Biosynthesis of the acetyl-CoA carboxylase-inhibiting antibiotic, andrimid in Serratia is regulated by Hfq and the LysR-type transcriptional regulator, AdmX

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Summary

Infections due to multidrug-resistant bacteria represent a major global health challenge. To combat this problem, new antibiotics are urgently needed and some plant-associated bacteria are a promising source. The rhizobacterium Serratia plymuthica A153 produces several bioactive secondary metabolites, including the anti-oomycete and antifungal haterumalide, oocydin A and the broad spectrum polyamine antibiotic, zeamine. In this study, we show that A153 produces a second broad spectrum antibiotic, andrimid. Using genome sequencing, comparative genomics and mutagenesis, we defined new genes involved in andrimid (adm) biosynthesis. Both the expression of the adm gene cluster and regulation of andrimid synthesis were investigated. The biosynthetic cluster is operonic and its expression is modulated by various environmental cues, including temperature and carbon source. Analysis of the genome context of the adm operon revealed a gene encoding a predicted LysR-type regulator, AdmX, apparently unique to Serratia strains. Mutagenesis and gene expression assays demonstrated that AdmX is a transcriptional activator of the adm gene cluster. At the post-transcriptional level, the expression of the adm cluster is positively regulated by the RNA chaperone, Hfq, in an RpoS-independent manner. Our results highlight the complexity of andrimid biosynthesis – an antibiotic with potential clinical and agricultural utility.

Introduction

The discovery of antibiotics is one of the main milestones in the history of medicine. However, excessive overuse of antibiotics has encouraged the emergence of multidrug-resistant bacteria, leading to a global increase in the spectrum of untreatable infections, which are currently responsible for around 50,000 annual deaths in Europe and the United States (Woodford et al., 2011; Blair et al., 2015). There is therefore an urgent need to identify new antibiotics, but efforts focussed on discovery and development of new antibiotics have met with only limited success (Lewis, 2013; Pidot et al., 2014). New platforms for antibiotic discovery include the generation of synthetic antimicrobials and development of species-specific antibiotics (Fischbach and Walsh, 2009; Lewis, 2013; Liu et al., 2013; Pidot et al., 2014). It has been estimated that 90% of microbial natural products, and more than 99% of the total number of secondary metabolites, remain to be discovered. Consequently, recent approaches to antibiotic discovery include screening of microbes from new ecological niches and attempts at exploitation of previously uncultured microbes (Fischbach and Walsh, 2009).

Natural products (and their synthetic derivatives) comprise most of the antibiotics used clinically (Newman and Cragg, 2007), many of which are based on non-ribosomal peptides and/or polyketides. Both families of secondary metabolites are synthesised by multifunctional enzymes, known as non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs), through sequential rounds of condensation of amino acids and acyl-CoA building units, respectively (Sattely et al., 2008; Hertweck, 2009). The great structural diversity of non-ribosomal peptides and polyketides results from the number of condensed building units and a range of pre- and post-assembly processing reactions (Sattely et al., 2008; Hertweck, 2009). This chemical diversity is consequently reflected in a broad spectrum of biological activities

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Due to the synthesis of NRPS- and PKS-based second-
et al., 2009; Mendes et al., 2009; Chowdhury et al., 2015). The biological synthesis of such metabolites can be energetically costly and so production is generally highly regulated (Coulthurst et al., 2005; Williamson et al., 2006; Liu et al., 2013). Biosynthetic gene clusters are often linked to their own regulatory genes (Chen et al., 2006; Zhao et al., 2010; Gurney and Thomas, 2011; Liu et al., 2013), the products of which are involved in sensing factors such as physiological state, population density and diverse environmental cues. As a result, the synthesis of the cognate secondary metabolite can be modulated appropriately. Quorum sensing regulatory circuits (Coulthurst et al., 2005; Williamson et al., 2006; Müller et al., 2009; Matilla et al., 2015), two-component systems (Sola-Landa et al., 2003; Haas and Défago, 2005; Williamson et al., 2006), orphan transcriptional regulators (Williamson et al., 2006; Lu et al., 2011; Klaponski et al., 2014) and post-transcriptional regulators (Vogel and Luisi, 2011; Romeo et al., 2013) can all be involved in the regulatory complexity of bacterial secondary metabolite control.

The rhizosphere is one of the most complex environments on earth, with many organisms interacting and competing for nutrients and space (Lugtenberg and Kamilova, 2009; Mendes et al., 2013). Many rhizosphere microbes have evolved the capacity to synthesize bioactive secondary metabolites that allow them to efficiently antagonize diverse niche competitors (Berg et al., 2002; De Vleesschauwer and Höfte, 2007; Raaijmakers et al., 2009;Pidot et al., 2014; Mousa and Raizada, 2015). Consequently, this defines the rhizosphere as a habitat with great potential for exploitation as a source of new natural products with pharmacological, chemotherapeutic and agricultural applications. Serratia plymuthica strains are near-ubiquitous in nature but have been commonly isolated from soil and the rhizosphere of many economically important crops (De Vleesschauwer and Höfte, 2007). Serratia plymuthica strains possess great potential as biocontrol agents by antagonizing the growth of plant-pathogens through the production of diverse bioactive secondary metabolites, siderophores and lytic enzymes (Alström, 2001; De Vleesschauwer and Höfte, 2007; Matilla et al., 2015). The strain used in this study, Serratia plymuthica A153, was isolated from the rhizosphere of wheat (Åstrom and Gerhardson, 1988) and it has been shown to possess bioactivity against fungi, oomycetes, bacteria and nematodes (Thaning et al., 2001; Matilla et al., 2012; Hellberg et al., 2015). These activities are mainly due to the synthesis of NRPS- and PKS-based secondary metabolites, such as the haterumalide, oocydin A (Thaning et al., 2001), and the polyamine antibiotic, zeamine (Hellberg et al., 2015).

