mutant strain behaved similarly to the parental strain (Figure 5), indicating that acinetobactin was synthesized. However, when we deleted both genes (*asbD* and *amoG*), we observed that the double mutant was almost unable to grow in iron-limiting conditions and the siderophore activity was very low (Figure 5). Overall, these results suggest that strain VT45.1 produces simultaneously acinetobactin and likely amonabactin. In addition, our

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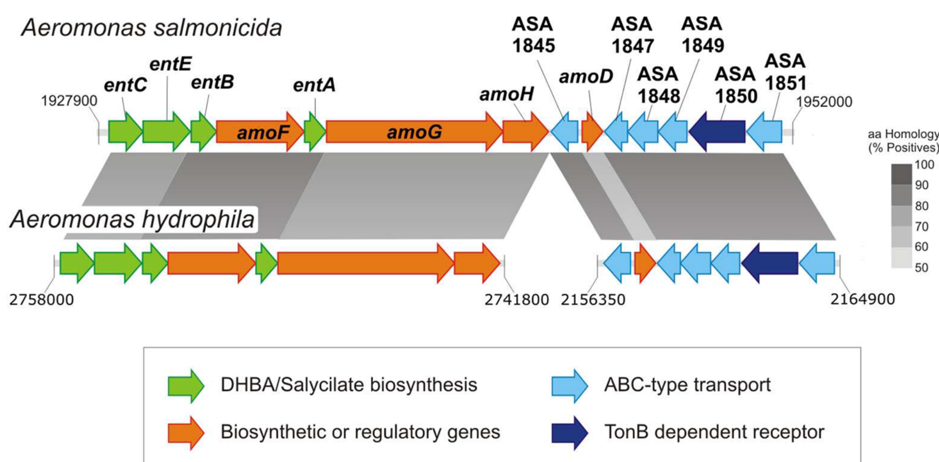

Figure 4. Genetic map of the amonabactin gene cluster in *A. salmonicida* and its comparison with *A. hydrophila*. Gray blocks link orthologous genes in each cluster. The percentage of amino acid sequence similarity in each protein is represented by different gray tones. DNA sequences used were those deposited in GenBank: *Aeromonas hydrophila* ATCC 7966 (accession no. NC_008570.1) and *Aeromonas salmonicida* A449 (accession no. CP000644).

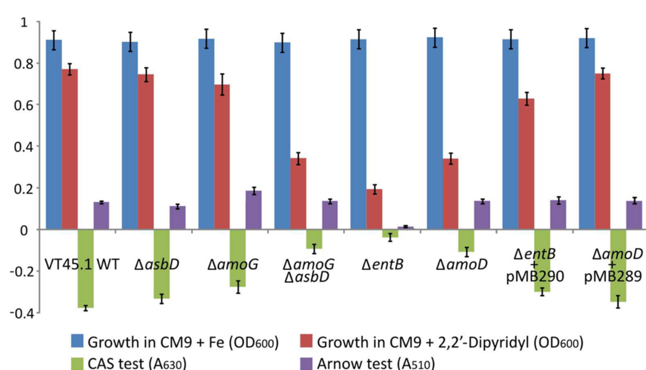

Figure 5. Growth after 18 h of incubation, DHBA production (Arnow test), and siderophore production (CAS assay) of strain VT45.1 (parental strain) and mutant strains for genes *asbD*, *amoG*, *entB*, and *amoD* and the double mutant *amoGasbD*. The results for complemented mutants of *entB* (Δ*entB*/pMB290) and *amoD* (Δ*amoD*/pMB289) are also shown. The bars represent mean values from triplicate experiments. Standard deviation among experiments is shown by error bars.

results clearly suggest that strain RSP74.1, despite harboring an amonabactin synthesis cluster, must likely carry an inactivating mutation in one or more of the genes involved in amonabactin biosynthesis.

