SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
MOLECULAR EVOLUTION
SOIL MICROBIOLOGY
BACTERIOLOGY
BACTERIAL GENETICS

Received
13 July 2012

Accepted
6 December 2012

Published
23 January 2013

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Mammalian cell entry genes in *Streptomyces* may provide clues to the evolution of bacterial virulence

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Understanding the evolution of virulence is key to appreciating the role specific loci play in pathogenicity. *Streptomyces* species are generally non-pathogenic soil saprophytes, yet within their genome we can find homologues of virulence loci. One example of this is the mammalian cell entry (*mce*) locus, which has been characterised in *Mycobacterium tuberculosis*. To investigate the role in *Streptomyces* we deleted the *mce* locus and studied its impact on cell survival, morphology and interaction with other soil organisms. Disruption of the *mce* cluster resulted in virulence towards amoebae (*Acanthamoeba polyphaga*) and reduced colonization of plant (*Arabidopsis*) models, indicating these genes may play an important role in *Streptomyces* survival in the environment. Our data suggest that loss of *mce* in *Streptomyces* spp. may have profound effects on survival in a competitive soil environment, and provides insight in to the evolution and selection of these genes as virulence factors in related pathogenic organisms.

Soil is a highly complex and competitive environment, in which interactions between multitudes of taxonomically diverse organisms occur. Evolutionary pressures in soil are high due to competition for resources, predation and continuously changing environments. Organisms with an ability to cope with these features have significant selective advantages over competitors. Gram-positive saprophytic soil bacteria from the genus *Streptomyces* have evolved many mechanisms that appear to offer competitive advantages in soil including the production of various small molecules, such as antibacterials, antifungals, and anti-helminthics[1–4], restriction-modification systems to cope with viral attack[5].

What is less clear is how streptomycetes cope with predation by eukaryotic soil organisms such as amoebae. However, the intrinsic mechanisms that allow soil organisms to interact with each other at the cellular and intracellular level are still poorly understood[4]. It is these mechanisms that may have led to the evolution of virulence traits in bacteria[6]. There has been much interest in the evolution of pathogenicity and how virulence factors in modern pathogens may have evolved through ancient interactions between bacteria and early eukaryotes. It seems likely that virulence mechanisms observed in pathogens are a coincidental product of selection in an unrelated host, with examples such as *Legionella* and *Mycobacterium* replicating in non-human environmental reservoirs[7–9]. It has also been suggested that because the evolution of true biological novelty is rare, virulence mechanisms have most likely evolved from the existing pool of genetic diversity. The main evidence supporting this hypothesis is the presence of virulence gene homologues in non-pathogens. One such locus is the mammalian cell entry (*mce*) gene cluster from *Mycobacterium tuberculosis* which is found in a variety of non-pathogenic actinomycetes[10].

The *mce* operon was first identified in *M. tuberculosis* when a whole genome library was cloned into a non-invasive strain of *Escherichia coli* and a clone was found to confer the ability to invade non-phagocytic HeLa cells[11]. In addition, *E. coli* transformed with this clone were preferentially taken up and able to persist in macrophages[11]. Subsequent investigation of the *M. tuberculosis* genome revealed that there were four *mce* operons[12]. Mutational studies of the *mce* operons showed that knockouts are attenuated in mouse infection models[13] yet hypervirulent in macrophage models[14], presumably due to the presence of the complete immune system in whole animals[15]. The *mce* operons are expressed at different stages of infection, with *mce1* expressed early during infection while *mce4* is expressed more strongly after a number of weeks[16,17].

Each *mce* operon encodes a core of two integral membrane proteins (YrbE1AB) and six putative secreted proteins (Mce1ABCDEF), which bear homology to ABC transporters and their substrate binding proteins[10]. The role of the *mce4* locus was conclusively determined by Mohn *et al.*, (2008), when it was shown that in *Rhodococcus jostii* RHA1, *mce4* encodes an ATP-dependant steroid transporter providing the first direct evidence of transport activity. These authors also showed that *mce4* activity was essential for growth on media containing a range of sterols as the sole carbon source. This suggests that the homologue of *mce4* in *Mycobacterium* spp. may facilitate uptake and utilisation of cholesterol from host cells during infection, a function which is known to be essential for successful cellular invasion and persistence[18,19] however there is significant divergence in amino acid sequence across the actinomycetes in the non-*mce* domains of the proteins, perhaps reflecting diversity in the substrate specificity.

Here we report the characterisation of the *mce* locus in the non-pathogenic soil saprophyte *Streptomyces coelicolor* A3(2) and how it influences interaction with other soil organisms. We show that the disruption of the *mce* cluster and characterisation of the mutants in amoebae and plant models, indicate these genes are likely to play a role in soil survival. In addition we report the regulation of these genes by the conserved two-component regulatory system MtrAB. Taken together these data suggest that loss of *mce* in *Streptomyces* spp. may have profound effects on survival in the soil environment, and provides insight in to the evolution and selection of the *mce* gene clusters as virulence factors in related pathogenic organisms.

Results

The *Streptomyces coelicolor mce* cluster. Analysis and annotation of the *S. coelicolor* genome identified a cluster of genes homologous to the *mce* (mammalian cell entry) cluster of *Mycobacterium tuberculosis*[11,20]. The cluster is 13.4 kb long and consists of nine core conserved genes, with two conserved *mas* (*mce* associated) genes in the downstream region, and one additional gene of unknown function[10] (Fig. 1). In *S. coelicolor* the core ABC transporter ATP-binding domain-containing protein (SCO2422) and the putative ABC transporter integral membrane proteins (SCO2421 and SCO2420; 50.8 and 55.4% identity respectively with their *M. tuberculosis* homologues) correspond to products of the genes in *M. tuberculosis* annotated as *yrbEA and yrbEB*. The next six genes in the operon (SCO2419-SCO2414) encode proteins containing the Mce motif (Pfam family: PF02470; 33–37% identity with their *M. tuberculosis* homologues) and are annotated as *mceABCDEF*. The gene products contain a putative substrate-binding domain and are predicted to deliver substrates to the membrane component of the ABC transporter[10]. Comparison of the *S. coelicolor* cluster amongst published complete genomes of *Streptomyces* (4) indicates conservation of a single copy of the *mce* operon[20–22]. The exception to this is the plant pathogen *S. scabies*, which lacks the *mce* cluster (www.sanger.ac.uk; strepdb.streptomyces.org.uk/), whilst the genome of *M. tuberculosis* has four copies of the operon and the decayed genome of *M. leprae* has only a single copy[12,23].