Our previous work showed that the strain A153, in addition to zeamine, produces an unidentified second antibacterial compound (Hellberg et al., 2015). In this study, we employed genome sequencing, comparative genomics and mutagenesis approaches to identify the genes involved in the biosynthesis of the unknown secondary metabolite. The regulation of the production of the antibacterial compound was also investigated and the results showed that the expression of the biosynthetic genes is tightly regulated at transcriptional and post-transcriptional levels. Different environmental cues controlling the transcription of the biosynthetic genes were also identified.

Results

Serratia plymuthica A153 produces the hybrid non-ribosomal peptide-polyketide antibiotic, andrimid

Characterization of the biocontrol rhizobacterium, S. plymuthica A153, showed that this strain possesses a strong bioactivity against Bacillus subtilis (Fig. 1A). The observed antibacterial activity was not associated with the production of other known bioactive secondary metabolites produced by A153, namely oocydin A (Matilla et al., 2012) or zeamine (Hellberg et al., 2015).

During the in silico analysis of the A153 genome sequence (Matilla et al., 2016) we identified at least five candidate biosynthetic PKS and NRPS gene clusters which could be responsible for the synthesis of the unknown antibacterial compound. To identify the genes responsible for this bioactivity, a random transposon insertion strain library was constructed and screened for mutants defective in antibacterial activity against Bacillus subtilis. Several transposon insertion mutants showing loss of antibacterial properties were isolated and all the insertions were transduced back into the wild type genetic background using the transducing phage øMAM1 (Matilla and Salmond, 2014) to confirm the link between transposon insertions and mutant phenotype. Random primed PCR confirmed that most of the transposons were located in a hybrid PKS/NRPS gene cluster described previously as responsible for the biosynthesis of the broad-spectrum antibiotic, andrimid (Figs. 1 and Supporting information Fig. S1) (Jin et al., 2006).

We had access to another plant-associated oocydin A producing strain, Serratia marcescens MSU97. This strain also showed strong antibacterial activity towards B. subtilis (Supporting information Fig. S2) and sequencing of its genome (Matilla and Salmond, unpubl. data) revealed that the andrimid (adm) gene cluster is also present in this plant epiphytic bacterium (Supporting information Fig. S3). We reported previously that MSU97 is recalcitrant to various
genetic tools (Matilla et al., 2012) and attempts at isolating mutants defective in the adm gene cluster of this strain were unsuccessful.

Comparative analyses of sequenced andrimid gene clusters

The biosynthesis of andrimid has been demonstrated in a broad range of bacteria (Fredenhagen et al., 1987; Long et al., 2005; Jin et al., 2006; Wietz et al., 2011; Sánchez et al., 2013). However, few adm gene clusters have been sequenced, including those of the marine bacteria, Vibrio nales SWAT-3 (PATRIC Genome ID 391574.12) and Vibrio coralliilyticus S2052 (Machado et al., 2015), and the plant-associated enterobacterium Pantoea agglomerans Eh335 (Jin et al., 2006; GenBank™ Accession No. AY192157.1). Additionally, our genomic analyses revealed that the adm gene cluster is also present in the recently sequenced rhizobacterium Serratia marcescens 90-166 (Supporting information Figs. S3 and S4) (Jeong et al., 2015).

Comparative analyses showed that the genomic context of the sequenced adm gene clusters in A153, MSU97, Eh335, S2052 and SWAT-3 is completely different and, consequently, the upstream and downstream predicted ends of the biosynthetic clusters were assigned based only on their homologies (Supporting information Figs. S3 and S4). These analyses allowed the identification of a gene, designated admV, located immediately upstream of admA. AdmV was not previously associated with andrimid biosynthesis (Jin et al., 2006) and in silico analyses did not shed light on its putative function. However, we found that the gene admV is conserved in all adm gene clusters (Supporting information Figs. S3 and S4). To further investigate its role in the synthesis of andrimid, we constructed an in frame deletion mutant defective in admV, thereby avoiding polar effects in the expression of the downstream adm genes. The resulting mutant strain no longer exhibited antibacterial activity and the bioactivity could be complemented by the in-trans expression of AdmV (Fig. 2).

The adm gene clusters of A153, MSU97, 90-166, Eh335, SWAT-3 and S2052 are 25.1, 24.9, 24.8, 24.7, 25.6 and 25.6 kb, respectively, and they are between 70.1% and 99.0% identical at the DNA level (Supporting information Fig. S4 and Table S1) suggesting that adm biosynthetic clusters may have been moved horizontally between the producing strains. In accordance with this hypothesis, the overall genomic G + C content of A153 (56.0%), MSU97 (58.9%), 90-166 (59.1%), SWAT-3 (44.5%) and S2052 (45.7%) is considerably different from the G + C content of their respective adm biosynthetic clusters, which are 45.8%, 47.6%, 46.27, 51.1% and 51.2%, respectively. Furthermore, remnant sequences of
transposable genetic elements were found flanking the gene clusters of all the producing strains, although located in different regions in these loci (Supporting information Fig. S3). In contrast, some remarkable differences were found between the six biosynthetic clusters. First, the intergenic regions of several adm contiguous genes are not conserved between the adm gene clusters (Supporting information Fig. S4) which could suggest differential regulation in the expression of the biosynthetic clusters. Second, the intergenic region between admS and admT is considerably larger in A153, MSU97 and 90-166, and we identified a putative ORF, admW, in these three Serratia strains (Supporting information Figs. S3 and S4). However, the in frame deletion of admW did not alter the antibacterial activity of A153 (Fig. 1A). Finally, a gene encoding a LysR-type transcriptional regulator was found upstream of admT in the adm gene clusters of Vibrionales SWAT-3 and Vibrio coralliilyticus S2052 (Supporting information Fig. S3).