Identification of Amonabactins in *A. salmonicida* VT45.1. From the results described above, there is evidence that VT45.1 produces another siderophore in addition to acinetobactin, and according to *in silico* predictions this siderophore might be amonabactin. Therefore, in order to demonstrate that amonabactin is being made by *A. salmonicida*, we used the mutant strain VT45.1Δ*asbD* (impaired in the synthesis of acinetobactin) to isolate the siderophore produced under iron-limiting growth conditions. A cell-free culture supernatant of this strain was subjected to a similar procedure described above for acinetobactin isolation. This method allowed us to isolate two pure compounds which were identified as amonabactin P750 (**2**) and amonabactin P693 (**3**) (Figure 2) by comparison of their NMR (Figure 3) and HRESIMS data with those reported to these compounds.[37] Furthermore, incubation of the cell-free culture supernatants of the VT45.1Δ*asbD* strain with $FeCl_3 \cdot 6H_2O$ and $GaBr_3$, posterior fractionation as described above, and HPLC−HRESIMS in the negative mode experiments allowed us to detect the Fe(III) complexes for amonabactin P750 (**2**), P693 (**3**), T789 (**4**), and T732 (**5**) and Ga(III) complexes for amonabactin P750 (**2**) and P693 (**3**) (Supporting Information Figures S6−S14; Table S1). The pure compounds isolated could be used as siderophores by *A. salmonicida* strain VT45.1Δ*asbD* as demonstrated by agar plate bioassays (data not shown). Thus, we could demonstrate that *A. salmonicida* produces also amonabactins as siderophores when growing in iron-limiting conditions.

Furthermore, to demonstrate that both siderophores are coproduced at the same time, we used supernatants from wild type strain VT45.1 to detect the simultaneous presence of acinetobactin and amonabactins. The results of LC-MS experiments show that both siderophores are present in the extracellular medium of cells grown under iron limitation (Supporting Information Figures S15 and S16). Thus, *A. salmonicida* VT45.1 and similar strains clearly produce, under laboratory conditions, acinetobactin and amonabactins simultaneously.

Acinetobactin and Amonabactin Synthesis Share Biosynthetic Enzymes. The synthesis of catechol siderophores begins with the conversion of chorismate in DHBA. Subsequently, the NRPS selects the different siderophore precursors to form the final structure.[4] In previous sections, we have shown that *asbD* and *amoG* encode essential NRPSs that participate respectively in acinetobactin and amonabactin biosynthesis. To convert chorismate into DHBA, *entABC* homologues are necessary. Once DHBA is synthesized, an EntE homologue begins the assembly process.[4] In the genome of *A. salmonicida* 449, we found homologues of all these genes only within the amonabactin cluster (Figure 4; Table 2), being absent in the acinetobactin cluster, which suggests that acinetobactin and amonabactin production share the DHBA synthesis pathway. To test this hypothesis, DHBA and siderophore production were analyzed in a Δ*entB* mutant from strain VT45.1.

Interestingly, when we deleted *entB* from the amonabactin cluster, the mutant was unable to produce any siderophore. In addition, analysis of the supernatants by the Arnow test revealed that DHBA production was completely abolished

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(Figure 5). Complementation of the mutation, by providing *in trans* an intact copy of *entB* gene, restored both DHBA and siderophore production (Figure 5). This demonstrates that synthesis of acinetobactin and amonabactin depends on the same set of genes necessary for DHBA synthesis and that there are not other genes that can be used instead. In fact, an *in silico* search on the genome of strain A449 does not show any putative orthologs for *entCEB* or *entA* genes.

