Transmembrane topology of the AbsA1 sensor kinase of *Streptomyces coelicolor*

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The sensor kinase AbsA1 (SCO3225) phosphorylates the response regulator AbsA2 (SCO3226) and dephosphorylates AbsA2~P. The phosphorylated response regulator represses antibiotic biosynthesis operons in *Streptomyces coelicolor*. AbsA1 was predicted to have an atypical transmembrane topology, and the location of its signal-sensing domain is not readily obvious. To better understand this protein and to gain insight into its signal response mechanism, we determined its transmembrane topology using fusions of *absA1* to *egfp*, which is believed to be the first application of this approach to transmembrane topology in the actinomycetes. Our results are in agreement with the *in silico* topological predictions and demonstrate that AbsA1 has five transmembrane domains, four near the N terminus and one near the C terminus. Unlike most sensor kinases, the largest extracellular portion of AbsA1 is at the C terminus.

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INTRODUCTION

Members of the genus *Streptomyces* produce a conspicuous number of secondary metabolites, including many molecules that are employed as antibiotics (Weber *et al.*, 2003). Typically, each species has the genetic capacity to produce 20–30 of these molecules, but may produce a much smaller number in the laboratory (Pelaez, 2006). Antibiotic biosynthesis, which has been extensively investigated in *Streptomyces coelicolor*, is subject to a complex battery of regulatory mechanisms (Bibb, 2005). These include genes that control both antibiotic production and sporulation, genes that control the production of several antibiotics, and pathway-specific regulators that control the production of just one antibiotic. In this work we continue our investigation into the two-component system encoded by the *absA* locus, which controls the production of at least four secondary metabolites produced by *S. coelicolor*.

The *absA* locus was discovered in a genetic screen for mutants that impaired the production of pigmented antibiotics (Adamidis *et al.*, 1990), and was shown to encode a two-component system consisting of the sensor kinase AbsA1 and the response regulator AbsA2. Evidence from two laboratories suggested that regulation by this locus involves repression by AbsA2. For example, a deletion of *absA2* confers the *pha* (precocious hyperproduction of antibiotics) phenotype, in which pigmented antibiotics are produced at elevated levels. It was subsequently found that some alleles of *absA1* confer the *pha* phenotype, while others impair antibiotic production

(Adamidis *et al.*, 1990; Anderson *et al.*, 2001; Sheeler *et al.*, 2005). For example, mutations in H202, the site of autophosphorylation, stimulate antibiotic production. Conversely, mutations in the 'X-box' (Fig. 1), which have been found to impair AbsA2~P phosphatase activity, inhibit antibiotic production (Sheeler *et al.*, 2005).

Most sensor kinases possess an N-terminal periplasmic or extracellular sensory domain consisting of 50–300 amino acids that is flanked by two transmembrane helices (Mascher *et al.*, 2006). These extracellular-sensing domains are diverse in sequence, consistent with the fact that they respond to different stimuli (Mascher *et al.*, 2006). The intracellular C-terminal domains phosphorylate response regulator target proteins, often altering their capacity to bind DNA and control target genes. Other sensor kinases have 2–20 N-terminal membrane-spanning domains connected by short intra- or extracellular linkers (Mascher *et al.*, 2006), and are believed to respond to stimuli either at the membrane interface (e.g. membrane-bound enzymes, membrane-integral components) or within the membrane (e.g. turgor pressure, mechanical stress). Still others are wholly cytoplasmic (e.g. CheA) and respond to intracellular signals or signals conveyed by other membrane proteins.

We applied transmembrane hidden Markov model (TMHMM), a computer-based membrane protein topology program (Krogh *et al.*, 2001), to the 571 amino acid residue AbsA1 protein. TMHMM predicted five membrane-spanning domains, four of which were proximal to the N terminus of the protein: TM₁ (amino acid residues 30–64), TM₂ (84–106), TM₃ (113–135) and TM₄ (140–162). A particularly unusual feature was an additional

Abbreviations: EGFP, enhanced GFP; Emλ, emission wavelength; Exλ, excitation wavelength; TMHMM, transmembrane hidden Markov model.