The *mce* locus is constitutively expressed throughout growth on solid medium. Semi-quantitative RT-PCR of the *mce* operon in *S. coelicolor* on solid YEME medium showed that the *mce* operon is highly expressed throughout the whole developmental life cycle relative to the multiplexed vegetative sigma factor *hrdB*, with no apparent developmentally linked transcriptional changes (Fig. 2.). The transcription of several *mce* genes was analysed by semi-quantitative RT-PCR and the level of transcription was comparable to *mceA*, therefore *mceA* was considered to be representative of the operon. On MS medium, the *mce* operon is repressed relative to the multiplexed *hrdB* control (Fig. 2). Interestingly, soya flour contains high levels of sterols[24], which suggests that high levels may repress expression of the *mce* genes. To test this hypothesis, RNA was isolated from *S. coelicolor* M145 and the *mce* null mutant grown for 36 hours on YEME medium and YEME medium supplemented with cholesterol (0.02 mg/ml). The addition of cholesterol to YEME medium repressed transcription of *mceA* (Fig. 2), suggesting that the *mce* cluster is only transcribed when low levels of sterols are available.

***mce* in *S. coelicolor* is expressed in an *mtrAB* dependent manner.** Nothing is known about the regulation of the *mce* gene cluster in *Streptomyces* spp. In *Mycobacterium* spp., the proximity of genes encoding GntR-like (*mce1* and *mce2*) and TetR-like (*mce3*) regulators to the *mce* gene clusters gives some clues to their regulation[25,26]. In *Streptomyces* spp., however, there is no obvious regulatory gene adjacent to the *mce* gene cluster. In *M. avium* the *mce* genes are down regulated in an *mtrB* mutant (*mtrB* encodes the sensor kinase in the MtrAB two-component system[27]. Semi-quantitative RT-PCR revealed transcription of *mtrA* in *S. coelicolor* is constitutive throughout growth relative to the vegetative sigma factor *hrdB* (Fig. 2). A *mtrA* null mutant was generated in order to determine whether MtrA modulates the transcription of the *mce* locus in *S. coelicolor*. Semi-quantitative RT-PCR showed that transcription of the *mce* locus was abolished in the Δ*mtrA* mutant,

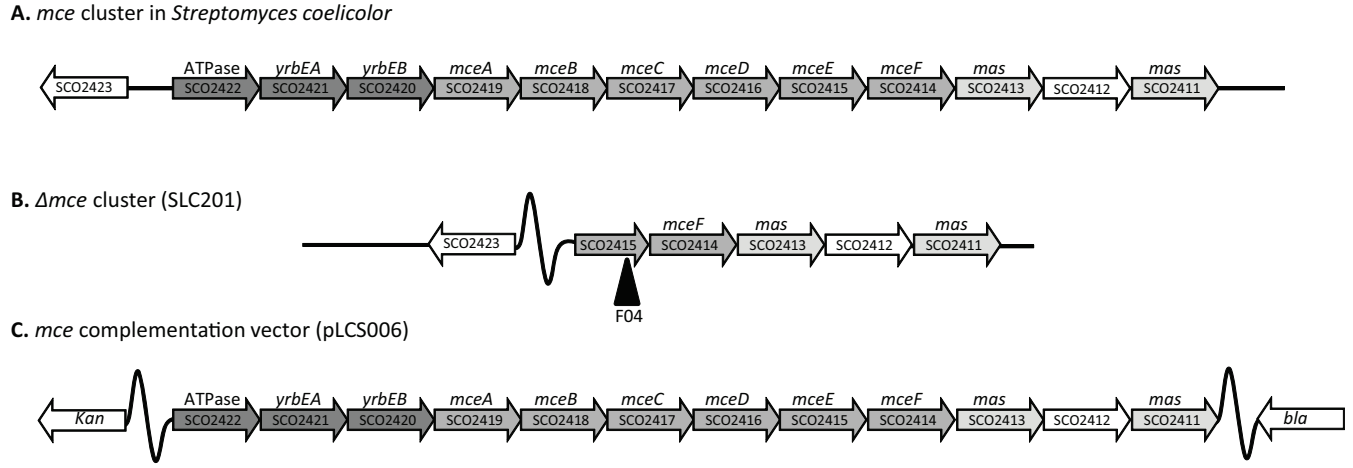

Figure 1 | (A) Organisation of the *mce* locus in *Streptomyces coelicolor*, including the SCO number designations (B) The genetic organisation of the Δ*mce* strain (SLC201) and the Tn*5062* (F04) insertion site (See materials and Methods) and (C) the organization of the complementation vector fragment in pLCS006.

