Phylogenetic analysis of the salinipostin \(\gamma\)-butyrolactone gene cluster uncovers new potential for bacterial signalling-molecule diversity

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Abstract

Bacteria communicate by small-molecule chemicals that facilitate intra- and inter-species interactions. These extracellular signalling molecules mediate diverse processes including virulence, bioluminescence, biofilm formation, motility and specialized metabolism. The signalling molecules produced by members of the phylum Actinobacteria generally comprise \(\gamma\)-butyrolactones, \(\gamma\)-butenolides and furans. The best-known actinomycete \(\gamma\)-butyrolactone is A-factor, which triggers specialized metabolism and morphological differentiation in the genus Streptomyces. Salinipostins A–K are unique \(\gamma\)-butyrolactone molecules with rare phosphotriester moieties that were recently characterized from the marine actinomycete genus Salinispora. The production of these compounds has been linked to the nine-gene biosynthetic gene cluster (BGC) \(spt\). Critical to salinipostin assembly is the \(\gamma\)-butyrolactone synthase encoded by \(spt9\). Here, we report the surprising distribution of \(spt9\) homologues across 12 bacterial phyla, the majority of which are not known to produce \(\gamma\)-butyrolactones. Further analyses uncovered a large group of \(spt\)-like gene clusters outside of the genus Salinispora, suggesting the production of new salinipostin-like diversity. These gene clusters show evidence of horizontal transfer and location-specific recombination among Salinispora strains. The results suggest that \(\gamma\)-butyrolactone production may be more widespread than previously recognized. The identification of new \(\gamma\)-butyrolactone BGCs is the first step towards understanding the regulatory roles of the encoded small molecules in Actinobacteria.

DATA SUMMARY

All sequences analysed in this paper were retrieved from publicly accessible databases including the Joint Genome Institute (JGI) Integrated Microbial Genomes & Microbiomes system (IMG)/MER and the National Center for Biotechnology Information (NCBI) databases, with all sequence accession information included in the supplementary dataset S1 (available via Open Science Framework: https://osf.io/4g3mn/). PCR sequences produced as part of this work can be accessed at NCBI GenBank (accession numbers MW321490–MW321495) and are also listed in Table S1 (available with the online version of this article). Additionally, all sequence alignment and tree files used for the phylogenetic analyses are available through the Open Science Framework: https://osf.io/4g3mn/ with DOI 10.17605/OSF.IO/4G3MN. Supplementary material can be found on Figshare at 10.6084/m9.figshare.14325233.

INTRODUCTION

Bacteria use chemical signalling molecules to regulate gene expression in a population-dependent manner. This process, known as quorum sensing, controls group behaviours including swarming, bioluminescence, virulence, biofilm formation, cell competence, DNA uptake, public-goods production and specialized metabolism. In many
Gram-negative bacteria, quorum sensing is mediated by acyl-homoserine lactone (AHL) autoinducers and their cognate receptors [1]. In some Gram-positive bacteria, autoinducing peptides and their respective transmembrane two-component histidine sensor kinases control similar group behaviours [2]. Among Actinobacteria, γ-butyrolactone signalling molecules regulate morphological development and specialized metabolite production. Given the importance of Actinobacteria for the production of antibiotics and other useful compounds, the discovery of new signalling molecules could facilitate the discovery of new natural products from the large number of ‘cryptic’ gene clusters detected in actinomycete genome sequences.

To date, the types of signalling molecules known to be produced by Actinobacteria include γ-butyrolactones [3–25], γ-butenolides [26–30], furans [31, 32], PI factor [33] and N-methylphenylalanyl-dehydrobutyrine diketopiperazine [34] (Fig. 1). Most of these were discovered from members of the genus *Streptomyces*. Sometimes referred to as actinobacterial ‘hormones’, signalling molecules are commonly

**Impact Statement**

Signalling molecules orchestrate a wide variety of bacterial behaviours. Among Actinobacteria, γ-butyrolactones mediate morphological changes and regulate specialized metabolism. Despite their importance, few γ-butyrolactones have been linked to their cognate biosynthetic gene clusters (BGCs). A new series of γ-butyrolactones called the salinipostins was recently identified from the marine actinomycete genus *Salinispora* and linked to the spt BGC. Here, we report the detection of spt-like gene clusters in diverse bacterial families not known for the production of this class of compounds. This finding expands the taxonomic range of bacteria that may employ this class of compounds and provides opportunities to discover new compounds associated with chemical communication.

