Characterization of SCO4439, a D-alanyl-D-alanine carboxypeptidase involved in spore cell wall maturation, resistance, and germination in *Streptomyces coelicolor*

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This work contributes to the understanding of cell wall modifications during sporulation and germination in *Streptomyces* by assessing the biological function and biochemical properties of SCO4439, a D-alanyl-D-alanine carboxypeptidase (DD-CPase) constitutively expressed during development. SCO4439 harbors a DD-CPase domain and a putative transcriptional regulator domain, separated by a putative transmembrane region. The recombinant protein shows that DD-CPase activity is inhibited by penicillin G. The spores of the SCO4439::Tn5062 mutant are affected in their resistance to heat and acid and showed a dramatic increase in swelling during germination. The mycelium of the SCO4439::Tn5062 mutant is more sensitive to glycopeptide antibiotics (vancomycin and teicoplanin). The DD-CPase domain and the hydrophobic transmembrane region are highly conserved in *Streptomyces*, and both are essential for complementing the wild type phenotypes in the mutant. A model for the biological mechanism behind the observed phenotypes is proposed, in which SCO4439 DD-CPase releases D-Ala from peptidoglycan (PG) precursors, thereby reducing the substrate pool for PG crosslinking (transpeptidation). PG crosslinking regulates spore physical resistance and germination, and modulates mycelium resistance to glycopeptides. This study is the first demonstration of the role of a DD-CPase in the maturation of the spore cell wall.

Streptomycetes are mycelial microorganisms characterized by their complex developmental cycles, including programmed cell death (PCD) and hyphae differentiation, which leads to aerial mycelium formation and sporulation¹². Streptomycetes are important industrial bacteria producing approximately two-thirds of clinical antibiotics, as well as a large number of eukaryotic cell differentiation inducers and inhibitors¹. Most of these bioactive compounds are specialized metabolites⁴, the production of which is regulated, at least in part, by hyphal differentiation⁵. *Streptomyces* development, is activated by extracellular signals, including nutritional stimuli or cell density (quorum sensing), and is regulated by complex signaling pathways that are only partially known⁶⁷. The best-characterized stages of *Streptomyces* development are those related to aerial mycelium and sporulation. Several key regulatory networks controlling these stages have been characterised (bald, sky or white pathways, reviewed in Flärdh and Buttner¹). Despite this, the regulation of aerial mycelium and sporulation remains incompletely understood, and new genes and proteins regulating these important processes, are still being discovered⁸.

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Stages preceding aerial mycelium, including spore germination and differentiation in liquid non-sporulating cultures, are even less characterized and comprehended⁹,⁻¹².

D-alanyl-D-alanine carboxypeptidases (DD-CPases) are members of the penicillin binding proteins (PBPs), a family of proteins inhibited by β-lactam antibiotics involved in peptidoglycan (PG) synthesis and remodelling. The PBPs constitute a family of acyltransferases with a common evolutionary origin and a common substrate (the D-Ala-D-Ala dipeptides present in the pentapeptide stems of PG precursors), that are collectively known as DD-peptidases. DD-peptidases include transglycosylases, which catalyze the polymerization of glycan chains composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM); DD-CPases, which remove the terminal D-alanine from muramyl pentapeptide; transpeptidases, which catalyze the cross-link formation between one D-Ala of one PG strand and one amino acid of the other strand; and endopeptidases, which cleave the cross-linked peptide side-chains."³. The DD-peptidases are classified on the basis of their molecular mass, amino acid sequence and enzyme activity at high molecular mass (HMM) and low molecular mass (LMM)¹¹⁻¹³. HMM DD-peptidases are usually bifunctional transglycosylases/transpeptidases (also classified as class A), or monofunctional transpeptidases (class B) anchored to membranes. LMM DD-peptidases are monofunctional carboxy-peptidases or endopeptidases, and the majority are also anchored to membranes (class C). LMM DD-peptidases are usually not essential, and they are not found in most studies and reviews on PBPs¹³.

Most bacteria have multiple PBPs with varying degrees of redundant activity. A phylogenetic analysis revealed that Actinobacteria have an average of five HMM DD-peptidases per genome; this number doubled only in streptomycetes (13 DD-peptidases in the case of S. coelicolor)³¹. Many DD-peptidases belong to the SEDS (shape, elongation, division, and sporulation) clusters of genes involved in PG synthesis and remodelling and are essential for growth, cell division, and cellular viability. DD-peptidases that are not included in the SEDS clusters are considered dispensable for growth and viability⁶, and their biological function remains poorly investigated. Streptomyces coelicolor harbors four SEDS clusters¹⁴, that include four transpeptidases (SCO2010, SCO2608, SCO3847 and SCO5301).

This work contributes characterizing the biological function of the largely ignored redundant and non-essential LMM DD-peptidases by studying the activity and role of SCO4439, a gene encoding a putative DD-CPase. SCO4439 is a very unusual DD-CPase that is highly conserved in streptomycetes and is therein fused with a putative transcriptional regulator domain (see below). SCO4439 was found to be slightly over-expressed during the aerial mycelium and sporulation stages¹⁵, however, its biological role remained unclear.

Results

Mutation of SCO4439 affects spore swelling during germination, increases spore resistance to acid/heat and reduces the glycopeptide resistance.