In addition, an *in silico* analysis of the catalytic domains present in the *asb* cluster shows the lack of a phosphopantetheinyl transferase (EntD homologue), an essential component for activation of PCP domains of the NRPS.[4] In the genome of *A. salmonicida*, there is a single copy of an *entD* homologue, and this gene is located within the amonabactin gene cluster (ASA1846, renamed to *amoD*). Interestingly, when we mutated this gene in strain VT45.1, the resulting phenotype was almost identical to the Δ*entB* mutant (Figure 5). Albeit the Δ*amoD* mutant produces levels of DHBA similar to the parental strain, it was unable to grow under iron limitation, and the amount of siderophores produced was equivalent to that of the double mutant Δ*amoGasbD*. Complementation of the mutation, by providing *in trans* a copy of the *amoD* gene, fully restored the wild type phenotype (Figure 5). All this suggests that *amoD* is an essential gene for acinetobactin and amonabactin synthesis and that it is the only gene encoding this function in *A. salmonicida*. It is noteworthy that the *entD* homologue is also absent from the gene cluster encoding the synthesis of pseudomonine in *Pseudomonas entomophila*, being located in a region of the genome unrelated to siderophore synthesis.[25]

On the basis of the results reported above, and on *in silico* predictions upon analysis of the protein sequences and domains, a proposed biosynthetic pathway for both siderophores in *A. salmonicida* is depicted in Figure 6. Once DHBA is made from chorismate using EntCBA enzymes, part of it would be used by AsbBCD proteins to synthesize acinetobactin from histidine and threonine. At the same time, DHBA could be used by enzymes AmoFGH for the synthesis of amonabactins from lysine and phenylalanine (amonabactins P) or tryptophan (amonabactins T)[37] (Figure 2).

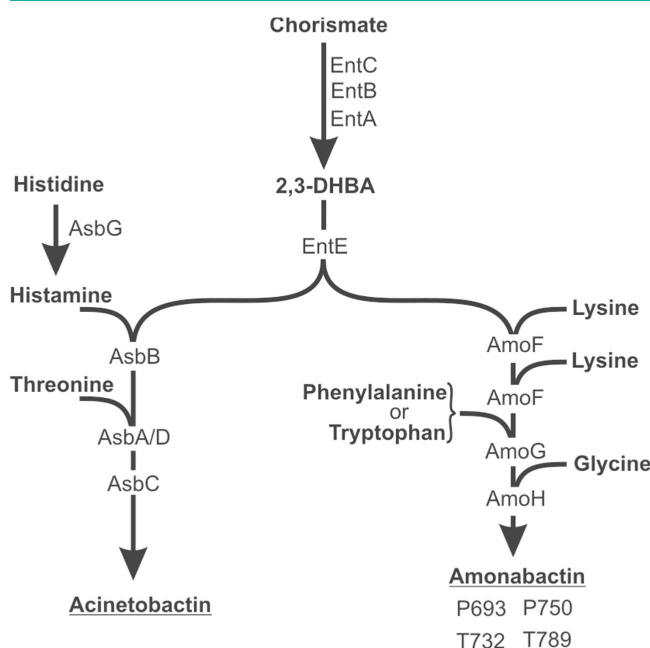

Figure 6. Schematic pathway for the biosynthesis of siderophores acinetobactin and amonabactin in *A. salmonicida* subsp. *salmonicida*. The enzymes putatively involved in each step and the main precursors for each siderophore are indicated.

Some Strains Harbor an Inactivated Copy of the *amoG* Gene. We have previously shown that single-gene mutations within the *asb* cluster in strain RSP74.1 abolish siderophore production,[15] suggesting that this strain produces only one type of siderophore encoded by this cluster. In the present work, we demonstrated that strain RSP74.1 produces acinetobactin as the only siderophore and, although harboring the amonabactin cluster, it is unable to produce amonabactin.

In order to find the reason for this impairment, we fully sequenced the RSP74.1 (accession number KM262645) and VT45.1 (accession number KM262646) amonabactin gene clusters and compared them with the homologous sequence in the genome of strain A449 deposited in GenBank. The results clearly showed that the RSP74.1 *amoG* gene (that encodes the main NRPS involved in amonabactin biosynthesis) has a deletion of 43 bp at the 3′ end of the gene (Supporting Information Figure S17) that would lead to a truncated AmoG protein. An *in silico* analysis of the resulting catalytic domains shows that this truncated protein would not have the PCP domain at the C-terminal end (Figure 7), making it unable to complete the synthesis of amonabactin. This domain is thus intact in strains A449 and VT45.1, and as shown above, strain VT45.1 clearly produces both amonabactin and acinetobactin.