Fig. 1. Schematic representation of the AbsA1 protein. AbsA1 is predicted to have five transmembrane domains, an intracellular N terminus and an extracellular C terminus. Triangles indicate the locations of the *egfp* fusions. A1ex_{60aa}-EGFP, A1ex_{137aa}-EGFP and A1ex_{571aa}-EGFP fusions are predicted to position EGFP on the extracellular side of the membrane, whereas A1in_{110aa}-EGFP and A1in_{218aa}-EGFP fusions are predicted to position EGFP on the intracellular side. The site of autophosphorylation (H202) and a residue involved in phosphatase activity (L253) are located in the H-box and X-box, respectively.

predicted membrane-spanning sequence, TM₅, C-terminal to the kinase/phosphatase domain (467–489). The protein is also predicted to have an intracellular N terminus (1–29), two extracellular loops (65–83 and 136–139) and an extracellular C terminus (490–571) (Fig. 1). The location of the AbsA1 sensory domain and the nature of the stimulus (assuming one exists) that it responds to are unknown.

The investigation of membrane protein topology in *S. coelicolor* is complicated by the absence of a *lacZ*–*phoA* fusion methodology, which is routinely used in *Escherichia coli* (Boyd *et al.*, 1993) and *Bacillus subtilis* (Piazza *et al.*, 1999). We therefore took advantage of the fact that enhanced GFP (EGFP) does not fold efficiently following passage through the Sec translocon, thereby allowing us to distinguish between fusions that position EGFP on the extracellular side of the cell (inactive) and fusions that position EGFP on the intracellular side of the cell (fluorescent) (Drew *et al.*, 2002; Feilmeier *et al.*, 2000). We constructed *absA1* C-terminal deletion fusions to *egfp* that positioned the fluorescent protein between each of the five predicted transmembrane domains, testing each fusion for fluorescence in *S. coelicolor*. We show that the experimental topological model of AbsA1 is in agreement with the predicted model.

METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. *E. coli* XL1-Blue was used to propagate all

plasmids. *E. coli* strains were grown at 37 °C in Luria–Bertani medium. Plasmids were introduced into *Streptomyces* by conjugation from *E. coli* ET12567/pUZ8002. Antibiotic concentrations used for plasmid selection were 100 µg ampicillin ml^{−1} and 50 µg apramycin ml^{−1}. Thiostrepton (30 µg ml^{−1}) was added to induce genes under the control of the *tipA* promoter. *S. coelicolor* strains were grown in liquid R5 medium (supplemented with 0.005% KH₂PO₄, 0.01 M CaCl₂·2H₂O, 0.3% L-proline, 7.5 µg uracil ml^{−1}, 50 µg histidine ml^{−1} and 6% PEG 8000), and on solid R2YE and MS agar (Kieser *et al.*, 2000).

Plasmids and primers. The primers and plasmids used in this study are listed in Tables 2 and 3, respectively. Primers were purchased from Sigma-Aldrich or the Mobix Laboratory at McMaster University. PCR was performed using Vent_R polymerase (New England Biolabs) and *Pfu* DNA polymerase (Stratagene). DNA sequencing was performed by the Mobix Laboratory.

Construction of a thiostrepton-inducible *egfp* reporter plasmid (*ptipAp-egfp*) for use in *Streptomyces*. The 110 bp *tipA* promoter was amplified by PCR with primers *tipAp*-forward and *tipAp*-reverse. The amplified *tipA* promoter was initially cloned into pPCR-Script Amp, sequenced, digested with *Eco*RI and *Xba*I, and ligated to pIJ8660 digested with the same enzymes to generate *ptipAp-egfp*. The construct was introduced into *S. coelicolor* J1501/pJRM10 by conjugation.

Construction of *absA1* C-terminal deletion fusions to *egfp*. The 180 bp *absA1* truncated gene was amplified by PCR with primers *absA1*-forward and A1ex_{60aa}-reverse. The 330 bp *absA1* truncated gene was amplified by PCR with primers *absA1*-forward and A1in_{110aa}-reverse. The 411 bp *absA1* truncated gene was amplified by PCR with primers *absA1*-forward and A1ex_{137aa}-reverse. The 654 bp *absA1* truncated gene was amplified by PCR with primers