Cosmid D6.2.B06_046 harboring a copy of SCO4439 interrupted by Tn5062 was used to obtain the S. coelicolor SCO4439::Tn5062 mutant using the methodology developed by Fernández-Martínez et al.⁶. The early stages of SCO4439::Tn5062 mutant spore germination were similar to those in the S. coelicolor wild strain (Fig. 1a–h); however SCO4439::Tn5062 mutant showed a clear and distinctive phenotype at later stages, consisting of a dramatic increase in spore swelling (Fig. 1k,l) with respect to the wild-type strain (Fig. 1j). The kinetics of spore germination in the S. coelicolor wild type and in the SCO4439::Tn5062 mutant were studied by using time-lapse confocal microscopy (Fig. 1m and Supplementary Movies 1 and 2). The spores of the S. coelicolor parental strain swelled until they reached a diameter of 2.3 ± 0.4 μm (5-hour culture), before the emergence of the germ tube, which coincided with a deswelling and a consequent reduction of the spore diameter (from 2.3 ± 0.4 μm at 5-hours to 1.2 ± 0.4 μm at 8-hours) (Fig. 1m, r). At early time points, the swelling of the spores of the SCO4439::Tn5062 mutant was slower than the swelling of the spores of the wild strain (compare 5-hour-time in Fig. 1m,n), but they continued to swell after the emergence of the germ tube, reaching a diameter that was the double that of the wild type spores (3.2 μm ± 0.4 μm) before deswelling and reducing the spore diameter (Fig. 1n,r). Another difference between the wild type and the SCO4439::Tn5062 mutant was that, after germination and deswelling, the cell membrane permeability inside the spores remained intact in the wild type strain (SYTO9 staining, Fig. 1o), but not in the SCO4439::Tn5062 mutant (PI staining, Fig. 1p). The increase in spore swelling affected 100% of the SCO4439::Tn5062 mutant spores at 15–18 hours (average diameter of 3.1 ± 0.4 μm, Fig. 1r).

Contrary to expectations⁶, DNA sequencing demonstrated that the insertion of Tn5062 into cosmid D6.2.B06_046 generated a deletion. Tn5062 was inserted at position 640 of SCO4439 and 669 of SCO4440, generating a loss of 1,641 bp, which affected the 5′-terminus of SCO4439 and most of the SCO4440 open reading frame (ORF) (schematized in Fig. 2a). The deletion of the 5′-terminus of SCO4439 may affect the expression of SCO4437 and SCO4438 (both located downstream of SCO4439), whereas the deletion of SCO4440 may affect the expression of SCO4441 and SCO4442 (Fig. 2a). To identify the gene responsible for the observed phenotype, plasmid pMS82⁷ was used to introduce different fragments of the SCO4437-SCO4442 chromosomal region, into the SCO4439::Tn5062 mutant strain (schematized in Fig. 2b). The only DNA fragments complementing the wild-type phenotype were those including SCO4439, as was the case for plasmid pBRB3 (compare Fig. 2c with Fig. 1k,l) (Supplementary Movie 3). Similar results were obtained in the SCO4439::Tn5062 mutant harboring plasmid pBRB2 but not in the mutant strain harboring plasmid pBRB1 (data not shown).

As introduced above, the SCO4439 gene was previously reported to be slightly over-expressed during aerial mycelium and sporulation in microarray-based transcriptomic analyses; however differences in expression were too low to be considered significant¹³ (Fig. 2e). In this work, RT-PCR analyses confirmed the expression pattern of SCO4439 (Fig. 2e).

The spore resistance profiles of the SCO4439::Tn5062 mutant and the wild type strains were compared (Fig. 3). Lysozyme treatment increases germination, and sonication kills 99% of the spores in the S. coelicolor wild strain. The SCO4439 mutation did not affect resistance to the lysozyme, to sonication or to freezing.
(Fig. 3a–c) but increased fivefold the spore resistance to acid and heating compared with the parental strain and the SCO4439::Tn5062 complemented mutant (Fig. 3d,e).

Mycelium resistance to glycopeptides (vancomycin and teicoplanin) was reduced in the SCO4439::Tn5062 mutant (minimum inhibitory concentrations of 110 and 0.7 μg/ml, respectively), in respect to the S. coelicolor wild type strain (minimum inhibitory concentrations 140 and 0.9 μg/ml) (Fig. 3f).

SCO4439 harbors two protein domains separated by a putative hydrophobic transmembrane region. SCO4439 encodes a multi-domain protein harboring a DD-CPase (conserved domain database Figure 1. Analysis of the germination stages in S. coelicolor wild type and in S. coelicolor SCO4439::Tn5062 mutant. (a–l) Confocal laser-scanning fluorescence microscopy analysis (SYTO9/PI staining) of the S. coelicolor wild type (left panel) and SCO4439::Tn5062 mutant (right panel). The same samples were observed using the fluorescence (left pictures) or interference contrast modes (right pictures). Bars indicate 8 μm (m,n) Time-lapse confocal microscopy (interference contrast mode) of the germination of spores from the wild type and of the SCO4439::Tn5062 mutant, respectively. Spore diameters are indicated. (o,p) Confocal laser-scanning fluorescence microscopy analysis (SYTO9/PI staining) and interference contrast images of spore swelling and spore deswelling stages in the wild type and SCO4439::Tn5062 mutant, respectively. (q) Time-lapse confocal microscopy of the germination of spores from the SCO4439::Tn5062 mutant harboring the SCO4439* mutated gene (SCO4439::Tn5062[pBRB3*] strain). Time-lapse experiments were limited to 12 hours (see Methods for details). Arrows indicate spores. Asterisks indicate swelled spores. (r) Quantification of the spore diameters (average ± SD) of the wild type, SCO4439::Tn5062 mutant, and SCO4439::Tn5062[pBRB3*] strain at 5, 8 and 15 hours.
accession COG1686), and a putative transcriptional regulator (conserved domain database accession PHA03307) (Fig. 4a). According to the Phobius software prediction (http://phobius.sbc.su.se/), these two domains are separated by a putative transmembrane region (Fig. 4a). The DD-CPase domain and the putative hydrophobic transmembrane domain are highly conserved in the *Streptomyces* genus (70% average similarity in *Streptomyces*) and actinomycetes in general (data not shown). The conservation of the transcriptional regulatory domain is substantially lower (44% average similarity in *Streptomyces*), and this domain is not present in other actinomycetes.