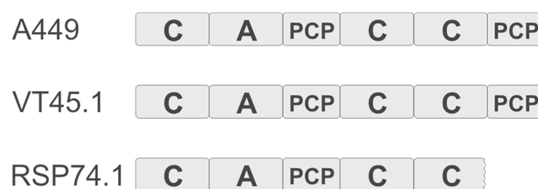

Figure 7. Catalytic domain prediction of the NRPS AmoG in strains A449, VT45.1 (produce amonabactin and acinetobactin), and RSP74.1 (produces only acinetobactin). C, condensation domain; A, adenylation domain; PCP, peptidyl-carrier protein.

To assess whether the presence of this deletion in the *amoG* gene is a unique feature of strain RSP74.1 or if it also occurs in other strains, we tested by PCR the presence of this 43 bp deletion in a collection of 20 *A. salmonicida* strains isolated from a variety of diseased fish. As a result, we found that this feature is present in several strains of this pathogen (Table 3). Interestingly, we could evidence the existence of a correlation between the origin of the strains and the presence of the 43 bp deletion, since all strains that lack this small fragment were isolated from marine fish or fish raised in seawater. Conversely, all the strains producing the two siderophores were isolated from freshwater-raised fish. This deletion was not detected in any of the genomes of *A. salmonicida* subsp. *salmonicida* deposited in the GenBank, suggesting that strains harboring the *amoG* deletion might have a clonal origin. The biological or ecological significance of this deletion that impairs amonabactin synthesis, and its possible restriction to the marine environments, would need further studies in order to find a plausible explanation.

■ CONCLUSIONS

From the results reported here and from the genomic data available at GenBank, we can conclude that the amonabactin

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Table 3. Strains of *A. salmonicida* Isolated from Different Hosts and PCR Analysis of the Presence of the 43 bp Deletion in *amoG* Gene[a]

strain	isolation source	intact (+) or disrupted (−) *amoG* gene	acinetobactin production[b]	amonabactin production[b]
isolated from fish raised in freshwater				
VT45.1	brown trout	+	+	+
Tambre	brown trout	+	+	+
Xella L14,8	brown trout	+	+	+
Ulla2002	Atlantic salmon	+	+	+
XellaR8G	brown trout	+	+	+
Carballiño vivas	brown trout	+	+	+
EO 0805	Atlantic salmon	+	+	+
MAO 1 + 1	brown trout	+	+	+
Neira 18_7_09	brown trout	+	+	+
Ulloa040528−01	Atlantic salmon	+	+	+
XellaR8P	brown trout	+	+	+
X70Riñón	Atlantic salmon	+	+	+
Río Avia	brown trout	+	+	+
007307R	Atlantic salmon	+	+	+
Xella L14,7	brown trout	+	+	+
InvernadoiroO+	brown trout	+	+	+
isolated from fish raised in seawater				
RSP74.1	turbot	−	+	−
SF3 1/03	turbot	−	+	−
RO11.1	turbot	−	+	−
PC884.1	turbot	−	+	−
RM272.1	turbot	−	+	−
EO 0303	Atlantic salmon[c]	−	+	−
R7 190.1	Atlantic salmon[c]	−	+	−

[a]Synthesis of acinetobactin and/or amonabactin is also indicated. [b]assessed by plate bioassays. [c]adults raised in seawater.