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**Fig. 1.** Actinobacterial AfsA homologue phylogeny, gene neighbourhoods and small molecule signalling products. Maximum-likelihood phylogeny of 22 AfsA homologues created with RAxML using a LG+I+G+F ProtTest model; branches are labelled with bootstrap support (500 replicates). Scale bar represents the mean number of amino acid substitutions per site. Coloured circles indicate the actinobacterial family. Gene neighbourhoods are drawn 5′ to 3′ when genome sequences were available and aligned by AfsA homologue (red); other genes are coloured by COG function. Coloured boxes delineate γ-butyrolactones (green), γ-butenolides (red), furans (blue) and others (yellow). Compounds mapped to the tree have been experimentally linked to their respective gene cluster (references are indicated in the insets). Those not mapped to the tree have not been linked to AfsA-containing gene clusters. Bracketed numbers are used in subsequent figures to refer to specific AfsA homologues and their associated signalling molecule products.
produced in low amounts and have proven difficult to isolate and characterize. Many of these molecules not only induce the production of specialized metabolites, but also regulate bacterial morphogenesis and control complex regulatory systems [15]. The first bacterial signalling molecule discovered was A-factor (autoregulatory factor, 2-isocaproyl-3R-hydroxy methyl-γ-butyrolactone) from the actinomycete Streptomyces griseus. It was shown to trigger sporulation and the production of the antibiotic streptomycin [3]. A-factor biosynthesis requires a γ-butyrolactone synthase and a reductase encoded by the genes afsA and bpra, respectively [15], and its elucidation revitalized the search to link signalling molecules to their biosynthetic genes [16, 32, 35–41]. Most of the biosynthetically characterized γ-butyrolactones, γ-butenolides and furans have been linked to afsA gene homologues via sequence similarity, biochemical verification or A-factor receptor binding assays. However, many afsA gene homologues observed in Streptomyces, Kitasatospora and Amycolatopsis genomes have yet to be linked to a small molecule [42–51]. Likewise, the Acl series of γ-butyrolactones reported from Streptomyces coelicolor has not been linked to an afsA homologue [3, 14, 18] (Fig. 1).

While most A-factor-like molecules have been identified from the genus Streptomyces, it remains possible that other actinobacterial taxa produce related signalling molecules. This includes the obligate marine actinomycete genus Salinispora, which comprises nine species: Salinispora tropica, Salinispora arenicola, Salinispora oceanea, Salinispora morocana, Salinispora cortesiana, Salinispora fennicola, Salinispora goodfellowii, Salinispora vitiensis and Salinispora pacifica [52, 53] isolated from marine sediments [54–57], seaweeds [55] and sponges [58, 59]. This genus has proven to be a prolific source of specialized metabolites [60] including the proteasome inhibitor salinosporamide A [61], which is currently in phase III clinical trials as an anticancer agent. Whole-genome sequencing of 118 Salinispora strains revealed 176 distinct biosynthetic gene clusters (BGCs), of which only 25 had been linked to their products [62]. In a subsequent study, a majority of Salinispora BGCs were shown to be transcriptionally active under standard cultivation conditions, suggesting that many of their small molecule products were being missed using traditional detection and isolation techniques [63]. Given that little is known about the regulation of specialized metabolism in this genus, it remains possible that signalling molecules play a role in the regulation of BGC expression.

Recently, a series of compounds known as salinistatins A–K with rare bicyclic phosphotriesters were identified from a Salinispora sp. RL08-036-SPS-B [64]. While these compounds were identified based on anti-malarial activity against Plasmodium falciparum, the γ-butyrolactone part of the salinistatin structure is reminiscent of Streptomyces A-factor [64, 65]. Salinistatin biosynthesis was linked to the spt gene cluster via a knockout of the afsA homologue spt9 in Salinispora tropica CNB-440, which resulted in the elimination of salinistatin production [63]. Subsequently, eight volatile bicyclic lactones, salinilactones A–H, were isolated and characterized from Salinispora arenicola CNS-205 [66, 67]. Two γ-butyrolactones, Sal-GBL1 and Sal-GBL2, were also recently characterized from multiple Salinispora strains [68]. The Sal-GBLs, salinilactones A–H and salinipostins A–K all share a bicyclic lactone motif and are proposed to originate from the same spt BGC [63, 66–68] (Fig. S1).

In this study, we set out to determine the distribution of Spt9 butyrolactone synthase homologues among sequenced bacteria and the diversity of BGCs in which they reside. We uncovered that salinipostin-like BGCs are widely distributed outside of the genus Salinispora and exhibit gene rearrangements and unusual gene fusions relevant to γ-butyrolactone biosynthesis. Finally, the evolutionary history of the salinipostin BGC indicates that it was horizontally transferred between Salinispora species at a location where they are known to co-occur.

**METHODS**

**Identification and distribution of Spt9 homologues**

The Pfam function of Spt9 was identified using the National Center for Biotechnology Information (NCBI) Conserved Domain Database prediction tool [69]. AnnoTree [70] was then used to determine the taxonomic distribution of the Spt9/afsA Pfam03756 A-factor biosynthesis hotdog domain-containing protein. The top 500 Spt9 homologues were identified using the Salinispora tropica CNB-440 319 amino acid Spt9 sequence as a blastp (2.6.0+) [71] query against the JGI Integrated Microbial Genomes and Microbiomes system (IMG)/MER sequence database (publicly available genomic sequence data integrated with JGI sequence data, all_img_core 2019) with an E value and sequence identity cut-off of 1×10⁻5 and >25%, respectively. Gene neighbourhoods were evaluated 20 kb upstream and downstream of all top Spt9 homologues. Sequences were grouped into actinobacterial family or gammaproteobacterial class. Also included were 22 previously characterized AfsA homologues, including 9 linked to the production of 34 γ-butyrolactone molecules, 1 linked to the production of two γ-butenolide molecules and 1 linked to the production of five furan molecules.