**The andrimid gene cluster consists of a large polycistronic unit**

The adm gene cluster in Serratia consists of 22 predicted ORFs and its genetic organization, together with the small intergenic distances between contiguous genes, suggests the presence of a single transcriptional unit (Figs. 3A and Supporting information Fig. S3). To further understand the biosynthesis of andrimid, we investigated its transcriptional regulation. With primers spanning the 3’ end of the upstream gene and the 5’ end of the contiguous downstream gene (Fig. 3A), we used reverse transcription-PCR (RT-PCR) to assess co-transcription of the adm genes in S. plymuthica A153. PCR products were obtained across all intergenic regions covering the complete adm gene cluster (Fig. 3B).
Temperature and carbon source regulate the transcription of the adm operon

The biosynthesis of secondary metabolites can be energetically costly and other secondary metabolites produced by Serratia strains have been shown to be highly regulated by various environmental cues (Williamson et al., 2006; Coulthurst et al., 2005). To shed light on the regulation of andrimid production in A153, we investigated the impact of different environmental parameters in the transcription of the adm operon.

At the optimal growth temperature (30 °C) for A153, andrimid production was abolished (Fig. 5A). However, as the temperature decreased, a gradual increase in the production of andrimid was observed, with higher production levels at 15 °C than at 25 °C (Fig. 5A). To examine whether the increase in andrimid production was reflected in the expression of the adm gene cluster, we evaluated the impact of temperature on the transcription of the biosynthetic cluster. β-galactosidase assays showed that the transcription was also thermoregulated (Fig. 5B). In accordance with this, no adm expression was observed at temperatures above 30 °C, confirming the tight correlation between andrimid production and transcription of the biosynthetic operon (Fig. 5B).

Serratia plymuthica A153 was originally isolated from the rhizosphere (Astrom and Gerhardson, 1988), a soil environment which is under the direct influence of plant root exudates. The composition of root exudates is chemically complex but quantitatively it consists mainly of carbon-based compounds, primarily organic acids and sugars (Uren, 2007; Badri and Vivanco, 2009; Suzuki et al., 2009). Thus, we investigated the production of andrimid in response to different carbon sources found in plant root exudates. Our results showed that andrimid is differentially produced depending on carbon source. Maximal antibacterial activity was observed in the presence of citrate, gluconate or glycerol whereas no activity was detected in the presence of arabinose or succinate as sole carbon sources (Fig. 6A). Although the growth rates of A153 varied between the tested carbon sources (Supporting information Fig. S5), the observed antibacterial activity was not correlated with the growth rate per se or the final optical density reached in each of the culture media. Consistent with these observations, higher transcriptional levels of the adm operon were observed in media containing citrate or gluconate, whereas no transcription was seen in arabinose media (Fig. 6B), confirming that both transcriptional activity and andrimid production, were carbon source-dependent.

Finally, we also evaluated the production of andrimid at different pH, aeration conditions and NaCl concentrations, but no impacts on antibacterial synthesis were observed (not shown).

The transcription of the adm biosynthetic cluster is growth phase dependent

We identified several mutants in which the Tn-KRCPN1 transposon generated β-galactosidase transcriptional fusions (Table 1). Because our RT-PCR analyses demonstrated the presence of an operonic adm biosynthetic cluster, we investigated transcription throughout growth in a Lac’ derivative of A153. Using a chromosomal fusion located in the first multidomain PKS/NRPS-encoding gene of the biosynthetic cluster (admK) our β-galactosidase assays showed that the transcription of the adm operon started in late-logarithmic phase of growth (Figs. 4A and 5B). Expression of the biosynthetic cluster correlated perfectly with the presence of andrimid in cell-free supernatants (Fig. 4B).

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The LysR-type regulator, AdmX, activates andrimid production

Analysis of the genomic context of the adm gene clusters in A153, MSU97, 90-166, Eh335, S2052 and SWAT-3 revealed a 2 kb region upstream of admV which was highly conserved in the Serratia strains (Supporting information Figs. S3 and S4). This region contained a gene encoding a putative LysR-type transcriptional regulator (LTTR; Supporting information Figs. S3 and S4). The family of LTTRs comprises one of the largest classes of transcriptional regulators in bacteria, functioning either as repressors or activators of the transcription of their target genes (Maddocks and Oyston, 2008). To investigate the potential role of the identified LTTR in andrimid
biosynthesis, we engineered an in frame deletion mutant in S. plymuthica A153. The resulting mutant did not inhibit growth of B. subtilis but antibacterial activity was fully restored by in trans expression of the LTTR encoding gene (Fig. 7A). These results suggested that the regulator, designated AdmX, is responsible for activating transcription of the adm gene cluster and β-galactosidase assays confirmed that expression of the biosynthetic cluster was abolished in an admX-deficient strain (Fig. 4A). These results were supported by qPCR analyses showing decreased adm transcript levels in the A153 ΔadmX strain (Supporting information Fig. S6).

To assess the transcription of admX throughout growth, we constructed a transcriptional fusion of the admX promoter to the reporter gene lacZ. β-galactosidase assays showed that the transcription of admX started in late-logarithmic phase of growth, correlating with transcription of the adm biosynthetic cluster (Supporting information Fig. S7). We explored the effect of AdmX on expression of its cognate gene. However, the transcription of admX

Fig. 5. The production of andrimid in Serratia plymuthica A153 is temperature-dependent and correlates with the expression of the adm gene cluster.
A. Halos of antibiosis against Bacillus subtilis of filter-sterilized supernatants of A153 strain JH6 (zeamine negative) grown in LB at different temperatures. The bioassays were repeated at least three times, and representative results are shown.
B. β-Galactosidase activity (filled symbols) throughout growth measured from the chromosomal fusion admK::lacZ in Serratia plymuthica A153 LacZ. Open symbols represent bacterial growth. Data are the mean and standard deviation of three biological replicates. Doubling times at 20, 25, 30 and 35 °C were 66.8 ± 0.4, 47.3 ± 0.7, 40.7 ± 0.4 and 40.5 ± 0.2 minutes respectively.