synthesis gene cluster is widespread among all species of the genus *Aeromonas*, while acinetobactin synthesis is restricted to *A. salmonicida*, being present in all strains and subspecies for which genomic data are available: *salmonicida*, *achromogenes*, *pectinolytica*, and *masoucida*. From the analysis of the genomic context of acinetobactin cluster, it seems clear that this gene cluster was likely acquired through horizontal gene transfer at some point during the speciation process of *A. salmonicida*, as demonstrated by its insertion between two genes, *fhuE* and *trmE*, that are common to all species of the genus *Aeromonas*. It is noteworthy that the acinetobactin cluster in *A. salmonicida* does not harbor the DHBA synthesis genes, it being the synthesis of acinetobactin dependent on the DHBA synthesis enzymes, as well as of the phosphopantetheinyl transferase encoded by the amonabactin cluster. This feature reinforces the idea that amonabactin is the ancestral siderophore of the species, and that acinetobactin was later acquired from other bacteria. The evolutionary advantage of having two siderophore systems is unclear. Although the strains of *A. salmonicida* having both clusters functional are highly virulent, the strains deficient in amonabactin synthesis were also isolated as causative agents of furunculosis. It has been suggested that the apparent redundancy of iron uptake mechanisms could be an advantage in changeable environments.[38,39] We could also speculate that each one of the siderophores will be preferentially used in specific environments with different iron availability, such as water (freshwater or seawater) and the host tissues. Experiments currently under way will help to determine whether both systems are actually expressed during infection or whether one of them is switched off at some stage inside the host. Future work will also elucidate the precise role of each one of these iron uptake systems in the biology and the virulence of *A. salmonicida* subsp. *salmonicida*.

METHODS

Bacterial Strains, Plasmids, and Media. Strains and plasmids used, as well as those derived from this study, are listed in Supporting Information Table S2. *A. salmonicida* subsp. *salmonicida* strains were grown at 22 °C in Tryptic Soy Agar and Broth (Pronadisa) supplemented with 1% NaCl (TSA-1 and TSB-1, respectively), as well as in M9 minimal medium[40] supplemented with 0.2% Casamino Acids (Difco) (CM9). *Escherichia coli* strains were routinely grown at 37 °C in Luria−Bertani (LB) medium (Pronadisa) or LB supplemented with the appropriate antibiotics. Ampicillin sodium salt (Ap) (Sigma−Aldrich) was used at 50 μg mL^{-1}, tetracycline (Tc) at 12 μg mL^{-1}, and gentamicin (Gm) at 15 μg mL^{-1} (final concentrations). All stocks were filter sterilized and stored at −20 °C. The iron chelator 2,2′-dipyridyl (TCI) was dissolved in distilled water to prepare a stock solution at 20 mM that was added to the sterile media at appropriated concentrations.

DNA Manipulations and Bioinformatics Tools. Total genomic DNA from *A. salmonicida* was purified with the Easy-DNA kit (Invitrogen). Plasmid DNA purification and extraction of DNA from agarose gels were carried out using kits from Fermentas (Thermo-Fisher). PCR reactions were routinely carried out in a T-Gradient Thermal Cycler (Biometra), with *Taq* polymerase BioTaq (Bioline). 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). The NCBI services (http://ncbi.nlm.nih.gov) were used to consult the DNA and protein sequence databases with BLAST algorithm.[41] Prediction of protein domains was carried out by using the Pfam database online facilities (http://www.sanger.ac.uk/Software/Pfam/).[24]

Construction of Mutants by Allelic Exchange. In-frame deletion of *asbD*, *amoG*, *entD* (*amoD*), and *entB* genes in *A.*

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salmonicida subsp. *salmonicida* VT45.1 were constructed as previously described.[15] PCR amplifications of two fragments of each gene and flanking regions (primers listed in Supporting Information Table S3), when ligated together, result in an in-frame (nonpolar) deletion. Each deleted allele construction was ligated into the suicide vector pKEK229,[42] which requires the *pir* gene product for replication and contains the *sacB* gene that confers sucrose sensitivity. The resulting plasmids were mated from *E. coli* S17−1-*λpir*[43] into *A. salmonicida* subsp. *salmonicida* VT45.1 wild type strain (Tc^R) and into previously constructed mutant strains, and exconjugants with the plasmid (Ap^R) integrated in the chromosome by homologous recombination were selected. A second recombination event was obtained by selecting for sucrose (15%) resistance and further checking for plasmid loss and for allelic exchange. This process led to the generation of *A. salmonicida* subsp. *salmonicida* MB158 (Δ*amoG*), MON120 (Δ*asbD*), MB168 (Δ*amoD*), and AVL1 (Δ*entB*) individual mutant strains and the double mutant MB160 (Δ*asbDamoG*) (Supporting Information Table S2). DNA sequencing of the region involved in the deletion was carried out to ensure that all mutations were in-frame. For *entB* and *amoD* gene complementation, the ORFs were PCR-amplified (primers listed in Supporting Information Table S3) with Hi-Fidelity Kapa Taq (Kapa), cloned into pHRP309 vector[44] and mobilized from *E. coli* S17−1 *λ-pir* into the respective *A. salmonicida* defective mutant. Gene *amoD* was cloned along with its own promoter (strain MB272), while *entB* was cloned under control of the *Vibrio anguillarum fvtA* promoter[45] (strain MB273).