Table 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
<i>S. coelicolor</i> strain		
J1501	<i>hisA1 uraA1 strA1 SCP1[−] SCP2[−] Pgl[−]</i>	Kieser <i>et al.</i> (2000)
<i>E. coli</i> strains		
ET12567	<i>dam-13::Tn9 dcm-6 hsdM</i>	MacNeil <i>et al.</i> (1992)
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1lac[F'</i> <i>proAB lacI^q ZΔM15 Tn10 (Tet^r)]</i>	Stratagene

Table 2. Primers used in this study

Primer	Sequence (5'–3')	Engineered site
<i>tipAp</i> -forward	GATATCGGCTGAGGGAGCCGAC	<i>EcoRI</i>
<i>tipAp</i> -reverse	TCTAGACTCCCTTCTCTGACGCCG	<i>XbaI</i>
<i>absA1</i> -forward	TCTAGAATGCACCGATGGCAG	<i>XbaI</i>
<i>Alex</i> _{60aa} -reverse	CATATGGAGCAGACACGCGGTCAG	<i>NdeI</i>
<i>Alin</i> _{110aa} -reverse	CATATGCAGTCTGCGGCCCCGCGAGGC	<i>NdeI</i>
<i>Alex</i> _{137aa} -reverse	CATATGGGTGGCGACCGGTGGATCGCCCTGGCCGACGGTGGCCAC*	<i>NdeI</i>
<i>Alin</i> _{218aa} -reverse	CATATGGCCGCCGCGAGCAGGGCC	<i>NdeI</i>
<i>Alex</i> _{571aa} -reverse	CATATGGAGCGTGTCTTGGCCATG	<i>NdeI</i>

*Underlined region encodes Asp-Pro-Pro-Val-Ala-Thr linker. Bold type, engineered restriction sites.

Table 3. Plasmids used in this study

Plasmid	Description	Reference or source
<i>S. coelicolor</i> plasmids		
pJRM10	Bifunctional replicon consisting of the SCP2-derived low-copy-number plasmid pIJ922 fused to pBluescript II SK(+); <i>tsr</i>	McCormick & Losick (1996)
pIJ8660	Derivative of the conjugative and integrative pSET152 containing <i>egfp</i>	Sun <i>et al.</i> (1999)
<i>ptipAp-egfp</i>	<i>ptipA</i> promoter in pIJ8660	This work
<i>ptipAp-Alex</i> _{60aa} - <i>egfp</i>	<i>absA1</i> [1–180 bp] fragment in <i>ptipAp-egfp</i> ; encodes <i>Alex</i> _{60aa} -EGFP	This work
<i>ptipAp-Alin</i> _{110aa} - <i>egfp</i>	<i>absA1</i> [1–330 bp] fragment in <i>ptipAp-egfp</i> ; encodes <i>Alin</i> _{110aa} -EGFP	This work
<i>ptipAp-Alex</i> _{137aa} - <i>egfp</i>	<i>absA1</i> [1–411 bp] fragment plus 18 nt encoding a 6 aa linker in <i>ptipAp-egfp</i> ; encodes <i>Alex</i> _{137aa} -EGFP	This work
<i>ptipAp-Alin</i> _{218aa} - <i>egfp</i>	<i>absA1</i> [1–654 bp] fragment in <i>ptipAp-egfp</i> ; encodes <i>Alin</i> _{218aa} -EGFP	This work
<i>ptipAp-Alex</i> _{571aa} - <i>egfp</i>	<i>absA1</i> [1–1713 bp] wild-type gene in <i>ptipAp-egfp</i> ; encodes <i>Alex</i> _{571aa} -EGFP	This work
<i>E. coli</i> plasmid		
pPCR-Script Amp	Phagemid cloning vector; variant of pBluescript II SK(+) containing a <i>SrfI</i> site for insertion of blunt-end amplicons	Stratagene

absA1-forward and *Alin*_{218aa}-reverse. The 1713 bp *absA1* gene was amplified by PCR with primers *absA1*-forward and *Alex*_{571aa}-reverse. All amplified genes were initially cloned into pPCR-Script Amp, sequenced, digested with *XbaI* and *NdeI*, and ligated to *ptipAp-egfp* digested with the same enzymes to generate *ptipAp-Alex*_{60aa}-*egfp*, *ptipAp-Alin*_{110aa}-*egfp*, *ptipAp-Alex*_{137aa}-*egfp*, *ptipAp-Alin*_{218aa}-*egfp* and *ptipAp-Alex*_{571aa}-*egfp*. The constructs were introduced into *S. coelicolor* J1501/pJRM10 by conjugation. Unlike the other four *AbsA1*-EGFP fusions, a 6 aa linker between *AbsA1* and EGFP was required for expression of *Alex*_{137aa}-EGFP.