Fig. 6. Effect of carbon source on the production of andrimid and expression of the adm gene cluster in Serratia plymuthica A153.
A. Halos of antibiosis against Bacillus subtilis of filter-sterilized supernatants of A153 strain JH6 (zeamine negative) grown in minimal medium with different carbon sources. The bioassays were repeated at least three times, and representative results are shown. All the carbon sources were used at a final concentration of 15 mM. With the exception of lactose, all carbon sources used are frequently found in plant root exudates. Bars, 5 mm.
B. β-galactosidase activity (filled symbols) throughout growth measured from the chromosomal fusion admK::lacZ in Serratia plymuthica A153 LacZ in minimal medium with different carbon sources. Open symbols represent bacterial growth. Data are the mean and standard deviation of three biological replicates. Growth and doubling times of A153 in all the carbon sources used are shown in Fig. S5.

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remained unaltered in an admX mutant strain (Supporting information Fig. S7). Finally, we also investigated whether andrimid thermoregulation is mediated by AdmX. Previous studies showed that the expression of another LTTR (PecT), and consequently the expression of its target genes, is thermoregulated (Héraut et al., 2014). However, no differences in transcripts levels of admX were observed (using qPCR analysis) at 25, 30 and 37 °C (data not shown).

**The chaperone Hfq regulates the expression of the adm gene cluster**

The biosynthesis of secondary metabolites is frequently regulated at post-transcriptional level and, in gammaproteobacteria, this is mainly mediated by the Csr/Rsm (Romeo et al., 2013) and Hfq (Vogel and Luisi, 2011) systems. To assess a potential role of these post-transcriptional regulatory systems, we analysed the production of andrimid in different genetic backgrounds in *S. plymuthica* A153. A mutant with a deletion in the non-coding sRNA, *csrB*, showed the same antibacterial activity as the wild type strain (not shown). However, in frame deletion of *hfq* led to complete loss of andrimid production (Fig. 7B) and this phenotype was partially complemented by the *in trans* expression of *hfq* (Supporting information Fig. S8).

β-galactosidase assays were performed to assess the expression of the *adm* gene cluster throughout growth in A153 Δhfq. The results showed that transcription of the andrimid operon was abolished in a *hfq*-deficient strain, indicating that the chaperone Hfq positively regulates expression of the *adm* biosynthetic cluster (Fig. 4A). qPCR analyses confirmed that the transcriptional levels of the *adm* gene cluster were reduced by 99.9% in an *hfq*-deficient background (Supporting information Fig. S6). To investigate whether Hfq regulates *admX* expression, qPCR experiments were also performed. However, *admX* transcript levels were unaltered in the Δhfq mutant (Supporting information Fig. S6).

The expression of the *adm* gene cluster starts in late-logarithmic phase of growth (Fig. 4) and so we investigated whether quorum sensing (QS) could be regulating andrimid production. A153 carries *sptI*, *sptR*, *splR* and *spsR* genes encoding LuxI- and LuxR-type proteins very similar to those of the three QS systems identified in the taxonomically related rhizobacterium, *S. plymuthica* G3 (Liu et al., 2011; Duan et al., 2012). However mutations in each of these four genes in A153 had no observable impacts on antibacterial activity (not shown). Similarly, the role of the stationary phase sigma factor, RpoS, in the production of andrimid was also investigated, but the antibacterial activity of an *rpoS* mutant was indistinguishable from that of the wild type strain (Fig. 7B).

**Discussion**

Since the golden age of antimicrobial discovery in the 1940–1960s, there has been a diminution in the rate of discovery of new antibiotics and this problem has been exacerbated by the progressive emergence worldwide of antibiotic-resistant bacteria (Lewis, 2013; Blair et al., 2015). However, there are many antibiotics described in the literature that, although not currently exploited, could prove to be lead molecules for drug discovery programmes.
leading to clinical or agricultural utility. The hybrid NRPS/PKS antibacterial compound, andrimid, was first isolated in the late 1980s from a brown planthopper intracellular symbiont belonging to the *Enterobacter* genus (Fredenhagen *et al.*, 1987). After its discovery, three related antibacterial compounds, moiramides A-C (Supporting information Fig. S1), were also isolated from *Pseudomonas fluorescens* (Needham *et al.*, 1994).

Andrimid is a nanomolar inhibitor of the bacterial acetyl-CoA carboxylase (Freiberg *et al.*, 2004), an enzyme responsible for the conversion of acetyl-CoA to malonyl-CoA in the first committed step for the synthesis of fatty acids (Harwood, 2007). The discovery of the andrimid target, together with its unusual chemistry, has stimulated interest in the biochemistry and genetics of the antibiotic. The andrimid gene cluster was first described almost a decade ago and a model for its biosynthesis proposed (Jin *et al.*, 2006). In this model, the function for most of the Adm proteins was predicted and, in some cases, later demonstrated biochemically (Fortin *et al.*, 2007; Magarvey *et al.*, 2008; Ratnayake *et al.*, 2011). However, the roles of the hypothetical proteins AdmB, AdmL and AdmN in the synthesis of andrimid remain unknown. In this study, we redefined the length of the *adm* gene cluster by identifying a new hypothetical protein encoding gene, *admV*, located immediately upstream of *admA* and forming part of the *adm* operon (Figs. 3 and Supporting information Fig. S3). Using mutagenesis and complementation analyses we demonstrated that *admV* is required for the biosynthesis of andrimid. Our bioinformatic analyses did not categorically clarify the function of *admV*. However, *in silico* studies predicted three alpha helices with a high content of positively charged amino acids at the C-terminal end, suggesting that *admV* may be a DNA binding protein.