To identify Spt9 homologues within the genus Salinispora, protein–protein BLASTP with an E value cut-off of 1×10⁻⁵ was used to search all public Salinispora genomes. PCR was used to confirm the integrity of the split spt BGC in Salinispora arenicola CNS-296 and the presence of a hypothetical gene in Salinispora pacifica CNS-143. PCR was performed by aliquoting 90 ng genomic DNA into a PCR mixture consisting of 2× Phusion green hot start II high-fidelity PCR master mix (1.5 mM MgCl₂, 200 µM each dNTP, 0.4 U Phusion enzyme; Thermo Scientific), 3% DMSO and 0.5 µM of each forward and reverse primer (primer pair A – 6F (5’-ATCGAACTGTTGC ATCGAATGGC-3’), 6dntransR (5’-GTAGCGGAGA AAGAAGCATC-3’); primer pair B – 6F, 6dntrans-IGR_R (5’-TGTTTATCAGAGTGCTCCC-3’); primer pair C – 6F, 7R (5’-GATCAGATAAAGAGGAGCGAG-3’)). PCR conditions were as follows: primer pair A (6F, 6dntransR),

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30 s of initial denaturation at 98 °C, followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 66 °C for 20 s and extension at 72 °C for 30–50 s, followed by a final extension for 7 min at 72 °C; primer pair B (6F, 6dntrans-1GR_R) same as the previous but with annealing at 65.6 °C for 20 s and extension at 72 °C for 69 s; primer pair C (6F, 7R), same as the previous but with annealing at 65.7–66 °C for 20 s and extension at 72 °C for 35–50 s, followed by a final extension for 5–7 min at 72 °C. The resulting products were visualized in a 0.8 % agarose gel run in 1× TAE (Tris-acetate-EDTA buffer) at 95–97 V for 30–60 min; then, excised, puriﬁed, Sanger sequenced in forward and reverse directions (Eton Bioscience), trimmed, and mapped to their respective genomes in Geneious v8.1.9 [72].

Identification of salinipostin-like BGCs
ClusterScout [73] searches were performed to identify salinipostin-like BGCs in sequenced genomes using the following Pfam functions: Spt1 Pfam00391, Pfam01326; Spt2 Pfam00501; Spt4 Pfam00550; Spt5 Pfam07993; Spt6 Pfam00334; Spt7 Pfam01040; Spt8 Pfam00296; Spt9 Pfam03756 (Fig. S1). It should be noted that antiSMASH v4 and v5 [74, 75] do not fully identify spt1–2 in the salinipostin butyrolactone BGC; thus, other methods were used to find spt-like BGCs. Independent ClusterScout searches were run with a minimum requirement of either 3, 4 or 5 Pfam matches, a maximum distance of <10 000 bp between each Pfam match, and a minimum distance of 1 bp from the scaffold edge. The boundaries of each match were extended by a maximum of 10 000 bp to help identify full biosynthetic operons. For some searches, the Spt9 Pfam was deﬁned as essential. MultiGeneBlast [76] was also used to query the contiguous Salinispora tropica CNB-440 salinipostin spt1–9 gene cluster against the NCBI GenBank Bacteria BCT subdirectory database. Finally, the string v11 database [77] was queried using Spt1–9 to identify signiﬁcant protein–protein interactions, gene neighbourhoods and gene co-occurrences within 5090 organisms. Biosynthetic clusters retrieved from each ClusterScout, MultiGeneBlast and string search were manually inspected for spt Pfams and gene organization.

Phylogenetic distribution of Spt9 homologues and salinipostin-like BGCs
A maximum-likelihood amino acid phylogeny was generated from the top 403 Spt9 homologues and an additional 22 experimentally characterized AfsA homologues. The sequences were aligned with MUSCLE [78] within the Mesquite system for phylogenetic computing [79] and analysed using ProtTest 3.4.2 [80] to determine an amino acid model for tree calculations. RAxML [81] was used to create a tree using ML+rapid bootstraps with 500 replicates. A second phylogeny was generated for the Spt9 homologues observed in salinipostin-like BGCs using the same parameters. The topologies of these trees and branch support were conﬁrmed using PhyML [82] with SMS Smart Model Selection (AIC model selection; BIONJ tree searching, NNI tree improvement and an aLRT SH-like fast likelihood method) [83].