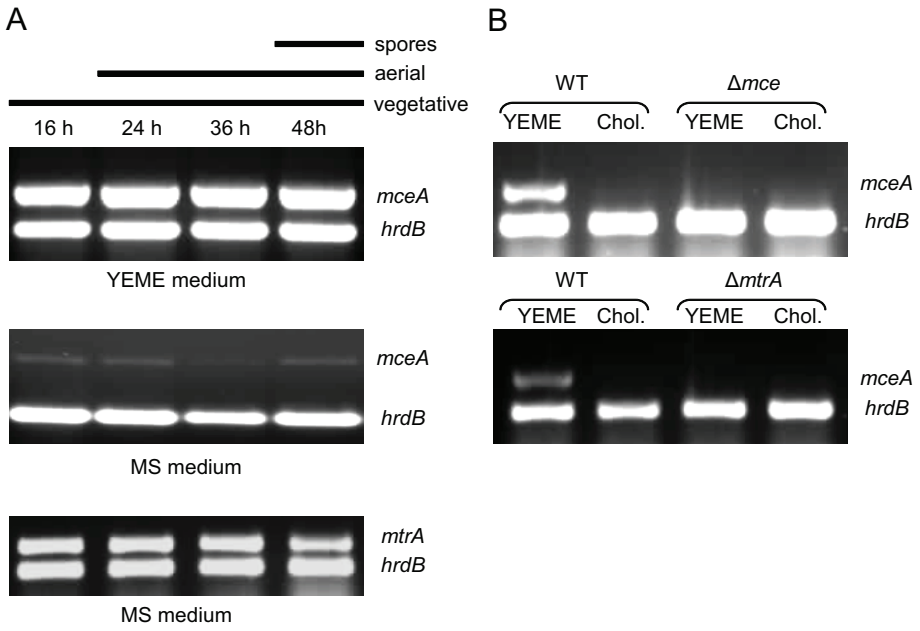

Figure 2 | (A) Transcriptional analysis of *mce* in *Streptomyces coelicolor*. RT-PCR of *mce*A, *hrdB* and *mtrA* during a developmental time course of *S. coelicolor* (M145) on solid YEME and MS media. The time points at which mycelium were harvested for RNA and the developmental stage of the culture was judged by microscopic examination and is indicated above. (B) Transcriptional analysis of *mceA* and *hrdB* on solid YEME with and without cholesterol in the wild-type (M145) and the Δ*mtrA* (SLC201) backgrounds.

even in the absence of cholesterol, conditions which stimulated relatively strong transcription of the *mceA* in the WT strain (Fig. 2), suggesting that MtrA is required for expression of the *mce* genes in *S. coelicolor*.

Mutational analysis of the *mce* cluster in *Streptomyces coelicolor*. Given the roles played by the *mce* genes during *M. tuberculosis* infection, a complete *mce* cluster deletion mutant was created to interrogate the function of the *mce* cluster in *S. coelicolor*. Screening the mutant on a range of carbon sources (glucose, sucrose, mannitol, glycerol, galactose, lactose, arabinose, sorbitol, Tween 20, steric acid, oleic acid, palmitic acid (all 10 g/L), cholesterol (0.02 mg/ml) and sitosterol (0.02 mg/ml)) showed no difference in growth between WT and the *mce* mutant strain (data not shown). *S. coelicolor* A3(2) is unusual among bacteria in that it produces an agarase (DagA) enabling the hydrolysis of agar[28]. To ensure that agar utilisation did not mask an inability for the *mce* mutant to utilise test substrates, we generated a Δ*dagA*-Δ*mce* strain and reanalysed the role of the *mce* locus in normal growth and differentiation. The Δ*dagA*−Δ*mce* strains grew equally well as the parental strain M145 on solid and in liquid cultures (data not shown).

The response of these strains to various stress conditions was also investigated, including 2% ethanol, growth at 37°C and 40°C, 70°C, 1 M NaCl, beta-lactam (Carbenicillin 100 μg/ml) and aminoglycoside (Gentamicin and Neomycin, 20 μg/ml) antibiotics, however the *mce* mutants grew equally well as the WT strain under the conditions tested. Interestingly, the *mce* mutants were more tolerant to SDS (10% w/v) and lysozyme (0.5 mg/ml) than WT in plate spot assays (Fig. 3A), suggesting the cell envelope of the *mce* null mutant differed from that of the parental strain. To further investigate any possible

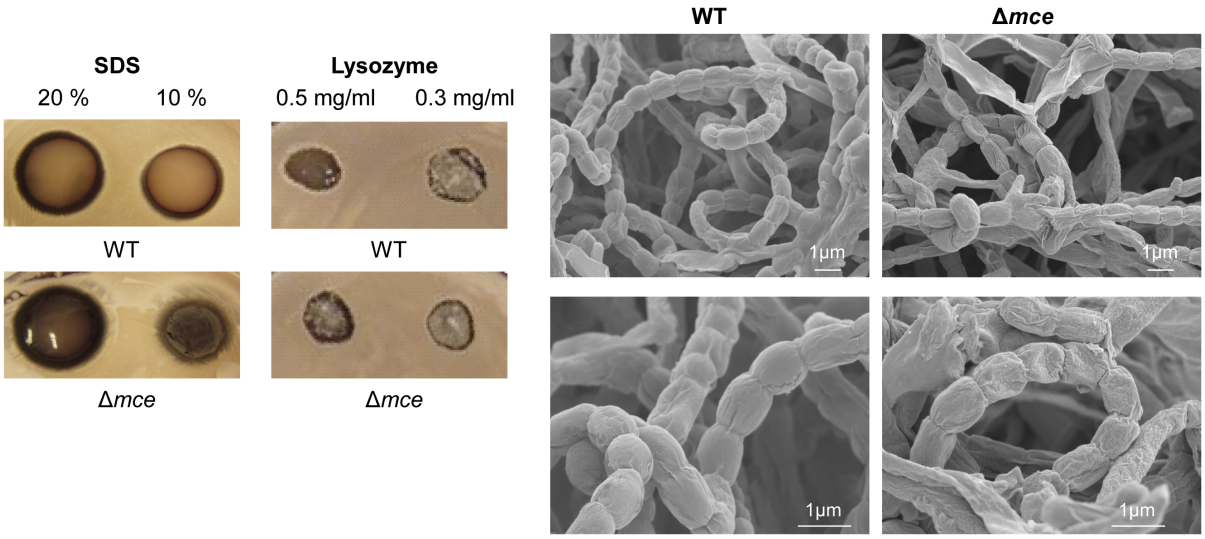

Figure 3 | (A) Resistance of wild-type (M145) and Δ*mce* mutant (SLC201) to SDS and lysozyme (concentrations indicated on figure). (B) Scanning Electron Microscopy of *S. coelicolor* wild-type (M145), and the Δ*mce* mutant (SLC201). Bars indicate 1 μm.