Fluorometric analysis of the *AbsA1*-EGFP fusions. Spores of *S. coelicolor* J1501/pJRM10 containing the *absA1-egfp* fusion plasmids (*ptipAp-Alex*_{60aa}-*egfp*, *ptipAp-Alin*_{110aa}-*egfp*, *ptipAp-Alex*_{137aa}-*egfp*, *ptipAp-Alin*_{218aa}-*egfp* and *ptipAp-Alex*_{571aa}-*egfp*) or the empty vector (*ptipAp-egfp*) were inoculated into 25 ml supplemented R5 medium containing 50 µg apramycin ml⁻¹ to OD₄₅₀ ~0.1. Spores of *S. coelicolor* J1501/pJRM10 were inoculated into medium lacking apramycin. After 31 h growth at 30 °C, cultures were induced by the addition of 30 µg thiostrepton ml⁻¹ (final concentration), and growth was continued for an additional 17 h. Three 1.5 ml aliquots of cells (~200 mg) were washed with 1 × PBS, pH 7.4 (135 mM NaCl, 1.3 mM KCl, 3.2 mM Na₂HPO₄ and 0.5 mM KH₂PO₄), resuspended in 200 µl PBS, and placed in a 96-well, flat-bottomed, polystyrene microtitre plate. A Tecan Safire plate-reader was used to obtain fluorometric measurements of each sample in triplicate. The

parameters were as follows: measurement mode=fluorescence bottom, excitation wavelength=488 nm, emission wavelength=508 nm, excitation bandwidth=5 nm, emission bandwidth=5 nm, and gain (manual)=90. The relative fluorescence was standardized to the expression levels of the *AbsA1*-EGFP fusions as determined by Western analysis of whole-cell lysates. Whole-cell lysates of the induced cultures were obtained by resuspending 200 mg cells in 1 ml P (protoplast) buffer (Kieser *et al.*, 2000) containing 2 mg lysozyme ml⁻¹. Samples were incubated at 30 °C for 1 h, and gently inverted after 30 min. Lysozyme-treated cells were pelleted (5 min, 13 000 r.p.m.; Heraeus Biofuge Pico) and resuspended in 1.5 × SDS-PAGE loading buffer (93.75 mM Tris/HCl, pH 6.8, 3% (w/v) SDS, 15% (v/v) glycerol, 0.015% (w/v) bromophenol blue) containing 62.5 µM DTT, and incubated at 95 °C for 15 min. The total amount of protein in each sample was standardized by running an aliquot on a 10% SDS-PAGE gel, staining with Coomassie brilliant blue and adjusting accordingly. Equivalent amounts of total proteins were resolved by 10% SDS-PAGE and subjected to Western analysis. Proteins were transferred to Immobilon-P PVDF membranes (Millipore) and probed with A.v. peptide antibody (BD Biosciences, catalogue no. 632376; rabbit polyclonal antibody that consists of a mixture of three different affinity-purified antibodies to GFP) at a 1:250 dilution. Horseradish peroxidase-coupled secondary antibody (PerkinElmer, Anti-Rabbit IgG (Goat) HRP-labelled, catalogue no. NEF812001E8) was used at a 1:10 000 dilution and detected by chemiluminescence using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, catalogue

no. NEL105). ImageQuant was used to determine the mean intensity corresponding to the expression levels of each fusion protein. To obtain the standardized fluorescence values, the relative fluorescence units (RFU) produced by a given fusion protein were divided by its mean intensity as determined by Western analysis.