To test whether the Salinispora salinipostin BGC was acquired as an intact gene cluster, Spt1–9 protein sequences from 116 Salinispora genomes were aligned with MUSCLE [78] and PhyML [82] was used to calculate a phylogenetic tree for each protein with automatic SMS Smart Model Selection (AIC model selection; BIONJ tree searching, NNI tree improvement and an aLRT SH-like fast likelihood-based method) [83]. The nine Spt1–9 protein trees were compared for congruency with a concatenated Salinispora species tree created using the following 11 single-copy protein sequences: DnaA, GyrB1, GyrB2, PyrH, RecA, Pgi, TrpB, AtpD, SucC, RpoB and TopA, as previously reported [84]. Spt1–9 protein sequences were also concatenated (3758 total amino acid characters), aligned with MUSCLE [78], and a maximum-likelihood tree calculated using SMS and PhyML with the previously described parameters.

RESULTS
Taxonomic distribution of Spt9 homologues
The γ-butyrolactone synthase AfsA is critical for the biosynthesis of the Streptomyces signalling molecule A-factor [15]. The identiﬁcation of the afsA homologue spt9 in the salinipostin (spt) BGC and its essential role in catalysing the γ-butyrolactone ring formation in salinipostin biosynthesis [63] led us to explore the distribution of Spt9 homologues among sequenced bacterial genomes. We ﬁrst identiﬁed that Spt9 belongs to the Pfam03756 ‘A-factor biosynthesis hotdog domain-containing protein’ family. It contains two AfsA-like hotdog fold superfamily domains and is distantly related to the FabA and FabZ β-hydroxyacyl-ACP dehydratases associated with fatty-acid biosynthesis in Escherichia coli [88]. Using AnnoTree [70], 1230 Spt9 Pfam03756 hits were identiﬁed out of the 27 000 reference genomes in the Genome Taxonomy Database (Fig. 2). Surprisingly, these sequences were distributed among 12 bacterial phyla, the majority of which are not known for the production of γ-butyrolactone signalling molecules. The phylum Actinobacteria contained 74% of the hits, Proteobacteria had 21% of the hits and the remainder were scattered across 10 additional phyla. Noticeably, 25% (911) of the 3579 Actinobacteria in the reference Genome Taxonomy Database contained the AfsA Pfam03756 compared to only 3% (256) of the 8882 Proteobacteria. These results inspired a more detailed analysis of Spt9 homologues among bacterial genome sequences.

Spt9 phylogeny and gene environment
We next conducted a BLASTP search to identify Spt9 homologues among the ~70 000+ bacterial genomes in the 2019 JGI IMG BLAST database [89]. The top 500 matches shared at least 25% amino acid identity with Spt9. After removing duplicate Salinispora sequences, 403 Spt9 homologues were further
analysed. We additionally included 22 AfsA homologues that have been bioinformatically or experimentally linked to the production of a diverse array of $\gamma$-butyrolactones, $\gamma$-butenolides and furans (Fig. 1). A maximum-likelihood phylogeny generated using these Spt9 homologues was incongruent with the established taxonomic relationships of the strains in which the sequences were detected (Figs 3 and S2). One prominent example includes the Salinispora Spt9 sequences, which are sister to a homologue in Streptomyces phaeofaciens. These sequences fall within a larger clade comprising diverse members of the Gammaproteobacteria (Citrobacter koseri and Dickeya sp.) and Actinobacteria (Cellulomonas cellacea and Rhodococcus sp.) as opposed to forming a clade with the family Micromonosporaceae to which Salinispora belongs. The 22 experimentally characterized AfsA homologues are restricted to one large clade in the phylogeny and distinct from the northern end of the tree, which contains the Salinispora Spt9 sequences (Fig. 3).

The large number of Spt9 homologues suggests considerable potential for the discovery of new $\gamma$-butyrolactone-synthase-mediated chemical diversity (Fig. 3). New biosynthetic routes are supported by the diverse gene environments in which these Spt9 homologues are observed. For example, antiSMASH 5 [75] analyses revealed that some Spt9 homologues are close to ketosynthase- and thiolase-encoding genes, suggesting

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**Fig. 2.** Distribution of Spt9 Pfam homologues across 27 000 bacterial genomes. Shaded taxa contain Spt9/AfsA Pfam03756 ($\alpha$-factor biosynthesis hotdog domain-containing protein) homologues as determined using AnnoTree. The phylogeny is from the Genome Taxonomy Database. Scale bar indicates the mean number of amino acid substitutions per site. Pie charts show the proportion of genomes in each taxon with a Spt9 homologue, with the total number of hits indicated. Pie chart sizing is proportional to the percentage of hits out of the 1230 detected across all taxa.
Fig. 3. Phylogeny and gene environments of AfsA and Spt9 homologues. Condensed maximum-likelihood phylogeny of the top Spt9 homologues (403, black) and experimentally characterized AfsA homologues (22, red) linked to known molecules. The tree was calculated with a WAG+I+G+F ProtTest model with 500 replicates in RAxML; branches are labelled with bootstrap support. Scale bar represents the mean number of amino acid substitutions per site. Taxonomically coherent clades are collapsed with the number of sequences indicated in parentheses. The *Pseudomonas* sp. RIT357 Spt9 homologue was used as an outgroup. Gene neighbourhoods are drawn 5' to 3' and aligned with the Spt9 homologue (red); genes are coloured by COG function as annotated by JGI IMG/MER. Shaded rectangles indicate actinobacterial family or gammaproteobacterial class (see the key) with circles proportional to the number of sequences in each familial clade. Representative chemical structures are shown [γ-butyrolactones – salinipostin A from *Salinispora tropica* CNB-440, A-factor from *Streptomyces griseus*; furan – methylenomycin furan MMF-1 from *Streptomyces coelicolor* A3(2)] and bracketed numbers correspond to the AfsA homologues and their associated compounds in Fig. 1. Stars indicate salinipostin-like BGCs.
they are part of larger polyketide synthase (PKS) gene clusters. Among *Salinispora* strains, six Spt9 homologues were observed outside of the *spt* BGC. Two of these were observed in *Salinispora oceaneensis* strains CNT-124 and CNT-584, each of which contains a *spt9* gene in a type II PKS BGC in addition to the *spt* BGC. The other four were observed in *Salinispora fenicalii* strains CNT-569 and CNT-942, which have *spt9* gene homologues in both a type II PKS BGC and a butyrolactone non-ribosomal peptide synthetase (NRPS) BGC while lacking the *spt* BGC.