Different fragments of the *SCO4439* gene were introduced into the *SCO4439::Tn5062* mutant strain, using pMS82 as the integrative carrier vector (see Materials and Methods for details and scheme in Fig. 4a). The only fragments that complemented the wild-type phenotype were those containing both, the transmembrane and DD-CPase domains (data not shown).

Amino acid sequence alignment of the *S. coelicolor* DD-CPase domain (Fig. 4b) showed an overall high identity with the orthologous proteins from the six model *Streptomyces* strains analyzed, including the triad of “SxxK”, “SxN” and “KTG” motifs that characterize the “SxxK” superfamily of penicillin-binding DD-peptidases. Interestingly, a replacement of Leu684 by Pro (randomly generated by PCR, see Methods) partially blocked the phenotype complementation in spore swelling in the *SCO4439::Tn5062* mutant (Fig. 1q). As discussed below, the maximum spore-swelling of the *SCO4439::Tn5062* mutant strain harboring the mutated *SCO4439* gene (*SCO4439::Tn5062 [pBRB3*] strain) was 3.9 μm (Fig. 1q), which is an intermediate value between the wild type (2.3 μm) and the *SCO4439::Tn5062* mutant (4.5 μm) spore diameters (Fig. 1m,n,q,r; Supplementary Movie 4).

**SCO4439** carboxypeptidase activity. The *SCO4439* protein and its mutated version *SCO4439* (mutation replacing Leu684 with Pro684, see above) were over-expressed in *E. coli*, and purified using His-tag affinity chromatography (Fig. 5a). The identity of the overproduced proteins was confirmed via peptide mass fingerprinting (data not shown).

The enzymatic activities of the purified *SCO4439* and *SCO4439* were assayed on D-Ala-D-Ala dipeptide and on the tripeptide Nα,Nε-diacetyl-l-Lys-D-Ala-D-Ala, which mimics the terminal portions of the PG pentapeptide precursors. *SCO4439* and *SCO4439* cleaved the D-Ala from the tripeptide and, to a lesser extent, from the dipeptide D-Ala-D-Ala. Thus, they showed a significantly higher DD-CPase activity than DD-dipeptidase activity (Fig. 5b). The enzyme activity of the mutated *SCO4439* was half that of the non-mutated protein (Fig. 5b and Figure 2).
discussed below). As expected, due to the presence of the canonical Ser-x-x-Lys motif present in SCO4439, the enzyme activity was inhibited by penicillin G (IC50 of 1 mM, Fig. 5c). As a control, no DD-CPase/DD-dipeptidase activity was detectable in the E.coli host transformed with the empty expression vectors (data not shown).

The DD-CPase/DD-dipeptidase activity of SCO4439 was then assayed in extracts from wild type S. coelicolor, the SCO4439::Tn5062 mutant, and the complemented mutant. The DD-CPase/DD-dipeptidase activity was always detectable in insoluble fractions (membranes and cell wall debris) from the S. coelicolor wild-type strain and the complemented mutant, but not in those from the S. coelicolor SCO4439::Tn5062 mutant (Fig. 5d,e). There was no detectable activity in the cytosolic fractions from any of the two strains (data not shown). Interestingly, the specific DD-CPase enzymatic activity was slightly higher at the early time points after germination (16 hours) than in the substrate (30 hours) or aerial (72 hours) mycelium stages (Fig. 5d). As expected, incubation of the insoluble fractions with penicillin G abolished the enzyme's activity (data not shown).

The spore PG crosslinking index (ratio between cross-linked glycine and total glycine) was estimated by adapting the 1-fluoro-2,4-dinitrobenzene (FDNB) method originally described for Bacillus19 to Streptomyces (see Methods for details) (Fig. 5f). As discussed below, the spores of the S. coelicolor SCO4439::Tn5062 mutant had 20% more crosslinking than the spores of the wild type strain. Interestingly, the PG crosslinking of the SCO4439::Tn5062 harboring the mutated SCO4439 gene (SCO4439::Tn5062 [pBRB3*]) were at an intermediate level in PG crosslinking between the SCO4439::Tn5062 mutant and the wild type strain (10% more crosslinking than the spores of the wild type strain; Fig. 5f).

**PG synthesis during spore swelling and germination.** Peptidoglycan synthesis during spore germination and swelling was analyzed using fluorescent BODIPY-vancomycin20 (Fig. 6). BODIPY-vancomycin preferentially stains nascent PG, the staining of which protrudes above the areas in which there is no PG synthesis20. No PG synthesis occurred during the spore germination and swelling early stages in the wild and complemented strains, at least in the amount detectable by BODIPY-vancomycin staining (Fig. 6). Only at the latest stages of spore swelling, some areas of PG synthesis become detectable in the swelled spores of the mutant strain (Fig. 6).

**Discussion**

The aim of this work was the characterization of SCO4439, a DD-CPase constitutively expressed during development (Fig. 2e), and whose enzymatic activity is present during all developmental stages analyzed (Fig. 5d). The best characterized DD-peptidases are those belonging to the SEDS genes involved in PG synthesis/remodeling which are essential for growth, cell division, and viability. However, with very few exceptions, the biological role
Figure 4. Structure of SCO4439 and orthologous proteins in the Streptomyces genus. (a) Scheme indicating the structure of the SCO4439 protein. Conserved database domain (CDD) references and their average similarities in the Streptomyces genus are indicated. SCO4439 fragments introduced in plasmids pBRB3, pBRB4, pBRB5 and pBRB6 are shown schematically. Fragments that complemented the phenotype in the mutant strain are highlighted in red. (b) Sequence alignment of the DD-CPase domain of SCO4439 (S. coelicolor) and their orthologs in other model streptomycetes. Conserved “SxxK”, “SxN” and “KTG” motifs that characterize the “SxxK” superfamily of DD-peptidases are indicated. An asterisk indicates the Leu684 whose replacement by Pro partially blocks complementation of the wild type phenotype in the SCO4439::Tn5062 mutant.

of other redundant DD-peptidases, not included in the SEDS clusters, such as SCO4439, remains unknown19,21. SCO4439 is not essential for growth; however its mutation resulted in a dramatic increase in both spore resistance to acid/heating and swelling during germination.