defects in cell morphology, scanning electron microscopy (SEM) was performed. SEM showed that spores produced by the *mce* mutant were wrinkled, more prone to collapsing and had abnormal appendages, indicative of spore germination on the spore chains (Fig. 3B). Analysis of the spore length also showed that the mean size of mutant spores was smaller (mean 0.7 μm +/− 0.05; n= 100) than WT (mean 0.9 μm +/− 0.05; n= 100).

A *Streptomyces coelicolor mce* mutant is virulent in an amoebae model. The *mce* cluster in *Mycobacterium tuberculosis* facilitates invasion and survival in macrophages[11] and recently there has been significant interest in the use of amoebae as surrogate macrophages[29]. Amoebae are abundant soil microorganisms, which likely predate and feed on streptomycetes in the soil. Therefore we developed a co-culture and plaque assay for investigating the role of the *mce* gene cluster in *S. coelicolor*-amoebae interactions.

Introduction of *S. coelicolor* M145 spores to *Acanthamoeba polyphaga* in liquid co-culture experiments resulted in predation of the spores by *A. polyphaga* (Fig. 4A). Repeating the experiment with *A. polyphaga* and the *mce* null mutant resulted in rapid germination of the spores in the *A. polyphaga* vacuoles and death of the amoeba within 24 hrs (Fig. 4A). Using a trypan blue viability assay it was possible to assess the number of viable amoebae after 24 hours in co-culture with WT *S. coelicolor* as 78% +/− 5%, however in co-culture with the *mce* null mutant strain this was reduced to 52% +/− 3%. The virulence observed in an *S. coelicolor mce* mutant is not unprecedented, since an *mce1* mutant of *M. tuberculosis* is hyper-virulent in a macrophage model[14]. Further screening of *S. coelicolor*-amoebae interactions was carried out using a modified plaque assay based on the method of Froquet et al.[30], which allowed a range of strains and conditions to be tested in parallel. The results of the aqueous co-culture were replicated using the plaque assay. The *mce* mutant showed reduced plaque formation in comparison to the parent, indicating the amoebae were killed following predation of the *mce* mutant spores (Fig. 4B). The observed phenotype could be complemented by the addition of the complete *mce* cluster on the integrating vector pLCS006 (Fig. 1 & Fig. 4B). In support of these data, the *mtrA* mutant, which does not express the *mce* cluster, also displayed a virulence phenotype and behaved indistinguishably from the *mce* mutant (Fig. 4B). Interestingly, we tested several additional genes from *S. coelicolor* that are virulence related in actinomycete pathogens in the amoeba model (*whiB*[31]; and[32]; *esxAB*[33]; *desD,* as an example of a siderophore[34], but no differences were observed when compared to the wild-type strain (Data not shown).

The *Mce* gene cluster is required for plant root colonisation. The likely substrate for the *Rhodococcus* and mycobacterial Mce proteins to import is sterols[35]. It is known that plants are a rich source of sterols such as sitosterol, stigmasterol and campersterol[36]. Given this rich source of phytosterols and the abundance of *Streptomyces* spp. in the soil[37], it is possible that the *mce* cluster aides the survival of streptomycetes in the rhizosphere by increasing access to soil resources. The role of the Mce importer was investigated in root colonisation assays using *Arabidopsis thaliana* Ecovar Columbia CL295 as a model. Plants inoculated with spores of *S. coelicolor* M145 grew normally, with uniform colonisation of the roots and bacterial numbers increased over a 7 day period (Fig. 5A & B). Plants inoculated with spores of the *mce* null mutant SLC201 showed reduced growth compared to WT inoculated plants and colonisation levels of the roots was also reduced (Fig. 5A & B). This phenotype could be recovered by complementation of the *mce* mutant with pLCS006 (Fig. 5A & B).