Despite incongruence with the species phylogeny, the gene environments surrounding some *Spt9* homologues are conserved. For example, many *Streptomyces* and *Kitasatospora* species in the centre of the tree have a *bprA* homologue (dark pink) next to the *spt9* homologues as required for A-factor biosynthesis in *Streptomyces griseus* (Fig. 3). The three sequences that share the highest similarity to *Spt9* in *Salinispora* (observed in *Streptomyces phaceofaciens*, *Dickeya* sp. and *Citrobacter koseri*) also contain *spt4* acyl carrier protein gene homologues (pale blue). Below the *Salinispora* Spt9 clade, conservation of two 3-oxoacyl (acyl-carrier-protein) synthases (light green), an acyl carrier protein (light blue, *spt4* homologues), a hydroxylase (pale yellow) and a 3-oxoacyl (acyl-carrier-protein) reductase (light blue) is observed across taxonomically diverse *Streptomyctecaeae*, *Nocardiopsaceae*, *Nocardiaecaeae*, *Thermomonosporaceae*, *Pseudonocardiaeae* and *Streptosporangaeae* strains. At the bottom of the tree, the Spt9 homologue in the gammapeptobacterial outgroup *Pseudomonas* sp. RIT357 shares conserved genes with other diverse families of bacteria including *Gammaproteobacteria*, *Nocardiaeae*, *Streptomyctecaeae* and *Pseudonocardiaeae* with a putative hydroxylase of the haloacid dehydrogenase (HAD) superfamily (light yellow), a cytochrome P450 (light teal) and a MFS (major facilitator superfamily) protein transporter (light yellow). While evidence of gene conservation around Spt9 homologues suggests some functional similarities in the *spt9* gene neighbourhoods across diverse bacterial families, the overall diversity of gene environments illustrates the potential for new routes of γ-butyrolactone, γ-butenolide and furan production among bacteria not known to produce these molecules.

**Targeted search for *spt*-like BGCs**

A number of *spt9* gene neighbourhoods outside of *Salinispora* caught our attention due to similarities with the salinipostin BGC (Fig. 3). To search more thoroughly for *spt*-like BGCs among sequenced genomes, we searched for *spt*-like BGCs using ClusterScout [73], MultiGeneBlast [76] and **v**11 [77]. These efforts led to the identification of 91 *spt*-like BGCs spanning six actinomycete families within the genera *Nocardia*, *Gordonia*, *Tsukamurella*, *Mycobacterium*, *Dictzia*, *Streptomyces*, *Kitasatospora*, *Rhodococcus* and *Kitzneria* (Fig. 4). All of these BGCs possess *spt1*, *spt5*, *spt6*, *spt7* and *spt9* homologues, with *spt9* towards the 3’ end of the cluster as seen in *Salinispora*. Notably, none of these *spt*-like BGCs contain the flavin-dependent oxidoreductase *spt8*, whose role is unknown in salinipostin biosynthesis, and none of the *spt*-like BGCs have been linked to the small molecules they encode.

A maximum-likelihood phylogeny of the Spt9 homologues observed in *spt*-like BGCs clearly delineates them from the AfA homologues linked to γ-butyrolactone, γ-butenolide and furan biosynthesis (Fig. 4). Compared with the nine gene salinipostin BGC identified in *Salinispora* (Fig. S1), we observed gene reorganizations and fusions in other bacteria (Fig. 4). Most notably, *spt2* and *spt3* are fused across the large clade bracketed by *Nocardia* and *Dictzia timorensis*, as well as two *Kitzneria* species and five *Streptomyces* species at the most southern part of the tree. This fusion is conserved across most BGCs except for those observed in *Salinispora*, four *Streptomyces* species and *Rhodococcus rhodnii* NRRL B-16535. A second gene fusion is observed between *spt6* and *spt9* in *D. timorensis*. Alignment of the *spt2*, *spt3*, *spt6* and *spt9* fused and individual genes reveals maintenance of the functional domains (Fig. S3). These gene fusions, also known as Rosetta gene fusions [90, 91], suggest a functional interaction between the encoded proteins in the biosynthesis of salinipostin-like γ-butyrolactone molecules. The gene fusions could have arisen from the single-domain *spt2*, *spt3*, *spt6* and *spt9* genes in the *Salinispora* sp. BGC and, thus, suggest some selective advantage for these co-localized biosynthetic genes to become fused.