DD-CPases are usually anchored to cell membranes at their N-terminus, and their active sites are exposed to the periplasmic space in which they catalyze the final stages of cell wall biosynthesis31. SCO4439 is very unusual, because in addition to the DD-CPase domain, it harbors an additional putative transcriptional regulator domain. Other DD-CPases, including most HMM DD-CPases, are multi domain proteins and harbor glycosyl transferase domains involved in cell wall maturation31. However, to our knowledge, SCO4439 is the first DD-CPase that is associated with a putative transcriptional regulatory domain. SCO4439 has a high molecular mass of 84 kDa, resulting in its classification as an HMM DD-CPase. However, the DD-peptidase domain of

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SCO4439 is homologous to LMM DD-peptidases and exhibits the DD-CPase activity that is peculiar to LMM DD-peptidases. The putative transmembrane domain of SCO4439 is located in the middle of the protein, separating the DD-CPase and the putative transcriptional regulator domains, which is also unusual. The (1) Phobius prediction of the protein structure, (2) the presence of DD-CPase activity only in S. coelicolor insoluble fractions (membranes and cell wall debris) and (3) the presence of the essential nature of the transmembrane domain for biological activity suggest that the DD-CPase domain is exposed to the periplasmic space, whereas the putative transcriptional regulatory domain is likely located on the cytosol side of the cell membrane. Further work is required to understand the biological function, if any, of the putative transcriptional regulatory domain located at the N-terminus of SCO4439. The presence of this domain in all SCO4439 orthologs suggests that it may have a relevant function. In contrast, its relatively low conservation (44% average similarity), its exclusive occurrence in streptomycetes, and finally, its unessential role in complementing the spore germination and resistance phenotypes in the SCO4439::Tn5062 mutant may indicate that this domain has not an essential function.

Spore germination comprises a succession of distinctive steps that were organized by Hardisson et al. into three stages: darkening, swelling, and germ tube emergence. The biomolecular mechanisms controlling these stages remain poorly characterized. There are two examples of proteins known to be involved in Streptomyces spore germination. NepA was described as a structural cell wall protein involved in the maintenance of spor dormancy in S. coelicolor. SsgA was identified as a protein marking cell-wall sites in which germination takes place. The phenotype of the SCO4439::Tn5062 mutant observed in this work indicates the existence of a new stage that includes the deswelling of the spores once they cannot resist further swelling. The occurrence of...
this stage was demonstrated via time-lapse microscopy in both the wild type and the 
SCO4439::Tn5062 mutant (Supplementary Movies 1 and 2; Fig. 1m,n). In the wild-type strain, spore deswelling coincided with germ tube 
emergence, whereas the spores of the 
SCO4439::Tn5062 mutant continued to swell after the emergence of the 
germtube (Fig. 7). In the partially complemented 
SCO4439::Tn5062 mutant (the strain complemented with the 
SCO4439* mutated gene), spore swelling persisted also after germ tube emergence, but the maximum swelling

Figure 6. Nascent-PG synthesis during germination. (a–d) S. coelicolor wild type. (e–j) 
SCO4439::Tn5062 mutant. (k–n), (SCO4439::Tn5062[pBRB3] complemented strain. GYM liquid cultures were stained with 
BODIPY-vancomycin, and observed at the confocal microscope. The interference contrast mode (left pictures) 
and fluorescent images (right pictures) are shown. Arrows indicate nascent PG. Developmental time points are 
indicated. Scale bars represent 4 μm.

Figure 7. Model for the biological function of SCO4439. (a) S. coelicolor wild type. (b) 
SCO4439::Tn5062. Classical germination stages: darkening, swelling and germ tube emergence24. The proposed spore deswelling 
stage is indicated. Green illustrates the membrane-intact cells (SYTO 9 staining); red indicates propidium 
iodide (PI) permeable cells (lysis). D-iGlx, D-iso-glutamine or D-iso-glutamic acid. See text for details.
This can be a consequence, that, at later time points, most of the mycelium suffers a programmed cell death. In some cases, glucose, yeast/malt extract were covered with cellophane disks, inoculated with 100 μl of a fresh spore suspension (1 × 10^7 viable spores/ml), and incubated at 30 °C. Spores were obtained from SFM solid cultures.

**Streptomyces** mutants created in germination such as SCO4439::Tn5062, represent a key tool that provides insight into this process. Up to now, the osmotic mechanism controlling spore swelling is largely unknown. Germ tube emergence is marked by SsgA27, and uncharacterized lytic enzymes regulate the splitting of the spore covers (Fig. 7)1,2. The dramatic swelling of the spores in the SCO4439::Tn5062 mutant after germ tube emergence suggests that the high osmotic pressure in the spore cytoplasm feeds this swelling. In the weaker spores of the wild-type strain (low PG crosslinking), spore swelling culminates with the emergence of the germ tube. However, spores of the SCO4439::Tn5062 mutant have higher PG crosslinking, and the swelling continues after the emergence of the germ tube. In this scenario, the cells likely still detect the high osmotic pressure that in normal conditions would indicate the absence of germination, thus they increase swelling and osmotic pressure to facilitate germination (Fig. 7). Further work is required to fully comprehend this phenomenon. Interestingly, some studies have already suggested a role for PBPs in spore germination in other sporulating bacteria such as *Bacillus*. Neyman and Buchanan and Buchanan and Gustafson31 showed that dacB mutants produce spores with unusual resistance to chemicals and heating in *Bacillus*.