Growth under Iron Limiting Conditions and Siderophore Production. To test the ability of *A. salmonicida* subsp. *salmonicida* mutants to grow under iron limited conditions, overnight cultures in LB of the parental and mutant strains were adjusted to an OD_{600} = 0.5 in a UV/vis spectrophotometer (Hitachi) and diluted 1:100 in CM9 medium containing 75 μM 2,2′-dipyridyl. Cultures were incubated at 25 °C with shaking at 150 rpm, and growth (OD_{600}) was recorded after 18 h of incubation. Siderophore production was measured using the chrome azurol-S (CAS) liquid assay.[46] Equal volumes of culture supernatants and CAS solution were mixed, and the A_{630} was measured in the spectrophotometer. In addition, the Arnow test[47] was used for spectrophotometric determination of DHBA concentration. A non-inoculated CM9 sample containing 2,2′-dipyridyl at an appropriate concentration was used as a spectrophotometric blank and as a negative control for CAS and Arnow assays. Siderophore production for purification and structural analysis (see below) was achieved by growing appropriate strains in 1 L of CM9 plus 50 μM 2,2′-dipyridyl in 2 L flasks with continuous shaking at 25 °C for 24 h. After incubation, bacterial cells were pelleted by centrifugation at 10 000 rpm in a Beckman J-21 high speed centrifuge. Supernatants were filtered through a continuous filtration cartridge with a 0.45 μm pore size membrane (Millipore) to remove any cell and organic debris. Siderophore activity present in supernatants was evaluated using the CAS assay.

Extraction, Purification, and Characterization of Siderophores. *Isolation of Acinetobactin (1).* One liter of cell-free culture broth of *Aeromonas salmonicida* subsp. *salmonicida* strain RSP74.1 was lyophilized to give 17 g. One gram of this material was dissolved in water (1 mL) and loaded in a OASIS HLB cartridge (Waters; 35 cm^3, 6 g), which was previously conditioned and equilibrated with 60 mL of acetonitrile (solvent B) and 60 mL of water (solvent A), each containing 0.1% TFA (v/v). It was eluted with the following mixtures of solvent A and solvent B: 1:0, 9:1, 8:2, 7:3, and 0:1. After washing the cartridge with 60 mL of solvent A and 30 mL of mixture of solvents A:B (9:1), the fraction eluted with 60 mL of a mixture of solvents A:B (8:2; 6.4 mg after evaporation to dryness) showed the presence of acinetobactin (**1**) by the (+)-ESI−MS displaying an intense peak at m/z 347 ($[M + H]^+$). Final purification of 2 mg of that fraction by HPLC using a Discovery HS F5 (100 × 4.6 mm, 5 μm) column with a mobile phase consisting of a 40 min gradient from 10 to 50% of solvent B at a flow rate of 1 mL min^{-1} gave 0.8 mg of pure acinetobactin (**1**) isolated as its TFA salt. From 4 g of the initial lyophilized material, repeating the same procedure, 13.4 mg of pure acinetobactin (**1**) were isolated. Compound confirmation was carried out by UV, ^1H NMR, and HRESIMS. Acinetobactin (**1**): $[\alpha]_D^{23}$ = −29.7 (c = 0.65, MeOH,). ^1H and ^{13}C NMR (Supporting Information Table S4). (+)-LR-ESIMS m/z: 347.14 $[M + H]^+$. (+)-HR-ESIMS m/z: 347.1362 $[M + H]^+$ (calcd. for $C_{16}H_{19}N_4O_5$, 347.1350).