Many of the *spt*-like BGCs differed in gene order compared to that observed in *Salinispora*, while others contained extra genes in the cluster including a nitroreductase (dark pink in Fig. 4). As noted, the flavin-dependent oxidoreductase *spt8* (Fig. S1) was unique to the *Salinispora* sp. BGC, yet has no proposed function in salinipostin [68] or salinilactone [66, 67] biosynthesis. Similarly, some *Streptomyces* *spt*-like BGCs did not contain *spt2* (AMP-ligase) and *spt4* (acyl carrier protein) homologues, which are proposed to help load and carry the Rα aliphatic sidechain during salinipostin biosynthesis, respectively [68]. These observations suggest additional structural diversity remains to be discovered. Furthermore, two BGCs found in *Kitasatospora cheerisanensis* and *Frankia* sp. contained *spt9* and *spt2* homologues next to a type I PKS, suggesting a potential role in PKS BGC regulation as observed in methylenomycin biosynthesis [31]. We observed that some of the 91 *spt*-like BGCs occur in different genomic locations within the same genus, which could support BGC migration or horizontal gene transfer. However, there is also evidence of vertical inheritance based on gene conservation in some strains. To investigate this further, we focused on the genus *Salinispora*, where BGC migration and transfer events have been previously reported [62].

**The *spt* BGC in the genus *Salinispora***

The salinipostin BGC (*spt1–9*) is highly conserved within the genus *Salinispora* [62]. Notably, only 5 of 118 strains with available genome sequences lack the *spt* BGC. These belong to the recently described species *Salinispora fenicalii*, *Salinispora goodfellowii* and *Salinispora vitiensis* (Fig. S4a). At the species level, the *spt* BGC is commonly observed in the same
Fig. 4. Phylogeny of Spt9 homologues within salinipostin-like BGCs. The maximum-likelihood phylogeny was calculated with a WAG+I+G+F ProtTest model with 500 replicates in RAxML; branches are labelled with bootstrap support. The branch length scale bar represents the mean number of amino acid substitutions per site. Gene neighbourhoods are drawn 5’ to 3’ and aligned with the Spt9 homologue (red). The names of AfsA homologues linked to the production of specific compounds are coloured in red. Gene fusions are shown with approximate transition points and neighbouring genes are coloured by COG function as annotated by JGI IMG/MER. Coloured rectangles indicate actinobacterial family or gammaproteobacterial class (see the key). Representative chemical structures are shown [γ-butyrolactones – A-factor from *Streptomyces griseus*, salinipostin A from *Salinispora tropica* CNB-440; γ-butenolide – SRB-1 from *Streptomyces rochei*; furan – methylenomycin furan MMF-1 from *Streptomyces coelicolor* A3(2)] and bracketed numbers correspond to the AfsA homologues and associated signalling molecule products in Fig. 1.
genomic environment (Fig. S5) within previously defined genomic islands (GIs) [62, 92]. For example, it occurs in GI 20 in Salinispora tropica [62] and GI 15 [62] in most Salinispora arenicola and Salinispora pacifica strains with notable conservation of upstream and downstream regions.

Variations in the spt BGC are observed in three Salinispora strains (Fig. S5). In Salinispora arenicola CNS-296, spt1–6 and spt7–9 are split onto different contigs and flanked by transposases. Targeted PCR amplifications of spt6 and the neighbouring transposase confirmed that the BGC is indeed split (Fig. S6a). Attempts to amplify a region between spt7 and the downstream hypothetical gene resulted in multiple PCR products likely due to multiple copies of the hypothetical gene in the Salinispora arenicola CNS-296 genome and were, thus, uninformative. However, primers spanning spt6–7 resulted in a product that was 3.2 kb larger than what was observed from a contiguous BGC in Salinispora arenicola CNQ-884 (Fig. S6b). Sequences obtained from the ends of this amplicon mapped poorly to spt6 (67% identity) and better to spt7 (99% identity), providing additional support for an insertion between spt6–7 in the Salinispora arenicola CNS-296 spt BGC. It remains to be determined whether Salinispora arenicola CNS-296 produces salinipostins. In contrast, the detection of spt genes on multiple contigs in Salinispora arenicola CNT-088 and Salinispora pacifica CNS-143 is likely due to poor genome assembly [93]. The hypothetical gene annotated between spt6–7 in Salinispora pacifica CNS-143 is also likely an error given that PCR products spanning spt6–7 in this strain and Salinispora arenicola CNQ-884, where the spt BGC is contiguous, yielded amplicons of the same size and with the same conserved spt6–7 domains (Fig. S6b).