The lack of the DD-CPase activity in SCO4439::Tn5062 mutant strain increases the pool of PG pentapeptide, the terminal D-Ala-D-Ala dipeptides of which are the molecular target of glycopeptide antibiotics52. Consequently, the mycelium of the SCO4439::Tn5062 mutant was more sensitive to vancomycin and teicoplanin than the mycelium of the wild-type strain (Fig. 3f). *S. coelicolor* resistance to vancomycin (but not to teicoplanin), was described to be due to the canonical set of vanRSHAX genes induced by vancomycin (but not by teicoplanin) that are responsible for replacing the terminal D-Ala-D-Ala dipeptides with the resistant D-Ala-D-Lac dipeptides53,54. Recent work demonstrated that other enzymes (VanY-like) contribute to glycopeptide resistance in actinomycetes by removing the last D-Ala from the PG-pentapeptide precursors54,55. Interestingly, these enzymes are membrane-associated LMW DD-CPases with a minor activity on dipeptides and are in some cases inhibited by β-lactams55,56.

The SCO4439 DD-CPase gene is constitutively expressed (Fig. 2e), whereas the specific DD-CPase enzymatic activity decreases during development (from 5 U/mg protein at 16 hours, to 3 U/mg protein at 72 hours) (Fig. 5d). This can be a consequence, that, at later time points, most of the mycelium suffers a programmed cell death12,13 disrupting cell membrane integrity and experiencing an increasing proteolytic activity. Loss of DD-CPase activity may be due to the increasing protein instability in the above conditions. Anyhow, the occurrence of other specific post-translational modifications regulating the DD-CPase activity cannot be ruled out.

Overall, this work demonstrates that the SCO4439 DD-CPase regulates the proportion of PG crosslinking in the spore cell walls, a process that is critical for the regulation of spore germination. The SCO4439 DD-CPase gene is constitutively expressed, and its activity is present in the *Streptomyces* vegetative hyphae. However, its biological role in the mycelium (beyond the increase of resistance to glycopeptide antibiotics) remains unknown. Knowledge of the biological role of the genes involved in antimicrobial resistance is important to understand the evolution of resistance in nature.

**Methods**

**Bacterial strains and media.** Bacterial strains are listed in Table 1. *Streptomyces coelicolor* M145 was the reference strain and was used to generate the mutants. Petri dishes (8.5 cm) with 25 ml of solid GYM medium (glucose, yeast/malt extract)37 were covered with cellophane disks, inoculated with 100 μl of a fresh spore suspension (1 × 10^7 viable spores/ml), and incubated at 30 °C. Spores were obtained from SFM solid cultures.

*Escherichia coli* strains were grown at 37 °C in solid (2% agar) or liquid 2xTY medium 39 supplemented with the appropriate antibiotics (Table 1).

**Disruption of SCO4439.** The transposon insertion single-gene knockout library created by Prof. P. Dyson’s research group18 was used for mutagenesis of SCO4439. Cosmid D6.2B06 was used to construct the SCO4439::Tn5062 mutant strain (Table 1). Gene disruption was carried out by obtaining double cross-overs via conjugation using *E. coli* ET12567/pPZ8002 as a donor strain and following the protocol described in Kieser et al.18. Mutant strains were confirmed using Southern blotting with chromosomal DNA digested with *SalI*. Southern hybridization was carried out using established procedures with the digoxigenin-labeled 3442-bp Tn5062 PvuII fragment from plasmid pQM506240 as a probe.

**Complementation of SCO4439::Tn5062 mutation.** The integrative plasmid pMS8241 was used to introduce different fragments from the SCO4437-SCO4442 chromosomal region into the SCO4439::Tn5062...
mutant. Fragments were amplified via PCR using Phusion High-Fidelity DNA Polymerase (Thermo), and were then cloned into pCR™-Blunt II-TOPO®. The sequences were checked via DNA sequencing using the M13 universal primers prior to subcloning them into pMS82. The following plasmids were constructed (Table 1): pBRB1 containing the SCO4440–SCO4442 fragment amplified with primers BRB1F/BRB1R; pBRB2 containing primers BRB2F/BRB2R; pBRB3 containing primers BRB3F/BRB3R; pBRB3* containing a mutation (Leu684 was changed by Pro) and cloned into pMS82/SpeI/EcoRV, Hyg®; pCR™-Blunt II-TOPO® Zero Blunt®TOPO®PCR Cloning Kit, Km®; pTOPO4439-P-N digested with NruI and SpeI (filled blunt) and religated, Km®; pTOPO4439-P-C digested with AfeI and NruI and religated, Km®; pTOPO4439-P-T-C digested with EcoRV/NdeI and cloned into pTOPO-4439-T-C digested with EcoRV/SpeI, Km®.

Table 1. Bacterial strains, plasmids and primers used in this study. The pairs of primers used to amplify fragments cloned in plasmids pBRB1–7 and pTOPO4439-P-N-T-C are described in the Materials and Methods section.
the SCO4437-SCO4439 fragment amplified with primers BRB2F/BRB2R; pBRB3 containing SCO4439 amplified with primers BRB2F/BRB3R. One of the amplified SCO4439 fragments cloned in pCR™-Blunt II-TOPO® had a mutation generated during the PCR that replaced Leu684 with Pro; this mutation was also cloned into pMS82 generating plasmid pBRB3*. In all cases, primers were designed to hybridize at least 250 bps before the ATG of the ORFs to encompass the promoter region.