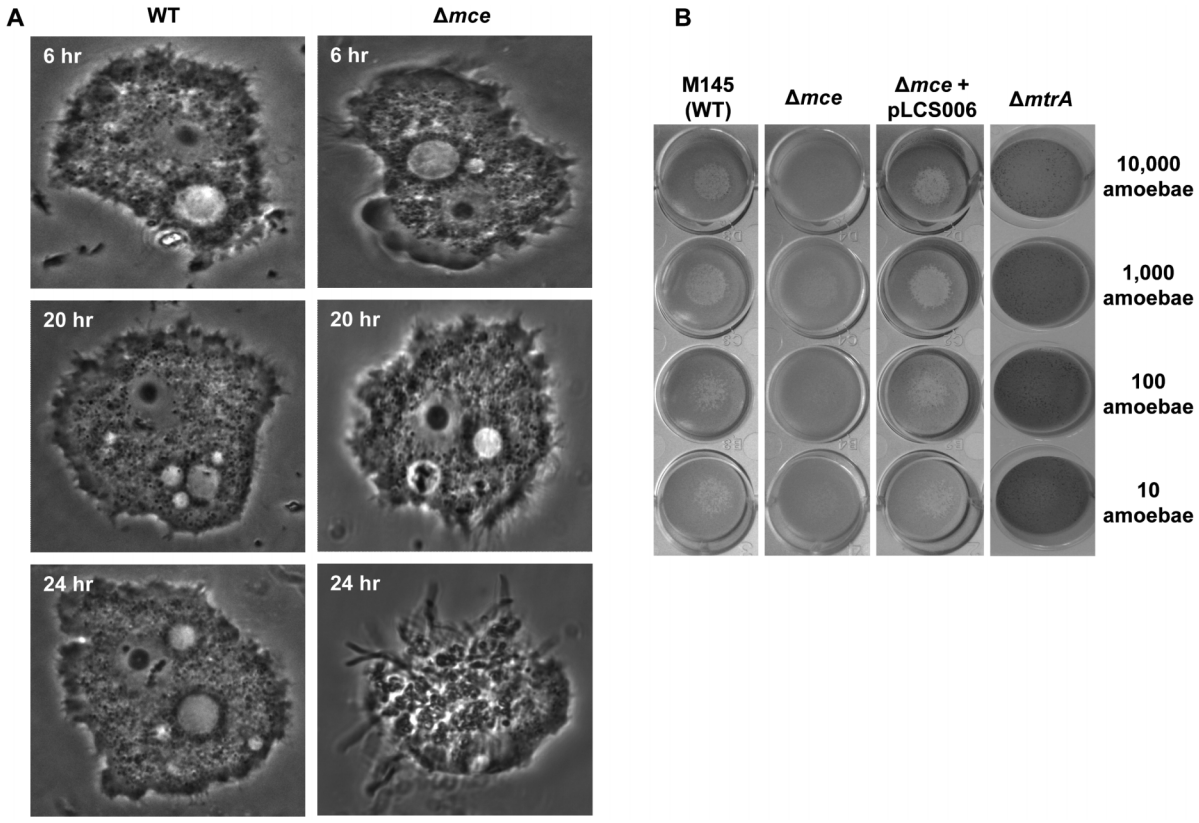

Figure 4 | (A) Co-culture experiments of *S. coelicolor* wild-type (M145) and Δ*mce* mutant with *Acanthamoeba polyphaga* over 24 h. (B) Amoeba plaque assay of wild-type *S. coelicolor* (M145), the Δ*mce* mutant (SLC201), the complemented Δ*mce* mutant (SLC201 + pLCS006), and the Δ*mtrA* mutant. The figure shows the number of amoebae (right hand side of the figure) seeded on to lawns of *S. coelicolor* strains on a 50% dilution of nutrient agar to equalize the growth of the bacteria and amoebae (optimised according to the method of Froquet et al., 2008[3]).