Proteinase protection assay of the AbsA1-EGFP fusion proteins. Spores of *S. coelicolor* J1501/pJRM10 containing the *absA1-egfp* fusion plasmids (*ptipAp-Alex_{60aa}-egfp*, *ptipAp-Alin_{110aa}-egfp*, *ptipAp-Alex_{137aa}-egfp*, *ptipAp-Alin_{218aa}-egfp* and *ptipAp-Alex_{571aa}-egfp*), or the empty vector (*ptipAp-egfp*), were inoculated into 100 ml supplemented R5 medium containing 50 µg apramycin ml⁻¹ to OD₄₅₀ ~0.1. After 31 h growth at 30 °C, cultures were induced by the addition of 30 µg thiostrepton ml⁻¹ (final concentration). Growth was continued for an additional 17 h at 30 °C. The cultures were harvested, washed with 50 ml 10.3 % sucrose, divided in half, and stored at -20 °C. One half of the sample was resuspended in 14 ml P buffer containing 2 mg lysozyme ml⁻¹, and divided between two 15 ml falcon tubes to allow efficient mixing and to aid in protoplast isolation. The cells were incubated in a 30 °C water bath for 1 h 30 min and gently inverted every 15 min. Protoplasts were gently removed from the surface of the digested cells, filtered through cotton wool, and centrifuged at 3000 r.p.m. for 10 min (Fischer Scientific Accuspin 1R). Protoplasts were gently washed with 1 ml P buffer to remove the lysozyme and centrifuged at 9000 r.p.m. for 3 min (Heraeus Biofuge Pico). Protoplasts were resuspended in P buffer and the total amount of protein in each sample was standardized by SDS-PAGE and staining with Coomassie brilliant blue. A 20 µl volume of either 50 mg proteinase K ml⁻¹ (BioShop) diluted in K buffer [50 mM Tris/HCl, pH 8.0, 5 mM CaCl₂, 50 % (v/v) glycerol] or K buffer (negative control) was added to 200 µl protoplasts (equivalent total protein concentrations) and incubated for 7 min in a 30 °C water bath followed by incubation at room temperature for 38 min. A 4 µl volume of 200 mM PMSF (Sigma) diluted in 100 % ethanol was added to stop the reaction. Protoplasts were centrifuged at 9000 r.p.m. for 3 min (Heraeus Biofuge Pico), washed with 500 µl 4 mM PMSF diluted in P buffer, and resuspended in 100 µl 1.5 × SDS-PAGE loading buffer containing 62.5 µM DTT. Samples were incubated at 95 °C for 10 min and stored at -20 °C. Prior to use, samples were reincubated at 95 °C for 3 min and centrifuged at 13000 r.p.m. for 30 s (Heraeus Biofuge Pico). A 50 µl volume of each sample was resolved by 10 % SDS-PAGE and subjected to Western analysis as described in the section above.

RESULTS

Establishing a GFP-based topological reporter system for *Streptomyces*

To identify the intra- and extracellular regions of AbsA1, we created gene fusions that positioned EGFP between each of its predicted transmembrane domains (Fig. 1). The *absA1-egfp* constructs were cloned into the single-copy integrating vector pIJ8660 under the control of the thiostrepton-inducible promoter (*tipAp*) (Murakami *et al.*, 1989). We introduced pIJ8660 (negative control; promoterless *egfp*), *ptipAp-egfp* (positive control; *egfp* under the *tipA* promoter), *ptipAp-Alex_{60aa}-egfp*, *ptipAp-Alin_{110aa}-egfp*, *ptipAp-Alex_{137aa}-egfp*, *ptipAp-Alin_{218aa}-egfp* and *ptipAp-Alex_{571aa}-egfp* into *S. coelicolor* strain J1501 containing pJRM10 [which confers resistance to thiostrepton (McCormick *et al.*, 1994)]. Spore suspensions

of each strain were prepared and used to inoculate supplemented R5 liquid media. After 31 h growth, thiostrepton was added to induce expression of the AbsA1-EGFP fusion proteins for 17 h.

Induced cells were harvested by centrifugation, washed with 1 × PBS and resuspended in 200 µl PBS. The cells were transferred to a 96-well microtitre plate and fluorescence was measured [excitation wavelength (Exλ), 488 nm; emission wavelength (Emλ), 508 nm]. In parallel, cells were lysed and subjected to Western analysis using anti-GFP antibodies to directly measure the expression level of each fusion (refer to Methods; data not shown). The expression levels of the fusions measured in this way were used to calculate the normalized fluorescence activity (Fig. 2a). *S. coelicolor* containing Alex_{60aa}-EGFP, Alex_{137aa}-EGFP and Alex_{571aa}-EGFP fusions produced background levels of fluorescence, whereas cells containing Alin_{110aa}-EGFP and Alin_{218aa}-EGFP fusions produced high levels of fluorescence that were comparable to *S. coelicolor* containing native, cytoplasmic EGFP.