*Isolation of Amonabactins P750 (**2**) and P693 (**3**).* One liter of a cell-free culture broth of *A. salmonicida* strain VT45.1Δ*asbD* was lyophilized to give 13.2 g. Part of this material (5.77 g) was dissolved in 6 mL of water, divided in three batches (2 mL each) and loaded into three OASIS HLB cartridges (35 cm^3, 6 g), which were previously conditioned and equilibrated with 60 mL of acetonitrile (solvent B) and 60 mL of H_2O (solvent A), each containing 0.1% TFA (v/v). After washing each cartridge with 60 mL of solvent A, several fractions were eluted with a gradient mixture of solvents A and B: 9:1, 8:2, 7:3, 1:1, and 0:1. Fraction eluted with 60 mL of a mixture of solvents A/B (1:1) gave 54.3 mg of residue after lyophilization. Final purification of this fraction was achieved by HPLC using a Discovery HS F5 (100 × 4.6 mm, 5 μm) column at a flow rate of 1 mL min^{-1} with a mobile phase consisting of a mixture of water (solvent A) and acetonitrile (solvent B), each containing 0.1% TFA (v/v): 15 min gradient from 10 to 60% of solvent B, 5 min gradient from 60 to 100% of solvent B, and finally 2 min isocratic period at 100% of solvent B. Fractions containing the first siderophore were pooled and dried *in vacuo* to afford 5.8 mg of amonabactin P750 (**2**). Other fractions containing the second siderophore were pooled and then purified by HPLC using a Discovery HS F5 (100 × 4.6 mm, 5 μm) column at a flow rate of 1 mL min^{-1} with a mobile phase consisting of an isocratic mixture of 35% of acetonitrile in water (v/v, each solvent containing 0.1% TFA) to give 0.5 mg of amonabactin P693 (**3**). The structures of both compounds were confirmed by UV, NMR, and HRESIMS, the spectral data of which are in concordance with those published for these compounds.[37] Amonabactin P750 (**2**): $[\alpha]_D^{20}$ = +5.8 (c = 0.14, MeOH). (+)-HRESIMS: m/z 751.3286 $[M + H]^+$ (calcd. for $C_{37}H_{47}N_6O_{11}$, 751.3297). Amonabactin P693 (**3**): $[\alpha]_D^{20}$ = +59 (c = 0.02, MeOH). (+)-HRESIMS: m/z 694.3048 $[M + H]^+$ (calcd. for $C_{35}H_{44}N_5O_{10}$, 694.3083).

*Analytical Detection of Amonabactins P750 (**2**), P693 (**3**), T789 (**4**), and T732 (**5**) and Their Fe^{III} Complexes.* One liter of cell-free culture broth from strain VT45.1Δ*asbD* was concentrated *in vacuo* to 0.5 L. A portion of 150 mL was transferred to a round-bottom flask; 100 mg $FeCl_3 \cdot 6H_2O$ was added, and the resulting mixture was gently stirred for 5 min. After incubation at 4 °C overnight, the solution was passed through two OASIS HLB cartridges (35 cm^3, 6 g), which were previously conditioned and equilibrated with 60 mL of acetonitrile (solvent B) and 60 mL of H_2O (solvent A) and, then, eluted with 30 mL using the following mixtures of solvent A/B: 9:1, 8:2, 7:3, 1:1, 3:7, and 0:1. All fractions were concentrated *in vacuo* to 2 mL and submitted to Arnow and siderophore assays. Fractions eluted with the mixture of solvents A/B (9:1 and 8:2) gave positive results to those assays. Then, they were analyzed by HPLC−HRESIMS in the negative mode using a Atlantis dC18 (100 × 4.6 mm, 5 μm) column (Waters) at a flow rate of 1 mL min^{-1} with a mobile phase consisting of a mixture of water and acetonitrile: 35 min gradient from 10 to 100% of acetonitrile, 2 min isocratic period at 100% of acetonitrile, and finally, 5 min from 100 to 10% of acetonitrile. Extracted mass experiments showed the presence of the corresponding $[Fe(Amonabactin)]^-$ and $[Fe(Amonabactin)]^{2-}$ complexes for amonabactin P750 (**2**), amonabactin P693 (**3**), amonabactin T789 (**4**), and T732 (**5**) (Supporting Information Figures S13, S14 and Table S1).