Andrimid has been shown to be a broad-spectrum antibacterial compound that acts on Gram-positive and Gram-negative bacteria. It was first isolated after it was shown to have potent activity against the different pathovars of the phytopathogen, *Xanthomonas campestris* (Fredenhagen *et al.*, 1987) but further research showed that it is also active against bacterial pathogens belonging to *Bacillus*, *Enterococcus*, *Escherichia*, *Salmonella*, *Staphylococcus*, *Vibrio* and *Yersinia* genera (Needham *et al.*, 1994; Singh *et al.*, 1997; Long *et al.*, 2005; Wietz *et al.*, 2010; Sánchez *et al.*, 2013). Resistance to andrimid is dependent on multi-drug efflux pumps (Freiberg *et al.*, 2004; Jin *et al.*, 2006) or the presence of andrimid-resistant acetyl-CoA carboxyltransferases (ACC) hypothesized to show decreased affinity for the inhibitor (Liu *et al.*, 2008). Interestingly, we showed that the plant-pathogen *Agrobacterium tumefaciens* and multiple rhizobacteria strains in the family Enterobacteriaceae (i.e., *Kluvyera*, *Pantoea*, *Serratia*, *Weeksella*, *Xenorhabdus*, and the emerging phytopathogen, *Dickeya solani*) (Supporting information Figs. S9 and S10) are sensitive to andrimid. However, the differential sensitivities to the antibiotic of these plant-associated strains could also indicate the presence of weaker intrinsic resistance mechanism(s) of different efficiencies.

NRPS and PKS constitute the main enzymatic source of secondary metabolites in bacteria; 36% of which are generated by hybrid NRPS/PKS gene clusters (Wang *et al.*, 2014). Secondary metabolites have been associated with important roles in the physiology and development of the bacterial host, but are also implicated in overcoming competitors in the same nutritional niche (Liu *et al.*, 2013; Pidot *et al.*, 2014; Wang *et al.*, 2014; Mousa and Raizada, 2015). The synthesis *in situ* of andrimid has been demonstrated in a sponge-associated bacterium (Oclarit *et al.*, 1994; Long *et al.*, 2005) and its production is favoured over that of other metabolites in conditions mimicking natural environments - suggesting an important ecophysiological role of andrimid (Wietz *et al.*, 2011). Consistent with this view, even sub-lethal concentrations of andrimid can promote a negative chemotactic response (in sensitive competitors) away from the antibiotic, thereby enhancing competitiveness of the producers (Graff *et al.*, 2013). In *S. plymuthica* A153, the synthesis of andrimid is carbon source-dependent and induced in the presence of sugars and organic acids commonly found in plant root exudates (Fig. 6). Carbon source has been shown to regulate antibiotic synthesis at both molecular and physiological levels and, in general, rapidly metabolized carbon sources are involved in repression of bacterial secondary metabolites (Sánchez *et al.*, 2010). However, our results did not show a correlation between preferred carbon sources and andrimid production (Figs. 6 and S5). Importantly, in A153, the production of the antibiotic zeamine is high in the presence of carbon sources which show low andrimid production (i.e., arabinose, sorbitol and succinate) whereas the synthesis of zeamine is repressed by carbon sources that stimulate andrimid production (i.e., citrate, gluconic acid and glycerol) (Fig. 6; Hellberg *et al.*, 2015). This "mirror image" metabolic regulation of andrimid and zeamine production when presented with different environmental carbon sources could be beneficial in maintaining the capacity of A153 to overcome bacterial competitors in its natural niche, the rhizosphere, where specific carbon source availability is likely to fluctuate.

Soil bacteria are subjected to daily and seasonal abiotic variations and temperature is considered a key abiotic factor influencing bacterial metabolic activity – which may therefore modulate inter- and intra-specific interactions between microbes. The results reported here show that andrimid synthesis in A153 is thermoregulated, with enhanced antibiotic production at lower temperature. Previous studies also showed that andrimid production in *Vibrio* (Long *et al.*, 2005; Wietz *et al.*, 2011) and *Serratia* strains (Sánchez *et al.*, 2013) is also thermosensitive. In
this study, we demonstrate that the observed thermoregulation is exerted at the transcriptional level (Fig. 5) and in an AdmX-independent fashion. During the analysis of the bioactive properties of A153, we showed that the production of the haterumalide, oocydin A, is also thermosensitive (Matilla and Salmond, unpubl. data) and this may indicate that a common control pathway is modulating the thermoregulation of the biosynthesis of secondary metabolites in A153. The ecological role of this thermosensitivity is unknown, although it could be related to competitiveness and efficient colonization of specific niches by the producing rhizobacterium, S. plymuthica A153.

To date, most of the published research on andrimid has focussed on its biochemistry, but the regulation of its biosynthesis has attracted little attention. The first adm gene cluster was identified in Pantoea agglomerans but no regulatory proteins were found in this biosynthetic cluster (Jin et al., 2006). However, our analysis of the genomic context of the andrimid gene cluster in A153, MSU97 and 90-166, revealed a LysR-type regulator-encoding gene, admX, which was highly conserved in these producing strains (Supporting information Figs. S3 and S4). Mutagenesis, complementation and gene expression analyses demonstrated that AdmX is a positive regulator of andrimid biosynthesis (Figs. 4 and 7A). AdmX is a 305 amino acid protein composed of a helix-turn-helix motif-containing DNA-binding domain and a probable effector binding domain at the N-terminal and C-terminal regions, respectively (Supporting information Fig. S11). By analogy with other LTTR structures (Ezezika et al., 2007; Monferrer et al., 2010; Devesse et al., 2011), AdmX is proposed to possess a potential effector binding site located in between the two lobes of the effector binding domain (Supporting information Fig. S11). Inspection of a homology model shows that this site is primarily composed of amino acids with hydrophobic side chains (Supporting information Fig. S11) and further investigations are necessary to identify candidate effector molecules.