Excitation (Exλ 320–500 nm, Emλ 508 nm) and emission (Emλ 500–550 nm, Exλ 488 nm) scans of the induced cultures revealed maximal absorbance and emission at 490 and 510 nm, respectively, for *S. coelicolor* containing Alin_{110aa}-EGFP, Alin_{218aa}-EGFP and EGFP (Fig. 2b, c). These values are in agreement with the published values for purified EGFP (Exλ_{max} 488 nm, Emλ_{max} 507 nm) (Sun *et al.*, 1999) and indicate that fusions predicted to position EGFP on the intracellular side of the cell (Alin_{110aa}-EGFP and Alin_{218aa}-EGFP) retained the fluorescence spectral characteristics of EGFP, whereas those that positioned EGFP on the extracellular side of the cell (Alex_{60aa}-EGFP, Alex_{137aa}-EGFP and Alex_{571aa}-EGFP) did not. This confirmation that the observed fluorescence matched the emission spectrum of EGFP was important because it ruled out the possibility that we were observing autofluorescence of molecules in *S. coelicolor*. Each of these measurements was repeated three times, and a representative result is shown. These results support the AbsA1 topology predicted *in silico*.

Proteinase K sensitivity of AbsA1-EGFP fusions

To confirm the intra- or extracellular location of our AbsA1-EGFP fusions, we used a proteinase protection assay. A similar approach, based on fusions to the small *Streptomyces tendamistat* protein, has successfully been used in *Streptomyces lividans* to determine the membrane topology of type I signal peptidases (Geukens *et al.*, 2001). Cultures expressing each of the AbsA1-EGFP fusions were used to prepare protoplasts, which were then treated with proteinase K, a non-specific serine protease. Proteinase K sensitivity was determined by Western analysis using anti-EGFP antibodies (Fig. 3). Under the conditions employed, only those EGFP fusions that, based on the fluorescence data, positioned the EGFP protein outside the cell (Alex_{60aa}-EGFP, Alex_{137aa}-EGFP and Alex_{571aa}-EGFP)

Fig. 2. Fluorescence activity of the AbsA1-EGFP fusions in *S. coelicolor*. (a) Normalized fluorescence activity of the AbsA1-EGFP fusion proteins in *S. coelicolor* mycelium from liquid-grown cultures. *S. coelicolor* containing A1in_{110aa}-EGFP and A1in_{218aa}-EGFP fusions produced levels of fluorescence comparable to those of *S. coelicolor* containing the EGFP protein only. Background levels of fluorescence were produced by *S. coelicolor* containing A1ex_{60aa}-EGFP, A1ex_{137aa}-EGFP and A1ex_{571aa}-EGFP fusions. (b) Excitation ($Ex\lambda$ 320–500 nm, $Em\lambda$ 508 nm) and (c) emission ($Em\lambda$ 500–550 nm, $Ex\lambda$ 488 nm) scans of the induced cultures. The excitation ($Ex\lambda_{max}$ ~490 nm) and emission ($Em\lambda_{max}$ ~510 nm) maxima for *S. coelicolor* containing A1in_{110aa}-EGFP, A1in_{218aa}-EGFP and EGFP are characteristic of those for the purified EGFP protein [$Ex\lambda_{max}$ 488 nm, $Em\lambda_{max}$ 507 nm (Sun *et al.*, 1999)].

Fig. 3. Proteinase K protection assay of the AbsA1-EGFP fusion proteins. Protoplasts of *S. coelicolor* containing the AbsA1-EGFP fusions were treated with proteinase K to determine the intra- or extracellular location of EGFP. Proteinase protection/sensitivity was analysed by Western analysis using anti-EGFP antibodies. The presence of a signal in the '+' proteinase K lane reflects a cytoplasmic location for EGFP, whereas the absence of a signal reflects an extracellular location for EGFP.

were sensitive to proteinase K. Those predicted to position EGFP inside the cell (A1in_{110aa}-EGFP and A1in_{218aa}-EGFP) were protected. This experiment was repeated twice and a representative result is shown. These results are consistent with the fluorescence data and validate our *in vivo*, *S. coelicolor*, *egfp* fusion approach to determine the topology of membrane proteins.