While conservation of the spt BGC within Salinispora supports vertical inheritance, the Spt9 phylogeny (Figs S4b and S5) reveals incongruencies with the established Salinispora species phylogeny [52, 62] (Fig. S4a) that are consistent with horizontal gene transfer. In one example, Spt9 sequences from Salinispora vitiensis CNS-055 and Salinispora cortesiana CNY-202 occur within the Salinispora oceanensis clade as opposed to outside of it (Figs S4 and S5). A more pronounced example is the placement of Salinispora tropica Spt9 sequences within the larger Salinispora arenicola clade, suggesting the former acquired the sequences from the latter. This transfer or recombination event appears to have involved the entire BGC, since all Salinispora tropica Spt1–8 sequences share a similar phylogeny (Fig. S4b). As predicted, a concatenated phylogeny of Spt1–9 (Fig. 5a) is incongruent with the Salinispora species phylogeny (Fig. S4a) and supports a horizontal exchange of the BGC between Salinispora arenicola and Salinispora tropica. Mapping the geographical origin of these strains onto the tree reveals that all 12 Salinispora tropica and the 6 most closely related Salinispora arenicola sequences all originated from ocean sediments collected in the Bahamas and the Yucatán (Fig. 5b). A closer examination reveals that the two species co-occur at four of the five collection sites (Fig. S7). This geographical proximity would provide opportunities for BGC horizontal gene transfer to occur.

**DISCUSSION**

Specialized metabolites that function as signalling molecules regulate important functional traits in bacteria. However, only a small number of bacterial signalling molecules have been identified to date. This may be because they are small in size, generally produced in low yields, and often lack activity in the bioassays commonly used to guide small molecule discovery. ∇-Butyrolactones represent an important class of signalling molecules produced by Actinobacteria (Fig. 1). The salinipostins, salinilactones, Sal-GBL1 and Sal-GBL2 were recently reported from the marine actinomycete genus Salinispora [64, 66–68], and bear structural similarities to previously characterized actinomycete ∇-butyrolactones.
Linkage between the biosynthesis of these compounds and the γ-butyrolactone synthase Spt9 encoded by the salinipostin spt BGC led us to more broadly explore the potential for signaling molecule production by assessing the distribution of this protein among sequenced bacterial genomes. Surprisingly, we detected Spt9 homologues across 12 diverse bacterial phyla, many of which are not known to produce γ-butyrolactones (Fig. 2). Despite the unexpectedly wide distribution of Spt9 homologues, only 285 of the ∼25 500 currently described microbial natural products in The Natural Products Atlas [94] contain a γ-butyrolactone moiety. Of these, only 14 have been linked to their respective BGCs in the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository [95] and only four of these, including the salinipostins in Salinispora, A-factor in Streptomyces griseus, SCB1-3 in Streptomyces coelicolor A3(2) and lactonamycin in Streptomyces rishiriensis, contain an Spt9 homologue. Thus, opportunities remain to identify the products of Spt9-containing BGCs and to establish formal links between these compounds and their biosynthetic origins. Our results suggest that the production of γ-butyrolactones and related compounds may be more common than previously recognized.

A phylogenetic tree of the top 403 Spt9 homologues, including experimentally characterized AfsA homologues, showed that the associated γ-butyrolactone, γ-butenolide and furen signalling molecules are restricted to a clade that is distinct from the majority of uncharacterized Spt9 sequences (Fig. 3). The Spt9 tree also showed major incongruencies with recognized actinobacterial and gammaproteobacterial classification, suggesting extensive horizontal gene transfer. The genomic environments around the Spt9 homologues were diverse, suggesting the potential production of considerable chemical diversity. It remains to be seen whether all of these Spt9 homologues catalyse γ-butyrolactone synthase-like reactions, especially when they are distantly related to experimentally characterized AfsA homologues. Heterologous expression to determine whether these Spt9 homologues perform the canonical AfsA condensation reaction that assembles a fatty acid ester (β-ketoacyl-DHAP ester) intermediate is a next step towards establishing their functionality [15].

Surprisingly, we discovered a large clade of Spt9 homologues in the genus Nocardia that occurred in operons with similar structure to the Salinispora spt BGC (starred in Fig. 3). To date, no small molecules isolated from Nocardia spp. have been linked to these BGCs. Differences between the Salinispora spt BGC and the 91 spt-like BGCs observed both in Nocardia and other genera (Fig. 4) include the absence of sp18 (a flavin-dependent oxidoreductase) and gene organization, with sp17 occurring after sp9. Several spt-like BGCs also have an additional nitratreductase gene between sp9 and sp17 (Fig. 4), suggesting the production of a γ-butyrolactone with a reduced nitrogen or nitro functional group. Other spt-like BGCs lack the AMP- ligase sp2 and the acyl carrier protein sp4, suggesting the products may lack the extended aliphatic sidechain observed in the salinipostins. These variations further support the production of new chemical diversity and provide opportunities to link structural changes to BGC evolution.