Three additional pMS82-derived plasmids were constructed containing different parts of the multidomain SCO4439 gene: one harboring the SCO4439 N-terminus and the other two harboring two regions of the SCO4439 C-terminus. The SCO4439 N-terminus truncated gene was generated in two steps: first, the whole SCO4439 gene was amplified with primers B2F/B3R and cloned into pCR™-Blunt II-TOPO®, selecting for the plasmid in which the C-terminus of the SCO4439 gene was oriented to the SpeI side of the pCR™-Blunt II-TOPO® (pTOPO4439); second, the DD-CPase domain was deleted by digesting pTOPO4439 with NruI and SpeI, the SpeI cohesive end was filled with the Klenow DNA polymerase, and the plasmid was religated to generate pTOPO4439-P-N. A stop codon (TAG) from the SpeI restriction site was formed in the correct ORF. Two C-terminus constructions were performed, one including the 5′-region of the gene (promoter and RBS) followed by the DD-CPase domain (SCO4439-P-C), and the second including the 5′-region followed by both the transmembrane and DD-CPase domains (SCO4439-P-T-C). SCO4439-P-C was generated by digesting pTOPO4439 with AfeI and NruI, and re-ligating the plasmid. pTOPO4439-P-C, lacked the AfeI-NruI fragment (the putative transcriptional regulatory and transmembrane domains) but conserved the 5′-region and the open reading frame. SCO4439-P-T-C was created in three steps: first the 5′-region was amplified with primers BRB2F/BRB6R and cloned into pCR™-Blunt II-TOPO® (pTOPO4439-P); second, the SCO4439 C-terminus (including the transmembrane and DD-CPase domains) was amplified with BRB3R/BRB6F and cloned into pCR™-Blunt II-TOPO®, and the plasmid in which the NdeI (introduced in primer BRB6F) orientated to the EcoRV side of the pCR™-Blunt II-TOPO® was selected, to generate pTOPO4439-T-C; and third, the promoter region was released from pTOPO4439-P with EcoRV-NdeI and cloned into pTOPO-T-C digested with the same enzymes to generate pTOPO4439-P-T-C, pTOPO4439-P-T-C conserved the open reading frame of the transmembrane and DD-CPase domains. The three truncated genes were subcloned into pMS82, generating plasmids pBRB4, pBRB5 and pBRB6 (Table 1).

The seven pMS82-derived plasmids (pBRB1-pBRB6 and pBRB3*) were independently conjugated into the SCO4439::Tn5062 strain as indicated above. The integration of these plasmids into the pMS82 integration site (gene SCO4848) was verified by PCR using primers SCO4848F (hybridizing with the SCO4848 gene) and pMS82R (hybridizing with pMS82). Plasmid integration was confirmed via the generation of a 617-bp amplicon.

**Viability staining.** Culture samples were obtained and processed for microscopy at various incubation durations, as previously described. The cells were stained with propidium iodide and SYTO 9 (LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen, L-13152). The samples were observed under a Leica TCS-SP8 confocal laser-scanning microscope at wavelengths of 488 nm and 568 nm excitation and 530 nm (green) or 640 nm (red) emissions. More than 30 images were analyzed for each developmental time point in a minimum of three independent cultures. For spore diameter quantification, the images were calibrated with Image J, and the diameter of at least 100 spores was quantified for each strain and developmental time point (Supplementary Figs S1 and S2). These images included pictures from at least three biological replicates.

**Time-lapsed (live) imaging.** Initially spores were incubated on GYM medium; after 6 hours of incubation, the samples were cut out and inverted into uncoated m-dishes (Ibidi GmbH). The lid was turned so it was supported on the vents, allowing gas exchange, and sealed off by two layers of Parafilm to prevent medium drying. The samples were incubated at 30°C and imaged with a Leica TCS-SP8 confocal laser-scanning microscope. Images were taken using the interference contrast mode (unstained samples) every 10 minutes for 12 hours. Time-lapse images were processed with Image J. Time lapse experiments were limited to 12 hours because prolonged incubations dry the culture medium and interfere with hyphal growth.

**RNA extraction and Real-Time Quantitative Reverse Transcription PCR (qRT-PCR).** Total RNA samples from three biological replicates of each developmental time point were obtained. Approximately 100 mg of mycelia (fresh weight) were scraped from the GYM-cellophane medium using a plain spatula. Five hundred microliters of RNA Protect Bacteria reagent (Qiagen) were added to the mycelia to provide immediate RNA stabilization. The extraction was carried out using the RNeasy Mini Kit (Qiagen). The lysis step was made using Fast-Prep (MP BioMedicals) with two 30-s force 6.5 cycles, with 1 minute on ice between each run. A phenol stabilization. The extraction was carried out using the RNeasy Mini Kit (Qiagen) and TURBO DNA-freeTM kit (Ambion) were performed to eliminate possible chromosomal DNA contamination. RNA integrity was verified using a 2100 Bioanalyzer (Agilent).

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**Resistance of spores to sonication, lysozyme, mild acid, heating and freezing.** Freshly prepared suspensions of spores were prepared at a concentration of 10⁶ spores/ml in sterile distilled water, and subjected to different treatments as detailed below. Germination of the spores prior to and after treatment were analyzed by plating and quantifying the number of colony-forming units. All quantifications were performed in triplicate, and the data correspond to the average ± SD of the replicates.

For sonication, 2 ml of spores were treated in an MSE Soniprep (six cycles of 15 seconds of sonication, 1 minute on ice). For lysozyme incubation, 1 ml of spores was treated with a concentration of 50 μg/ml freshly prepared...
lysozyme (Sigma-Aldrich, L6876) and incubated at 37 °C for 30 minutes. For mild acid treatment, 0.2 ml of spores were incubated with 0.1 N of HCl for 5 minutes at 25 °C; acid was neutralized via 20-fold dilution in 50 mM potassium phosphate buffer (pH 7.1). For heating, the spores were heated at 55 °C for 90 minutes. For freezing, the spores were stored at −20 °C for 24 hours.