DISCUSSION

As a step towards understanding signal transduction by AbsA1, we have solved the transmembrane topology of the protein by constructing AbsA1 C-terminal deletion fusions to EGFP. To the best of our knowledge, this is the first time that EGFP fusions have been used to this end in an actinomycete – a previous effort involving the DrrB protein of *Streptomyces peucetius* involved expression of the protein in *E. coli* (Gandlur *et al.*, 2004). We suggest that the approach we have taken could be generally applicable to streptomycete membrane proteins, especially those that do not express or target efficiently to membranes in a heterologous host such as *E. coli*.

The topology of AbsA1 is interesting for several reasons. The lack of a large extracellular sensory domain near the N terminus excludes AbsA1 from the most common class of extracellular-sensing histidine kinases. The presence of an

Fig. 4. AbsA1 and AbsA1-like proteins: topology and C-terminal amino acid sequence alignment. (a) Schematic representation of the transmembrane topology of AbsA1 and AbsA1-like proteins as predicted by the TMHMM program. Numbered blocks represent transmembrane domains. Horizontal lines below the transmembrane domains represent intracellular regions, whereas lines above the transmembrane domains represent extracellular regions. With the exception of SGR_4840, all of the sensor kinases are predicted to have an intracellular N terminus. (b) Amino acid sequence alignment of the C-terminal extracellular domains of AbsA1 and AbsA1-like proteins. The alignment was created using CLUSTAL W, and an 80% cut-off was used to shade the alignment. Proteins: *S. coelicolor* AbsA1 (SCO3225) and SCO7711, *S. griseus* SGR_3297 and SGR_4840, *S. kasugaensis* KasX, *Saccharopolyspora erythraea* SACE_7062 and *Nocardia farcinica* nfa37240.

extracellular C-terminal domain suggests that this sequence is important for signal response. Indeed, while the sequence of the C-terminal domain does not display similarity to any known motif, and is classified as an

unknown region in the SMART database (Schultz *et al.*, 2000), it does appear to be conserved. A BLAST search identified six uncharacterized, putative two-component system sensor kinases (*S. coelicolor* SCO7711, *Streptomyces*

Fig. 5. Schematic representation of the transmembrane topology of AbsA1, and a proposed signal response mechanism based on the stimulus interacting with the C-terminal domain. For simplicity, only the monomeric form of the protein is shown. In the presence of the stimulus the C-terminal domain undergoes a conformational change, resulting in AbsA1 autophosphorylation and subsequent repression of antibiotic production.

griseus SGR_3297 and SGR_4840, *Streptomyces kasugaensis* KasX, *Saccharopolyspora erythraea* SACE_7062 and *Nocardia farcinica* nfa37240) that have a predicted topology similar to that of AbsA1 (Fig. 4a). All are predicted to have minimal N-terminal extracellular extensions ranging in size from ~3 to 9 amino acid residues and an extracellular C-terminal domain ranging in size from ~85 to 95 amino acid residues. An amino acid sequence alignment of the extracellular C-terminal domains of these proteins with that of AbsA1 reveals the conservation of several residues (Fig. 4b). We note that these include cysteine residues that, perhaps due to the oxidizing environment of the extracellular milieu, form disulfide bonds that play a role in the structural integrity or signal response mechanism of these proteins. Indeed, our preliminary work suggests that the C-terminal domain of this protein is important for modulating the kinase and phosphatase domains of the intracellular portion of AbsA1, perhaps as depicted in Fig. 5.

Finally, it is intriguing that all of these AbsA1-like sensor kinases are from actinomycetes. These Gram-positive filamentous bacteria produce diverse secondary metabolites and encounter extreme environmental conditions. Strikingly, three of these proteins appear to be in or very close to biosynthetic gene clusters for secondary metabolites: *kasX* is in the kasugamycin biosynthetic gene cluster (Ikeno *et al.*, 2006), and the genes for SGR_3297 and SCO7711 are adjacent to genes for, respectively, a non-ribosomal peptide synthase (SGR_3286) and a polyketide synthase (SCO7716). They therefore resemble the *absA1* gene (SCO3225), which is within the cluster that encodes the non-ribosomal peptide synthase subunits for a calcium-dependent antibiotic (SCO3230–3232).