In general, LysR transcriptional regulators activate the expression of their target genes while negatively autoregulating their own transcription (Maddocks and Oyston, 2008). LTTRs have been shown to regulate genes involved in metabolism, virulence, motility, chemotaxis, quorum sensing and biofilm formation (Maddocks and Oyston, 2008). However, only a limited number of LTTRs have been found to regulate the biosynthesis of secondary metabolites – such as actinorhodin (Mao et al., 2013), phenazines (Klaponski et al., 2014), pyoluteorin (Li et al., 2012), ralfuranones (Kai et al., 2014) and undecylprodigiosin (Mao et al., 2013). LTTRs are broadly distributed within the prokaryotic kingdom, suggesting dissemination and acquisition by horizontal gene transfer (HGT; Maddocks and Oyston, 2008). In accordance with this notion, sequences reminiscent of transposases were found flanking admX (Supporting information Figs. S3 and S4) and the G + C content of the admX genes in A153, MSU97 and 90-166 is considerably higher than that of their respective adm gene clusters. Although AdmX seems to be restricted to the adm biosynthetic clusters present in Serratia strains, BLAST analyses showed that AdmX is up to 84% identical (90% similar) to an orphan LysR-type regulator highly conserved within Enterobacter and Klebsiella genera, again suggesting that this LTTR-encoding gene may have been acquired by HGT. Interestingly, we found an LTTR-encoding gene immediately upstream of admT in the adm gene clusters of Vibrio coralliilyticus S2052 and Vibrionales SWAT-3 (Supporting information Fig. S3), perhaps indicating an important regulatory role in the expression of the adm gene clusters in these strains. It has been proposed that the fragmented andrimid biosynthetic pathway reflects a recent evolutionary origin (Magarvey et al., 2008) and so the insertion of the LTTR-encoding genes in the adm gene clusters of Vibrio and Serratia may represent a step forward in the evolution of these biosynthetic clusters.

The post-transcriptional regulation of the adm gene cluster in A153 was also investigated and our results showed that the RNA binding protein, Hfq, positively regulates the expression of the andrimid operon. Previous studies of Serratia strains showed that the synthesis of secondary metabolites such as a carbapenem antibiotic (Willf et al., 2011), prodigiosin (Willf et al., 2011) and pyrrolnitrin (Zhou et al., 2012) is regulated by Hfq, and we recently showed that Hfq positively regulates the expression of the oocydin A (Matilla et al., 2015) and zeamine (Hellberg et al., 2015) gene clusters in S. plymuthica A153. It is known that Hfq also stimulates rpoS translation (Vogel and Luisi, 2011) and the deletion of hqf results in reduced rpoS transcripts levels in A153 (Matilla et al., 2015). However, contrary to our observations on regulation of oocydin A in A153 (Matilla et al., 2015), Hfq-mediated regulation of andrimid is independent of RpoS (Fig. 7B), as observed in other Serratia strains for the biosynthesis of zeamine (Hellberg et al., 2015), carbapenem (Willf and Salmond, 2012) and prodigiosin (Willf and Salmond, 2012) antibiotics.

In summary, we have shown that the plant-associated bacterium, Serratia plymuthica A153, produces the broad spectrum antibacterial compound, andrimid. Comparative genomics, molecular genetics and transcriptional approaches led us to re-define the borders of the adm gene clusters and identify new genes involved in the biosynthesis and regulation of andrimid. Further, in vivo assays expanded the spectrum of bacterial strains which are sensitive to andrimid. For the first time, the regulation of the biosynthesis of andrimid was investigated at transcriptional and post-transcriptional levels. Future research will provide information about the effectors recognized by AdmX and such knowledge, coupled with the work described in this study, may encourage the use of plant-
Experimental procedures

Bacterial strains, culture media and growth conditions

Bacterial strains used in this study are listed in Table 1 and Supporting Information Table S2. Serratia, Bacillus, Agrobacterium, Dickeya, Kluvyeria, Pantoeea, Weeksella, Xanthomonas, Xenorhabdus, Yersinia and their derivative strains were grown routinely at 30 °C, unless otherwise indicated, in Luria Broth (LB; 5 g yeast extract l−1, 10 g Bacto tryptone l−1 and 5 g NaCl l−1) or minimal medium (0.1%, w/v, (NH4)2SO4, 0.41 mM MgSO4, 40 mM K2HPO4, 14.7 mM KH2PO4, pH 6.9–7.1) with glucose (0.2%; w/v) as carbon source, unless otherwise indicated. Escherichia coli strains were grown at 37 °C in LB. Escherichia coli DH5x was used as a host for gene cloning. Media for propagation of E. coli J2163 were supplemented with 300 μM 2,6-diaminopimelic acid. When appropriate, antibiotics were used at the following final concentrations (in μg ml−1): ampicillin, 100; chloramphenicol, 25; kanamycin, 25 (E. coli strains) and 50 (Serratia strains); streptomycin, 50; tetracycline, 10. Sucrose was added to a final concentration of 10% (w/v) when required to select derivatives that had undergone a second crossover event during marker-exchange mutagenesis. Bacterial growth (OD600 nm) was measured on a Unicam HeI:ios spectrophotometer at 600 nm, 1 cm path length.

In vitro nucleic acid techniques and bioinformatic analyses

Plasmid DNA was isolated using the Anachem Keyprep plasmid kit. For DNA digestion, the manufacturer’s instructions were followed (New England Biolabs, Roche and Fermentas). Separated DNA fragments were recovered from agarose gels using the Anachem gel recovery kit. Ligation reactions and total DNA extraction were performed as previously described (Sambrook et al., 1989). Competent cells were prepared using calcium chloride and transformations were performed by standard protocols (Sambrook et al., 1989). Phusion® high fidelity DNA polymerase (New England Biolabs) was used in the amplification of PCR fragments for cloning. PCR reactions were puriﬁed using the Anachem PCR Clean-up kit. PCR fragments were veriﬁed by DNA sequencing that was carried out at the University of Cambridge DNA Sequencing Facility (Cambridge, UK) or at the Institute of Parasitology and Biomedicine Lopez-Neyra (CSIC; Granada, Spain). Sequence comparison analyses were performed employing the wgVISTA online tool (Frazer et al., 2004). Open reading frames (ORFs) in the andrimid gene clusters were predicted using Glimmer 3.0 (Delcher et al., 1999). Blast analyses were used for the functional gene assignment. Protein domain organization was identiﬁed using the NCBI conserved domains database. Multiple sequence alignments were carried out with ClustalW2 (European Bioinformatics Institute). Artemis software (Wellcome Trust Sanger Institute) was used to visualize genomic sequences.