Analytical Detection of Amonabactins P693 and P750 As Their Ga^{III} Complexes. One liter of cell−free culture broth from strain VT45.1Δ*asbD* was concentrated *in vacuo* to 300 mL, adding 61 mg of $GaBr_3$ and then, incubated at 4 °C overnight. The resulting solution was passed through five OASIS HLB cartridges (35 cm^3, 6 g) using a similar procedure as described before. Analysis by HPLC−HRESIMS in the negative mode of the fractions eluted from the HLB cartridges with a mixture of solvents A:B (9:1) and (8:2) showed the presence of the corresponding $[Ga(Amonabactin)]^-$ and $[Ga(Amonabactin)]^{2-}$ complexes for amonabactin P750 (**2**) and amonabactin P693 (**3**) (Supporting Information Table S1).

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Detection of Acinetobactin and Amonabactins in VT45.1 Wild Type Strain. One liter of a cell-free culture broth of VT45.1 wild-type strain was lyophilized to give 14.8 g of material. One gram of this material was submitted to the same SPE methodology described above, and the resulting fractions were analyzed by HPLC-HRESIMS in the positive mode. The fraction eluted with a mixture of solvents A/B (8:2; 29 mg after evaporation to dryness) showed the presence of acinetobactin (**1**; Supporting Information Figure S15). The fraction eluted with a mixture of solvents A and B (1:1; 5.5 mg after evaporation to dryness) showed the presence of amonabactin P750 (**2**) and amonabactin P693 (**3**) (Supporting Information Figure S16).

Monitoring of Active Fractions. Siderophore activity in the different fractions was followed by the CAS test (see above) and by biological assays. For the CAS assay, equal volumes of 1:10 diluted fractions and CAS solution were mixed, and the A_{630}[46] was measured. Biological activity was detected as previously described[9,48] by testing the ability of fractions or purified compounds to promote the growth of *A. salmonicida* indicator strains inoculated in CM9 agar with 130 μM 2,2′-dipyridyl (inhibitory concentration). All fractions and compounds to be tested were adequately dissolved in aqueous solutions at a concentration of 1 mg mL^{-1}, and 10 μL was used to impregnate blank paper disks (6 mm ⌀) that were deposited in the CM9 agar plates. As an indicator strain, we used strain MON15 (RSP74.1Δ*asbD*)[15] for the detection of acinetobactin and amonabactin since this strain does not produce any of these siderophores but can use both of them since it has intact the two outer membrane receptors (FstB and FstC). A growth halo of the indicator strain around a particular sample indicates siderophore activity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00624.

> Figures S1−S5, chemical characterization of acinetobactin; Figures S6−S14, chemical characterization of amonabactins; Figures S15 and S16, detection of simultaneous production of acinetobactin and amonabactins in wild type strain VT45.1; Figure S17, partial DNA sequence of gene *amoG* showing a deletion of 43 bp present in some strains; Table S1, identification of the amonabactin Ga(III) and Fe(III) complexes; Table S2, strains and plasmids used; Table S3, primers used for mutant construction, complementation, and strain screening; Table S4, NMR spectral data for acinetobactin-TFA salt (PDF)

Accession Codes

GenBank accession codes for amonabactin cluster are KM262645 for strain RSP74.1 and KM262646 for strain VT45.1.

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Notes

The authors declare no competing financial interest.

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