**Determination of the minimum inhibitory concentration (MIC).** Minimum inhibitory concentrations (MICs) of teicoplanin and vancomycin (Sigma-Aldrich V1130 and T0578) were determined in GYM by adding increasing concentrations of glycopeptides. The inoculum was 10⁶ spores/plate, and the plates were incubated at 30 °C until colonies appeared. The MIC was the lowest concentration of the antibiotic that inhibited the visible growth of *S. coelicolor*⁴⁴,⁴⁵. The experiments were performed in triplicate, and were highly reproducible with a variation of zero.

**SCO4439 and SCO4439* gene overexpression and protein purification.** The SCO4439 and SCO4439* (SCO4439 mutated in Leu684) genes were amplified with primers BRB7F/BRB7R from pBRB3/pBRB3* and cloned into the expression vector pET11a (Novagen) to generate pBRB7/pBRB7*. A His® tag was included at the 5′-terminus of the BRB7F primer. The SCO4439/SCO4439* genes were overexpressed in *E. coli* JM109 (DE3) using the MagicMedia *E. coli* Expression Medium (Invitrogen K6803). The expression was performed at 18 °C for 36 hours following the manufacturer’s protocol. The cells were harvested via centrifugation, resuspended in buffer A (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, complete EDTA-free Protease Inhibitor Cocktail Tablets from Roche, pH 7.4) and ruptured using Fast-Prep (MP™ Biomedicals) with < 106 μm beads (Sigma-Aldrich, G8893) and three 20-s force 6.5 cycles, with 1 minute on ice between each run. Finally, the samples were centrifuged at 7,740 × g for 15 minutes at 4 °C. The resulting supernatant fraction was centrifuged at 100,000 × g for 1 hour at 4 °C, and the supernatant was used for protein purification.

Recombinant His®-SCO4439/His®-SCO4439* were purified using 1 ml HisTrap HP affinity columns from GE Healthcare (reference 17-5247-01). Buffer A, described above, was used as a binding buffer; the elution buffer was similar but contained 500 mM imidazole. The protein was purified using an Amersham Pharmacia FPLC (LCC 500 plus controller and two P500 pumps). Purification was performed at 4 °C using a flow of 1 ml per minute, a 20 ml linear elution gradient, and collecting fractions of 500 μl. Fractions were analyzed via SDS-PAGE Coomassie gels, and those containing the overproduced protein were combined, quantified by Bradford,⁴⁶ and used for further experiments.

**Mass spectrometry analysis.** The identity of the overproduced protein was confirmed via peptide mass fingerprinting. The overproduced purified His®-SCO4439 protein was manually excised from a 1D Coomassie gel, and the proteins were digested following the method of Havlis et al.,⁴⁷ and analyzed using a 4800 Proteomics Analyzer matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (AB Sciex). Protein identification was performed using Mascot v. 2.2.04.

**Assay of DD-dipeptidase and DD-carboxypeptidase activities.** Enzymatic activities were assayed as reported previously⁵⁵,⁵⁶ by measuring the release of D-Ala from commercially available dipeptide (D-Ala–D-Ala, 10 mM; Sigma-Aldrich, A0912) and tripeptide (N,N-diacetyl-L-Lys–D-Ala–D-Ala, 10 mM; Sigma-Aldrich, D9904) in the reaction buffer (100 mM Tris-HCl, pH 7.2), together with different amounts of the purified recombinant His®-SCO4439 or His®-SCO4439*. The release of D-Ala was followed spectrophotometrically with a D-amino acid oxidase (Sigma-Aldrich, A5222)-peroxidase (Sigma-Aldrich 77332) coupled reaction that oxidized the colorimetric substrate 4-aminoantipyrine (Sigma-Aldrich 06800) to chinonemin in the presence of phenol solution (Sigma-Aldrich P4557).⁵⁵,⁵⁶ One unit of enzyme activity is defined as the amount of enzyme that produced 1 μmol D-Ala per minute from the tripeptide as substrate; this value must be halved when the dipeptide is used as substrate. To measure the inhibition of DD-C-Pase/DD-dipeptidase activity, the protein was incubated with increasing concentrations (from 0 to 5 mM) of penicillin G (Sigma-Aldrich, P3032) for 15 minutes at 37 °C and then added to the assay mixtures. All measurements were performed in triplicate, and the data correspond to the means ± SD.

**Cellular fractioning.** *S. coelicolor* and the SCO4439::Tn5062 mutant were grown in solid GYM medium as described above for 16, 30 and 72 hours at 30 °C. The mycelium collected from cellophane discs was suspended in 2 ml of 0.9% NaCl per gram of cells (wet weight). All of the following manipulations were carried out at 0 to 4 °C, and all solutions contained proteinase inhibitors (0.19 mg/ml phenylmethanesulfonyl fluoride and 0.7 g/ml pepstatin, both purchased from Sigma-Aldrich P7626 and P5318), unless otherwise stated. Mycelia were fragmented by sonication with a Sonics Vibra-Cell VCX 130. Sonication was carried out for 5 minutes on ice with cycles of 30 seconds with an amplitude of 90% (90% of 60 Hz), and breaks of 10 seconds. The samples were then centrifuged at 39,000 × g for 15 minutes, and the supernatants (cytoplasmic fractions) were collected. Alkaline extraction of the insoluble fraction (membranes and cell wall debris) was carried out by adapting a previously developed protocol for extracting membrane-bound proteins in enterococci by Kariyama et al.⁴⁴ and recently adapted to Streptomyces by Binda et al.⁴³. The sedimented pellets were resuspended in ice-cold distilled water; immediately prior to centrifugation (28,000 × g for 15 minutes at 4 °C), the pH was adjusted to 12 by adding an appropriate volume of 2.5 N NaOH. Immediately after centrifugation, the supernatants (resuspended insoluble fractions) were neutralized to pH 7 by adding 0.5 M sodium acetate (pH 5.4)⁵⁵,⁵⁶,⁴⁵. Enzymatic activities in the cytosolic fractions and the re-suspended insoluble fractions were assayed as previously reported⁵⁵,⁴⁵.