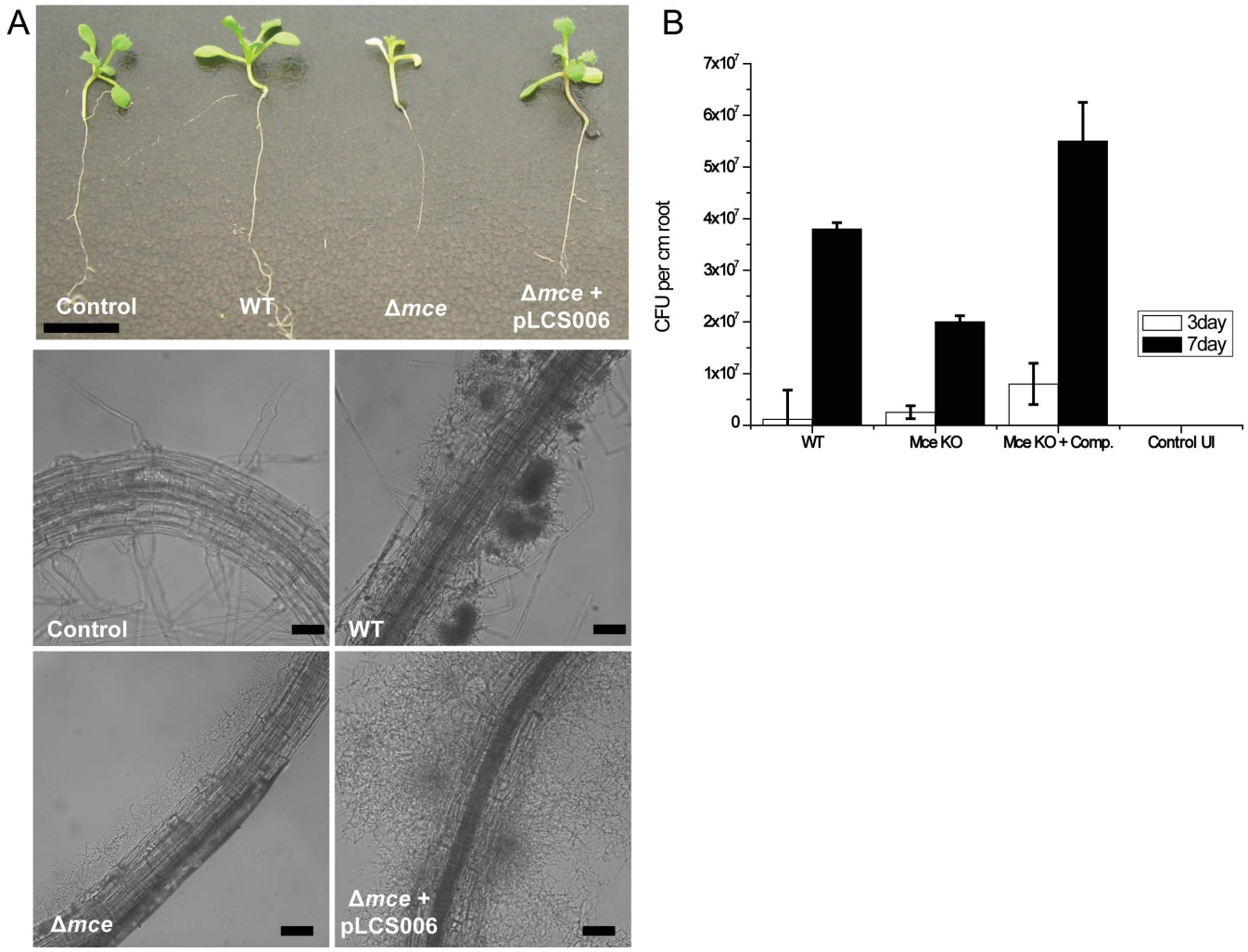

Figure 5 | Colonisation of *Arabidopsis* roots by wild-type *S. coelicolor* (M145), the Δ*mce* mutant (SLC201), the complemented *mce* mutant (Δ*mce* + pLCS006) showing whole plants and microscopy of roots (A) and colony counts of colonised roots at 3 and 7 days post inoculation. Error bars represent the standard deviation of 3 replicates from 3 independent experiments.

Discussion

This study investigated the function of a known pathogenicity locus in the non-pathogenic soil actinomycete *S. coelicolor* through mutagenesis and screening under a range of conditions. The *mce* locus was not required for growth on a range of carbon substrates and *mce* mutants were not altered in a range of tests against deleterious agents, except for an increased resistance to lysozyme and SDS in the *mce* null mutant compared to wild-type *S. coelicolor*. It has recently been shown in some *Rhodococcus* spp. that the *mce* loci encode ABC-like importer systems[10,35], and it is possible that the reduction in the number of transporters/porins may stabilise the cell envelope and result in increased resistance to SDS (membrane) and lysozyme (cell wall) stressors. Scanning electron microscopy also indicated changes to the spore surface in the *mce* mutant, as its spores were more wrinkled in appearance than those of the parent and in addition showed premature germination. Interestingly, such precocious germination has also been observed in mutants lacking the chitobiose transporter sugar binding protein DasA[38], as well as in other *S. coelicolor* mutants with defective cell envelopes, including an *lsp* (lipoprotein signal peptidase) mutant which is defective in lipoprotein biogenesis[39]. In mycobacteria, Cangelosi *et al.*[27], noted that the *mtrA* mutants of *M. avium* have defective cell walls and given that the *mce* gene clusters in both *S. coelicolor* and *Mycobacterium* spp. are down regulated in *mtrAB* mutants, this indicates some commonality in the regulons of the MtrAB two-component system within the actinomycetes, which is not surprising because the MtrAB two-component system, and its accessory lipoprotein LpqB, are highly conserved in actinomycetes[40].

Amoebae are often used as surrogate macrophages for infection studies[29] and streptomycetes are likely to encounter and be predated by amoebae in their natural soil environment. The *mce* locus is required for intracellular survival in macrophages in *M. tuberculosis*[11], therefore we hypothesised that wild-type and *mce* mutant strains of *S. coelicolor* might behave differently in an amoeba model. We were able to observed predation of *Streptomyces coelicolor* spores by *A. polyphaga* in both the plaque assay and co-culture experiments. Intriguingly, this study shows that a *S. coelicolor mce* mutant has a virulent phenotype against *A. polyphaga*, with the spores germinating and resulting in the death of amoebae. This is in stark contrast to WT *S. coelicolor*, which is predated successfully by *A. polyphaga* (Fig. 4A). The virulence phenotype observed in *S. coelicolor mce* is not unprecedented since an *mce1* mutant of *M. tuberculosis* is hypervirulent in a range of models, most likely due to more rapid growth in the host[14,15]. The reason for *S. coelicolor mce* null mutant virulence is not yet fully understood, but they clearly survived predation better than wild-type strains when tested in two separate amoeba models. Two key phenotypic differences may contribute to this; the precocious germination phenotype and increased survival in the

Table 1 | Strains and plasmids used in this study

Strain or plasmid	Genotype/comments	Source or reference
Strains		
S. coelicolor A3(2)		
M145	Prototrophic, SCP1$^-$ SCP2$^-$	Kieser *et al.*, 2000
SLC201	M145 derivative with a deletion of the *mce* operon (see Fig. 1.)	This work
Δ*mtrA*	M145 Δ*mtrA::apr*	This work
Plasmids		
pIJ10702	Cosmid backbone replacement integrating at phage ϕC31 *attB* site.	Foulston and Bibb, 2010
pNRT4	Kanamycin (*aphII*) containing *E coli* shuttle vector	Herron, University of Strathclyde, Unpublished
pLC006	Cosmid 8A2 digested to 22 kbp, with the Supercos1 backbone swopped with pIJ10702 and pNRT4 ligated to confer kanamycin resistance for counter selection.	This work

phagosome due to increased lysozyme resistance. Growth of the *S. coelicolor Δmce* mutant was impaired, however, in plant root colonisation assays, suggesting that the ability to utilise the multitude of nutrient sources from plant root exudates is a selective advantage in the rhizosphere, perhaps representing two conflicting selective pressures for streptomycetes in soil. *Streptomyces* spp. are well established as rhizosphere bacteria, where they likely offer protection to plant roots against fungal infection[4,37]. Plants are known to be a major source of sterols, sugars, polysaccharides, amino acids, and fatty acids in the environment[41,42] and offer a significant resource in the rhizosphere, suggesting they may form a mutually beneficial protective symbiosis with plant roots. Whilst the only known substrate for the Mce transporters is sterols, from the work of Mohn et al.[35], the duplication and diversification of these *mce* clusters in certain actinomycetes (such as rhodococci and mycobacteria) may have lead to the organisms increasing the repertoire and diversity of substrates transported by Mce. In pathogens the ability to utilise abundant host carbon sources such as sterols for intracellular growth during infection or persistence would be a significant selective trait[35]. In *Streptomyces* spp. we do not know the nature of the substrates transported by the *mce* cluster, however based on homology we predict that its substrates would be similar to the sterols transported by the mycobacterial Mce transporters. Transcription of the *mce* was reduced in medium containing sterol, suggesting that higher levels of sterols repress *mce* gene expression. However it is possible that with six substrate-binding proteins (encoded by *mceABCDEF*) the Mce transporter may be responsible for transporting multiple substrates, perhaps of a related chemical nature.