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REFERENCES

- Adamidis, T., Riggle, P. & Champness, W. (1990). Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic biosynthesis but not sporulation. *J Bacteriol* **172**, 2962–2969.
- Anderson, T. B., Brian, P. & Champness, W. C. (2001). Genetic and transcriptional analysis of *absA*, an antibiotic gene cluster-linked two-component system that regulates multiple antibiotics in *Streptomyces coelicolor*. *Mol Microbiol* **39**, 553–566.
- Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. *Curr Opin Microbiol* **8**, 208–215.
- Boyd, D., Traxler, B. & Beckwith, J. (1993). Analysis of the topology of a membrane protein by using a minimum number of alkaline phosphatase fusions. *J Bacteriol* **175**, 553–556.
- Drew, D., Sjostrand, D., Nilsson, J., Urbig, T., Chin, C. N., de Gier, J. W. & von Heijne, G. (2002). Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. *Proc Natl Acad Sci U S A* **99**, 2690–2695.
- Feilmeier, B. J., Iseminger, G., Schroeder, D., Webber, H. & Phillips, G. J. (2000). Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. *J Bacteriol* **182**, 4068–4076.
- Gandlur, S. M., Wei, L., Levine, J., Russell, J. & Kaur, P. (2004). Membrane topology of the DrrB protein of the doxorubicin transporter of *Streptomyces peucetius*. *J Biol Chem* **279**, 27799–27806.
- Geukens, N., Lammertyn, E., Van Mellaert, L., Schacht, S., Schaerlaekens, K., Parro, V., Bron, S., Engelborghs, Y., Mellado, R. P., Anné, J. & other authors (2001). Membrane topology of the *Streptomyces lividans* type I signal peptidases. *J Bacteriol* **183**, 4752–4760.
- Ikeno, S., Aoki, D., Hamada, M., Hori, M. & Tsuchiya, K. S. (2006). DNA sequencing and transcriptional analysis of the kasugamycin biosynthetic gene cluster from *Streptomyces kasugaensis* M338–M1. *J Antibiot (Tokyo)* **59**, 18–28.
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). *Practical Streptomyces genetics*. Norwich, UK: The John Innes Foundation.
- Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567–580.
- MacNeil, D. J., Gewain, K. M., Ruby, C. L., Dezeny, G., Gibbons, P. H. & MacNeil, T. (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61–68.
- Mascher, T., Helmann, J. D. & Uden, G. (2006). Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**, 910–938.
- McCormick, J. R. & Losick, R. (1996). Cell division gene *ftsQ* is required for efficient sporulation but not growth and viability in *Streptomyces coelicolor* A3(2). *J Bacteriol* **178**, 5295–5301.
- McCormick, J. R., Su, E. P., Driks, A. & Losick, R. (1994). Growth and viability of *Streptomyces coelicolor* mutant for the cell division gene *ftsZ*. *Mol Microbiol* **14**, 243–254.
- Murakami, T., Holt, T. G. & Thompson, C. J. (1989). Thiostrepton-induced gene expression in *Streptomyces lividans*. *J Bacteriol* **171**, 1459–1466.
- Pelaez, F. (2006). The historical delivery of antibiotics from microbial natural products – can history repeat? *Biochem Pharmacol* **71**, 981–990.
- Piazza, F., Tortosa, P. & Dubnau, D. (1999). Mutational analysis and membrane topology of ComP, a quorum-sensing histidine kinase of *Bacillus subtilis* controlling competence development. *J Bacteriol* **181**, 4540–4548.
- Schultz, J., Copley, R. R., Doerks, T., Ponting, C. P. & Bork, P. (2000). SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res* **28**, 231–234.
- Sheeler, N. L., MacMillan, S. V. & Nodwell, J. R. (2005). Biochemical activities of the *absA* two-component system of *Streptomyces coelicolor*. *J Bacteriol* **187**, 687–696.
- Sun, J., Kelemen, G. H., Fernandez-Abalos, J. M. & Bibb, M. J. (1999). Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**, 2221–2227.
- Weber, T., Welzel, K., Pelzer, S., Vente, A. & Wohlleben, W. (2003). Exploiting the genetic potential of polyketide producing streptomycetes. *J Biotechnol* **106**, 221–232.

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