Also of note are the spt2–spt3 and spt6–spt9 gene fusions observed in the genera Nocardia, Gordonia, Tsukamurella, Mycobacterium, Dietzia and Streptomyces. Both pairs of fused genes appear functional based on the maintenance of conserved functional domains (Fig. S3). D. timorensis is the only strain with both spt2–spt3 and spt6–spt9 fusions, and is sister to the large clade containing the other gene fusions. Protein fusions can arise when clustered genes are co-transcribed and co-translated, providing evidence of functional interaction and, perhaps, a selective advantage over individual proteins [90, 91, 96]. The gene fusions observed in the spt-like BGCs appear to represent the evolution of more complex, multifunctional proteins in these strains. The spt2–spt3 and spt6–spt9 gene fusions are similar to recently described multi-domain enzyme fusions in the desiferroamine (des) BGC, where they are hypothesized to contribute to chemical diversification [96]. Two additional fusions involving an AfsA/Spt9 homologue were observed within trans-AT PKS modules associated with gladiofungin and gladiostatin biosynthesis, where AfsA functions for unprecedented offloading and butenolide formation [97, 98]. Identifying unusual gene fusions such as these represents an exciting new avenue for genome-mining-driven natural product discovery [96, 99].

Conservation of the spt BGC in the 116 Salinispora genomes examined here suggests it was present in a common ancestor of the genus. Yet, the Salinispora spt phylogeny is incongruent with the established species phylogeny (Figs 5a and S4). These incongruencies are likely due to horizontal gene transfer events, which have been identified as important avenues of BGC transfer and diversification, especially in Actinobacteria [100]. The most apparent of these events is represented by the clustering of the Salinispora tropica Spt9 sequences within the Salinispora arenicola clade. All 12 Salinispora tropica Spt1–9 sequences share the same evolutionary history (Fig. S4), providing evidence that the horizontal gene transfer affected the entire spt BGC (Fig. 5a). Co-localization of Salinispora tropica and Salinispora arenicola in Bahamian and Yucatán sediments provides spatial opportunities for these exchange events to occur (Figs 5b and S7). BGC exchange is well documented in the genus Salinispora and has been linked to gene gain, loss, duplication and divergence in lineage-specific patterns [62, 84, 101]. It remains unknown whether the Salinispora arenicola spt locus that appears to have replaced the ancestral version or been acquired de novo in Salinispora tropica provides a selective advantage or affects the compounds produced. The apparent interspecies exchange of spt adds to growing evidence that this process occurs between both closely and distantly related bacteria, as seen in the granaticin, coronafacoyl phytotoxins, tunicamycin, foxicin, antimycin, streptomycin and bicyclomycin BGCs [100]. Overall, acquisition of the spt BGC by Salinispora tropica highlights the importance of understanding the functional roles of its products and the effects of these exchange events on population and species-level dynamics.
The spt BGC has been linked to the production of both the salinipostins A–K [63, 64, 68], Sal-GBL1 and Sal-GBL2 [68], and the salinilactones A–H [66, 67], which share structural similarities to the A-factor family of γ-butyrolactone signalling molecules. Additionally, Salinispora arenicola and Salinispora pacifica produce two additional AHLs that have yet to be linked to their biosynthetic origins [102]. AHLs are the most common class of autoinducer signalling molecules produced by Gram-negative bacteria; thus, Salinispora appears to employ both γ-butyrolactone and homoserine lactone signalling molecules. While further studies are needed to understand the ecological functions of the spt products, there is evidence that lactone signalling molecules affect microbial community organization and function [103], and can elicit specialized metabolite production [40, 104, 105]. Thus, the small molecule products of spt BGCs may regulate the expression of other biosynthetic pathways in Salinispora. In support of this, spt9 was detected within Salinispora PKS and NRPS gene clusters. This is reminiscent of the methylenomycin BGC in Streptomyces coelicolor A3(2), where methylenomycin furan (MMF) signalling molecules induce methylenomycin production [106]. Additionally, we identified an spt-like BGC neighbouring the recently identified cyphomycin PKS BGC in a Brazilian sp. ISID311 isolated from the fungus-growing ant Cephalomyrmex sp. [107]. None of the genes in this spt-like BGC have been linked to cyphomycin biosynthesis [107], which suggests they encode a different small molecule that may have a regulatory role. The recently reported total synthesis of salinipostin [108] and the identification of molecules from orphan spt-like BGCs could support future studies to explore the roles of signalling compounds in regulating actinomycete specialized metabolism.

Our results reveal unexplored biosynthetic potential related to γ-butyrolactone signalling molecules in bacteria. The γ-butyrolactone synthase spt9 is broadly distributed among diverse bacteria and observed in a wide range of gene environments suggesting the potential for unrealized chemical diversity. Experimentally characterized γ-butyrolactone, γ-butenolide and furan BGCs are largely restricted to the genus Streptomyces, yet spt-like BGCs are observed among bacterial genera that are not widely recognized for the production of signalling molecules. Evidence of gene fusions and gene gain/loss in the newly described spt-like BGCs suggest that new chemical diversity awaits discovery within this unusual class of compounds.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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