Examining regulation of the *mce* genes in *S. coelicolor* revealed that it is part of the MtrAB regulon, indicating that there is at least some overlap in the genes regulated by MtrA in different actinomycete genera. This two-component system is relatively poorly understood, but appears to regulate some essential processes in actinomycete growth (cell wall remodelling and cell division), survival and pathogenicity[27,40,43,44]. Intriguingly the *mce* cluster has been lost from the plant pathogen, *S. scabies*, which maybe due to the presence of additional plant specific virulence factors, providing a route to plant derived nutrients[45].

The evolution of virulence almost certainly draws on existing pools of genetic diversity and novel functions are achieved through selection and alterations in regulation, leading to the co-option of genes for roles in pathogenicity. Here we have shown that the *mce* locus in the non-pathogenic *S. coelicolor* has functions that mediate interaction of these organisms with plants and amoebae, suggesting that these genes are important in the soil environment. This locus however is a known virulence factor for the human pathogen, *M. tuberculosis*. The evolution of this locus, through duplication and divergence (*M. tuberculosis* has four copies of the *mce* operon) has almost certainly contributed to virulence in mycobacteria. The presence of these genes across the actinomycete lineage[10] and the standard G+C content of the *mce* genes in each organism (Clark and Hoskisson, unpublished) suggest that this locus is ancient in the actinomycete lineage. We therefore hypothesise that the *mce* locus was present in the ancient actinomycete lineage (streptomycetes split from the main lineage approximately 440 million years ago)[46]; and niche specialization (soil in *Streptomyces* spp., or soil and ultimately eukaryotic hosts in mycobacteria) has lead to divergence of the respective clusters in each actinomycete genus throughout evolution. Understanding the selection that leads to these changes in function are key to our appreciation of the evolution of virulence in bacteria and could have a profound impact on our understanding of the emergence of pathogenic strains.

Methods

Strains, plasmids, and growth conditions. The strains of *S. coelicolor* A3(2) and its derivatives used are summarised in Table 1. All strains were cultivated at 30°C on mannitol-soya flour (MS) agar[47] or solid YEME medium containing 0.02 mg/ml cholesterol as indicated[28]. Conjugation of plasmids from the *E. coli* strain ET12567 (*dam*$^-$ *dcm*$^-$ *hsdS*), which contained the plasmid pUZ8002 as a driver for transfer[48]. *Acanthamoeba polyphaga* was maintained on peptone yeast glucose (PYG) medium in 25 ml tissue culture flasks at 20°C[49].

Construction of a *mce* cluster deletion mutant and complementation. A derivative of cosmid 8A2[50] carrying a Tn*5062* insertion in SCO2415 (8A2.1.F04; provided by Dr Lorena Fernández-Martínez and Professor Paul Dyson) was used to generate an *mce* cluster deletion mutant based on the *in vitro* transposition method[51]. Construction of the *mce* cluster deletion mutant was achieved through partial digest of cosmid 8A2.1.F04 using BamHI to excise the region between and including SCO2422 and SCO2415, but retaining the Tn*5062* insertion in SCO2415, resulting in the formation of cosmid pLCS001. This cosmid was subsequently introduced into *S. coelicolor* M145, by conjugation from *E. coli* ET12567/pUZ8002. Mutants exhibiting the double-crossover phenotype (apramycin resistant, kanamycin sensitive) were confirmed by Southern hybridisation, creating strain SLC201 (Δ*mce* cluster).

The complementation vector pLCS006 was constructed using cosmid 8A2.2.G07, which contains a Tn5062 insertion in SCO2423 (a gene adjacent to the *mce* cluster). Partial digestion with *Kpn*I, followed by partial digestion with *Eco*RI and self re-ligation, resulted in the excision of sections of the cosmid insert not containing the *mce* genes. The resulting cosmid was reduced from 50.1 kb to 22.3 kb while maintaining the supercos1 backbone and associated antibiotic resistance markers. To create an single copy integrating vector for complementation, this cosmid was then used to transform hyper-recombinant *E. coli* strain BW25113/pIJ790 along with a 5247 bp *SspI* fragment of plasmid pIJ10702 (a gift from Prof Mervyn Bibb and Dr Lucy Foulston, John Innes, UK) containing φC31 integrase and *attP* site[52]. The resulting recombinant plasmid was digested and ligated with plasmid pNRT4 (provided by P. R. Herron, University of Strathclyde) that contains a kanamycin resistance gene (*aphII*) to facilitate selection in apramycin resistant strains. The resulting cosmid derivative (pLCS006) was introduced into ET12567/pUZ8002 and introduced into *S. coelicolor mce* mutant strains by conjugation.

Generation of an mtrA mutant strain. The *mtrA* (*SCO3013*) null mutant was created by PCR-targeted mutagenesis[53]. A disruption cassette consisting of an o*riT* and the apramycin resistance gene, *aac(3)IV* from pIJ773[53] was generated by PCR amplification with primers SCO3013KOF (5′-GTGCCAGGTCACGCCAGGT-AACGATTAGCTAATGGGATGATTCCGGGGATCCGTCGACC-3′) and

SCO3013KOR (5′-GACCCGGGCGCCGAAGCGGCACTGTCCCTGGCC-ATGTCATGTAGGCTGGAGCTGCTTC-3′) that included the start or stop codons of *mtrA* and 36 nt sequence homologous to the upstream or downstream sequence of the *mtrA* open reading frame. The resulting PCR product was used to mutagenize *S. coelicolor* cosmid StE33 as previously described[53]. Mutagenized cosmid was verified by restriction digest and introduced into *E. coli* ET12567/pUZ8002 and conjugated into *S. coelicolor* M145 as previously described[28]. Transconjugants were selected for resistance to apramycin and sensitivity to kanamycin. The integrity of the *mtrA* mutant strain was verified by PCR using primers RFS16 (5′-GCTATCCGCTCGCGGTG-3′) and RFS17 (5′-GAAGAGACGGGAGCCGAC-3′).