Random transposon mutagenesis

Random transposon mutagenesis of S. plymuthica A153 using Tn-KRCPN1 was performed by biparental conjugation mating using Escherichia coli J2163, as described previously (Matilla et al., 2012). In total, three thousand kanamycin-resistant insertion mutants were screened for their antibacterial activity against Bacillus subtilis using dual drop culture bioassays. Auxotrophic mutants were discarded and insertion mutations were transduced into the wild type strain A153 using phage λMAM1 (Matilla and Salmond, 2014). The insertion site of transposon Tn-KRCPN1 in mutants of interest was determined using random primed PCR following the method described previously (Matilla et al., 2012) and using primers described in Supporting Information Table S3.

Antibacterial assays

Antibiotic activity was tested using agar lawn assays. Briefly, indicator plates for andrimid production contained a 0.8% LB agar (LBA) top lawn containing 200 μl of an overnight culture of the bacterial strain to test. Five microliters of overnight cultures of the andrimid-producing strains were spotted on the surface of the indicator agar lawn and incubated for 48 h at 25 °C, unless otherwise indicated. To determine andrimid levels in bacterial supernatants, culture samples were taken, bacterial cells were pelleted by centrifugation (10,000 x g, 10 min), and the supernatant was ﬁltered (0.2 μm). Three hundred microliters of the filter-sterilized supernatant were added to wells cut into the LBA plate and incubated at 25 °C for 24 h. All experiments were repeated at least three times.

Construction of strains and plasmids

Chromosomal mutants of Serratia plymuthica strains were constructed by homologous recombination using derivative plasmids of the suicide vector pKNG101. These plasmids, which are listed in Supporting Information Table S4, were conﬁrmed by DNA sequencing and they carried mutant in-frame deletions for the replacement of wild type genes in the chromosome. Primers used in this study are listed in Supporting Information Table S3. In all cases, plasmids for mutagenesis were transferred to S. plymuthica strains by triparental conjugation using E. coli CC118:pir and E. coli HH26 (pNJ500) as helper. The plasmids for the construction of the in-frame deletion mutants were generated by amplifying the up- and downstream ﬂanking regions of the gene, or domain to be deleted. The resulting PCR products were digested with the enzymes speciﬁed in Supporting Information Table S4 and ligated in a three-way ligation into pUC18Not, previously cloned into the marker exchange vector pKNG101. The in-frame deletion mutant strains ANDX, ANDV and ANDW were generated using plasmids pMAMV175, pMAMV191 and pMAMV192, respectively. Mutant strains defective in hfq, A153H and A153HL, were constructed using plasmid pMAMV193. All relevant mutations were conﬁrmed by PCR and sequencing.

For the construction of the complementing plasmids, the genes were ampliﬁed using primers described in Supporting Information Table S3 and cloned into pTRB30. All the inserts

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were confirmed by PCR and sequencing. Complementing plasmids were used to transform A153 by electroporation.

Genetic complementation assays
Complementation of mutations was carried out by the introduction of a wild type copy of the corresponding mutated gene in trans on plasmid pTRB30. For the complementation assays, LBA containing the appropriate antibiotic (to maintain the plasmid) and isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.1 or 1 mM were added to holes punched in Bacillus subtilis bioassay plates. Then, 5 µl of overnight cultures of the selected strains were spotted on the surface of the LBA containing the antibiotic and IPTG and were incubated at 25 °C for 2 days.

Generalized transduction
The generalized transducing viunalikevirus, φMAM1, was used for transduction of chromosomal mutations, as described previously (Matilla and Salmond, 2014).

β-Galactosidase assays
Expression of the lacZ reporter gene was performed using the fluorogenic substrate 4-methylumbelliferyl β-D-galactoside (Melford Cat No. M1095) at a final concentration of 0.125 mg ml⁻¹, as described previously (Ramsay, 2013). Samples were measured in a SpectraMax Gemini XPS fluorescence microplate reader ( Molecular Devices) using the following settings: excitation 360 nm, emission 450 nm, cut-off 435 nm, reading every 30 s for 20 min at 37 °C. β-Galactosidase activity was expressed as relative fluorescent units s⁻¹ and normalize to the OD₆₀₀ nm of the corresponding sample. Alternatively, β-galactosidase activity was measured as described previously (Miller, 1972) using 2-Nitrophenyl β-D-galactopyranoside (ONPG; Sigma–Aldrich Cat No. N1127) as substrate. All the transcriptional fusion assays were carried out using S. plymuthica A153 LacZ (control) or derived mutants.

RNA extraction, cDNA synthesis, reverse transcription-PCR (RT-PCR) and quantitative real time PCR analyses
RNA was extracted from early stationary phase cultures grown in LB medium using an RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically and RNA integrity was assessed by agarose gel electrophoresis. Genomic DNA contamination was eliminated by treating total RNA with Turbo DNA-free (Ambion). The synthesis of cDNA was performed using random hexamers (GE Healthcare) and SuperScript II reverse transcriptase (Invitrogen) in a 30 µl reaction with 2 µg of total RNA and incubation at 42 °C for 2 h. As negative control the reaction was performed omitting the reverse transcriptase. For the RT-PCR analysis, the equivalent of 50 ng of total RNA was subjected to PCR amplification using primers to amplify across the junctions (Supporting Information Table S3). Positive and negative control PCR reactions were performed using genomic DNA and no-RT cDNA samples, respectively, as templates. PCR conditions consisted of 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 62 °C, and extension for 40 s at 72 °C. qPCRs were performed as described previously (Burr et al., 2006) using primers specific for admX and admV (Supporting Information Table S3). qPCR amplifications were performed using an MyQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad). To confirm the absence of contaminating genomic DNA, control PCRs were carried out using no RT cDNA samples as templates. Melting curve analyses were conducted to ensure amplification of a single product. The relative gene expression was calculated using the critical threshold (ΔΔCt) method (Pfaffl, 2001) and using 16S rRNA as the internal control to normalize the data.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Structures of andrimid and moiramide B. The structure of andrimid consists of an unsaturated fatty acid chain, a pyrrolidinedione ring, a valine and glycine derived 3-ketoamide and the amino acid β-phenylalanine. Chemical synthesis studies showed that the fatty acid chain and β-phenylalanine are involved in bacterial cell penetration of the antibiotic, whereas the pyrrolidinedione head and the 3-ketoamide moiety are responsible for the antibacterial activity (Pohlin, *et al.* 2005; Freiberg, *et al.* 2006).