**FDNB determination of spore PG crosslinking.** The protocol described by Atrih et al.⁴⁸ to analyze the PG crosslinking in the spores of *Bacillus* was adapted to *S. coelicolor*. The protocol for PG extraction was modified as follows: spores were collected from solid SFM media⁴⁶, the concentration of spores used for extraction
was 3 mg (dry weight) per ml of extraction buffer; FDNB treatment was performed using 200 μl of the extracted spore cell wall. This protocol works for the analysis of the PG from Streptomyces spores, but not for mycelium, perhaps due to the difficulty to homogenize the dense pellets of the mycelium, making PG poorly accessible to the extraction and FDNB treatment.

Glycine and diaminopimelic acid (Dpm) were measured via high-performance liquid chromatography using pre-column derivatization with o-phthaldehyde (OPA) and UV detection (338 nm). The chromatographic equipment used included the Agilent 1100 HPLC System: a G1312A binary pump, G1329A autosampler, and G1315B-Diode Array Detector. Data collection and integration were performed using Software Chem Station LC 3D. The column used was a 250 × 3.9 mm, 100 Å, Symmetry C18 (5 μm) (WAT046980) from Waters. The binding buffer (10 mM Na₂HPO₄, 40 mM boric acid, pH 8.15) and the elution buffer (MeOH:ACN:H₂O; 45:45:10, v/v/v) were filtered (0.45 μm) prior to use. Samples were eluted in an increasing gradient of elution buffer (20% for 1.9 minutes, 70% for 13 minutes; 100% for 2.7 minutes) with a flow of 1 ml/minute. The column temperature was 40 °C, the injection volume 20 μl, and the detection of the amino acids was at 338 nm. Pure glycine and Dpm (both from Sigma-Aldrich) were used as standards.

The crosslinking index defined by Atrih et al. is based on the difference between the Dpm measured in the FDNB-treated and untreated samples. FDNB treatment is performed prior to PG hydrolysis and blocks NH groups of the Dpm residues that have not formed crosslinks. NH groups blocked with FDNB cannot react with the derivatizing reagent used for HPLC UV detection. S. coelicolor differs from Bacillus, and PG crosslinking is formed by Gly instead of Dpm. Consequently, in this work, the crosslinking index was calculated as the ratio between the cross-linked Gly (Gly detected in the FDNB-treated samples) and total Gly (Gly detected in the non-treated samples). Dpm was used to normalize the glycine measurements (expressed as a ratio to Dpm).

**Bioinformatic analyses.** Transmembrane topology of the SCO4439 gene was analyzed using Phobius software (http://phobius.sbc.su.se/).

Orthologous sequences to SCO4439 from other streptomyces were obtained from the databases at the National Center for Biological Information (http://www.ncbi.nlm.nih.gov). The accession numbers of the selected sequences were: WP_016326920 (S. lividans), NP_824958 (S. avermitilis), YP_006878621 (S. venezuelae), WP_013002845 (S. scabies), YP_001825771 (S. griseus), and WP_003961441 (S. clavuligerus). The DD-CPase domains of the proteins were aligned using MUSCLE software, and amino acid similarities were estimated by using Lalign software (http://www.ch.embnet.org/software/LALIGN_form.html).

**Fluorescent vancomycin staining.** Nascent PG synthesis was stained in Streptomyces liquid cultures growing in GYM medium, inoculated with spores at a concentration of 1 × 10⁶ viable spores/ml, and incubated at 30 °C and 200 rpm for 5 and 8 hours. The samples were stained as previously described. BODIPY-vancomycin (Invitrogen V34850) was mixed with an equal amount of unlabeled vancomycin (Sigma SBR00001). The vancomycin and BODIPY-vancomycin mixtures were added to the cultures at final concentrations of 1 μg/ml and incubated for 20 minutes. Cells were fixed for 15 minutes at room temperature using PBS (0.14 M NaCl, 2.6 mM KCl, 1.8 mM KH₂PO₄ and 10 mM Na₂HPO₄) containing 2.8% paraformaldehyde and 0.0045% glutaraldehyde, and observed under a Leica TCS-SP8 confocal laser scanning microscope at 505 nm excitation and 513 nm emission wavelengths.

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Acknowledgements

This research was funded by an ERC Starting Grant (Strp-differentiation 280304). Our thanks to Beatriz Gutiérrez Magán (Universidad de Oviedo, Dpto. Biología Funcional, Área de Microbiología) for laboratory assistance, Paul Dyson and Meirwyn Evans (Swansea University) for providing the disrupted cosmids, Carlos Barreiro and Mar Calonge (INBIOTEC, Leon) for the mass spectrometry and amino acid analyses, Maggie Smith (University of York) for providing the pMS82 plasmid, and Nature Publishing Group Language Editing service for proof-reading the text.
Author Contributions
B.R., P.Y., M.T.L.G., N.G.Q. and E.B. performed the experiments; B.R., P.Y., E.B., F.M. and A.M. assisted with data analysis; all authors contributed to the critical discussion of the manuscript; B.R., F.M. and A.M. conceived, designed the work and wrote the manuscript. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Rioseras, B. et al. Characterization of SCO4439, a D-alanyl-D-alanine carboxypeptidase involved in spore cell wall maturation, resistance, and germination in *Streptomyces coelicolor*. 
Sci. Rep. 6, 21659; doi: 10.1038/srep21659 (2016).

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