Amoeba assays. *Amoeba-bacteria co-culture assay*. *Acanthamoeba polyphaga* cultures were grown to log phase (3–5 days) in PYG at 20°C and cells were harvested by centrifugation (500 × *g* for 3 minutes). Cells were re-suspended in phosphate buffered saline (PBS) and live cell titres (assessed by trypan blue exclusion) were adjusted to the required number of cells. The amoebae and spores of *S. coelicolor* were combined in a 24-well plate at a multiplicity of infection (MOI) of 1 : 1 or 1 : 10, with approximately 1×10^5 number of microorganisms per well. Plates were incubated at 21°C, with cells harvested for microscopy at the indicated time intervals.

Phagocytic plaque assay of amoebae killing. The method of Froquet et al.[30], was adapted for analysing the interaction between *A. polyphaga* and *S. coelicolor*. Aliquots (0.75 ml) of nutrient agar in varying concentrations (Nutrient broth at 100–10% of the manufacturer's recommended concentration with 15 gl^{-1} bacteriological agar; which allows for equalisation of the growth of the bacteria with the amoebae) were placed in each well of a 24-well plate and dried in a laminar flow hood. *S. coelicolor* spores were diluted to 10^8 spores/ml with PBS and 50 µl the diluted suspensions were transferred to the surface of the agar and allowed to dry; this ensures a confluent lawn of bacterial growth. Amoebae cells were harvested as described above and diluted to a concentration of 200×10^4, 20×10^4, 2×10^4 or 0.2×10^4 live cells per ml with PBS. Amoebae suspension (5 µl) was dispensed into the centre of each well. Plates were incubated at room temperature for 2–5 days and observed daily for signs of plaque formation.

Plant Cultivation and root colonisation assays. Root colonisation assays were performed using *Arabidopsis thaliana* (Ecovar Columbia CL295). Seeds were surface sterilized (15% sodium hypochlorite solution for 15 minutes), washed in sterile distilled water and germinated on Murashige and Skoog agar medium supplemented with 2% sucrose in Petri dishes[54]. Plants were grown at 21°C with a 16-h photoperiod for seven days. Plants for colonization experiments were transferred to Magenta boxes (8 plants per box; Sigma), containing Murashige and Skoog agar medium as above and allowed to equilibrate for 24 hrs on the surface of the medium before inoculation. This allowed the plant roots to be separated more easily for colony counting. Plants roots were inoculated with *S. coelicolor* strains (1×10^6 spores) or mock-inoculated with sterile 20% glycerol. Plants were harvested 3 days and 7 days and colonization of plant roots was assessed by microscopy.

To enumerate bacterial colonization, 1 cm sections of root were removed under sterile conditions and homogenized in 500 µl sterile distilled water, serially diluted and counted by plating on to nutrient agar (Difco).

RNA isolation and RT-PCR of the *mce* and *mtrA* genes. RNA samples were isolated throughout the lifecycle of wild-type and mutant strains of *S. coelicolor* as previously described[55]. Aliquots (50 µg) of DNase-treated RNA were reverse transcribed and immediately subjected to 25 cycles of PCR amplification using the One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instruction. Control reactions in which reverse transcriptase was omitted were also performed. The following primers were used for amplification of *mceA* (forward 5′-TCCGACCCGGTGGTCGTCGAGA-3′; Reverse 5′-GTGAAGTCGGTGAGCGCCGTGA-3′), *mtrA* (forward 5′-GACACCGCACTGGCCGAGA -3′; Reverse 5′- GTAGCCCCAGACCTGCTCGA -3′) and the vegetative sigma factor *hrdB* was used as a control in a multiplex PCR for constitutive expression and amplification using the following primers (forward 5′-GAGGCGACCGAGGAGCCGAA-3′; Reverse 5′-GCGGAGGTTGGCCTCCAG-CA-3′).

Microscopy. Light microscopy and scanning electron microscopy were performed as described previously[56].

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Acknowledgements

LCC was funded by a PhD scholarship from University of Strathclyde. We would also like to acknowledge the support of the Society for General Microbiology for a Presidents Fund grant to PAH to undertake work at the Instituto de Agricultura Sostenible, Córdoba, Spain. MIH acknowledges financial support from the Royal Society and the University of East Anglia to work on MtrAB and RFS is supported by MRC grant G0801721. We would also like to thank Dr Maria Sanchez-Contreras, Prof Paul Dyson, Prof Mark Buttner, Dr Paul Herron and Dr Paco Baron-Gomez for strains/plasmids. We would also like to thank Michael Ambrose, John Innes Centre, for contributing the *Arabidopsis* seeds. We thank Vartul Sangal for helpful comments on the manuscript.

Author contributions

L.C.C. and P.A.H. conceived the study and carried out experimental work with contributions from P.P., J.W., R.F.S., M.I.H., G.P.W.; L.C.C. and P.A.H. wrote the manuscript with contributions from P.P., J.W., R.F.S., M.I.H., G.P.W.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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

How to cite this article: Clark, L.C. *et al*. Mammalian cell entry genes in *Streptomyces* may provide clues to the evolution of bacterial virulence. *Sci. Rep.* **3**, 1109; DOI:10.1038/srep01109 (2013).