Fig. S2. Antibacterial activity of *Serratia marcescens* MSU97 against *Bacillus subtilis*.

Fig. S3. Schematic representation of the andrimid gene clusters of *Serratia marcescens* MSU97, *S. marcescens* 90-166, *Pantoea agglomerans* Eh335, *Vibrio corallilyticus* S2052 and *Vibrio nales* bacterium SWAT-3. Numbers below the arrows represent the intergenic distance between contiguous genes, with negative numbers indicate overlapping genes.

Fig. S4. DNA homology between the andrimid gene cluster of *Serratia plymuthica* A153 and the andrimid gene clusters of the other producing strains. A, Schematic representation of the *adm* gene cluster in *Serratia* strains. B-F, Alignments representing the percentage of DNA homology between the *adm* gene cluster of A153 and those of *S. marcescens* MSU97 (B), *S. marcescens* 90-166 (C), *Pantoea agglomerans* Eh335 (D) *Vibrio corallilyticus* S2052 (E) and *Vibrio nales* SWAT-3 (F). Alignments were performed using wgVISTA (Frazer, *et al.* 2004).

Fig. S5. Growth of *Serratia plymuthica* A153 in minimal medium with different carbon sources. Growth curves showing the doubling time in sorbitol (181.2 ± 1 min), mannitol (153 ± 1 min), fructose (145.8 ± 2 min), galactose (115.2 ± 1 min), mannose (181.6 ± 2 min), lactose (413.4 ± 6 min), xylose (208.2 ± 2 min), succinic acid (142.8 ± 1 min), maltose (145.2 ± 1 min), sucrose (106.2 ± 1 min), glucose (115.2 ± 1 min), glycerol (121 ± 1 min), gluconic acid (96.6 ± 1 min), arabinose (235.2 ± 2 min) and citrate (158.7 ± 3 min) as sole carbon source. Data are the mean and standard deviation of three biological replicates. The assays were done at 25 °C with shaking at 200 rpm.

Fig S6. Impact of Hfq (A) and AdmX (B) on the expression of *admV* and *admX*. Quantitative real-time PCR was used to measure transcript levels of *admV* (grey bars) and *admX* (white bars) in *Serratia plymuthica* A153, and derivative strains. The values showed the average expression relative to wild type expression. The arrow in Fig. 4A indicates the time point when the samples for qPCR were taken. The
data are the mean and standard deviation of three biological replicates.

**Fig. S7.** AdmX transcription correlates with the expression of the andrimid gene cluster. Transcription of the admX (PadmX::lacZ; pMAMV244) promoter fusion throughout growth in *Serratia plymuthica* A153 strains. β-Galactosidase activity (filled symbols) and growth curves (open symbols) were determined in LacZ (red) and ΔadmX (blue) in LB medium at 25 °C. A153 wt harbouring the empty reporter plasmid (black) was used as negative control in the assays. Data are the mean and standard deviation of three biological replicates.

**Fig. S8.** Genetic complementation of *Serratia plymuthica* A153 strain A153H. Expression of *hfq* in trans in A153 Δhfq restored andrimid production and therefore the antibacterial activity against *Bacillus subtilis*. Induction of Hfq expression was done by addition of 0.1 mM of IPTG. The bioassays were repeated at least three times, and a representative figure is shown. Pictures were taken after 48 h of incubation at 25 °C.

**Fig. S9.** Sensitivities of different bacterial strains to the antibiotic andrimid. Bioactivities of *Serratia plymuthica* A153 and the non-andrimid producing mutant of A153, VN2, against ecologically different bacterial strains. For the assays, an indicator top agar lawn was prepared as described in “Experimental procedures,” and 5 μl overnight cultures of the A153 strains were spotted on the surface of the bacterial indicator agar lawns. The bioassays were repeated three times, and representative results are shown. Pictures were taken after 48 h of incubation at 25 °C. The strains used are described in Table 1 and supplementary Table S2.

**Fig. S10.** Andrimid shows antibacterial activity against *Bacillus subtilis*, *Dickeya solani* and *Xanthomonas campestris* pv. *campestris*. Recovery of viable *Bacillus*, *Dickeya* and *Xanthomonas* cells grown in the presence of A153 JH6 (andrimid positive, zeamine negative) and A153 XJH6 (andrimid and zeamine negative) supernatants. The values showed the percentage of viable cells in the presence of JH6 supernatants relative to the number of viable cells in the presence of XJH6 supernatants. For the assays, overnight bacterial cultures of *Bacillus*, *Dickeya* and *Xanthomonas* were adjusted to an optical density at 600 nm (OD600) of 0.1 and grown at 30 °C with orbital shaking (225 rpm). At an OD600 of 0.4, 10 mL of the bacterial culture was removed and pelleted by centrifugation at 4,000 x g for 10 min at room temperature. The pellet was resuspended in 5 ml of 2X LB and 5 ml supernatants of an overnight culture of A153 JH6 or A153 XJH6 were added to the bacterial culture. Samples were taken after 5 and 10 h of incubation and the number of colony forming units (CFU) were determined. Data are the mean and standard deviation of three biological replicates.

**Fig. S11.** Homology model of AdmX. The model was generated by the Geno3D modeling algorithm (Combet *et al.*, 2000) and the structure of the BenM transcriptional regulator (PDB ID 3K1N) as template. The site for the binding of potential effector molecules is indicated.

**Table S1.** Identity at DNA level of the andrimid gene clusters between producing strains.

**Table S2.** Additional bacterial strains used in this study.

**Table S3.** Oligonucleotides used in this study.

**Table S4.** Plasmids used in this study.