Acyl depsipeptide (ADEP) resistance in Streptomyces

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ADEP, a molecule of the acyl depsipeptide family, has an antibiotic activity with a unique mode of action. ADEP binding to the ubiquitous protease ClpP alters the structure of the enzyme. Access of protein to the ClpP proteolytic chamber is therefore facilitated and its cohort regulatory ATPases (ClpA, ClpC, ClpX) are not required. The consequent uncontrolled protein degradation in the cell appears to kill the ADEP-treated bacteria. ADEP is produced by Streptomyces hawaiensis. Most sequenced genomes of Streptomyces have five clpP genes, organized as two distinct bicistronic operons, clpP1clpP2 and clpP3clpP4, and a single clpP5 gene. We investigated whether the different Clp proteases are all sensitive to ADEP. We report that ClpP1 is a target of ADEP whereas ClpP3 is largely insensitive. In wild-type Streptomyces lividans, clpP3clpP4 expression is constitutively repressed and the reason for the maintenance of this operon in Streptomyces has been elusive. ClpP activity is indispensable for survival of actinomycetes; we therefore tested whether the clpP3clpP4 operon, encoding an ADEP-insensitive Clp protease, contributes to a mechanism of ADEP resistance by target substitution. We report that in S. lividans, inactivation of ClpP1ClpP2 production or protease activity is indeed a mode of resistance to ADEP although it is neither the only nor the most frequent mode of resistance. The ABC transporter SclAB (orthologous to the Streptomyces coelicolor multidrug resistance pump SCO4959–SCO4960) is also able to confer ADEP resistance, and analysis of strains with sclAB deletions indicates that there are also other mechanisms of ADEP resistance.

INTRODUCTION

The Clp ATP-dependent proteases in bacteria are generally involved in the elimination of denatured or misfolded proteins. However, they also degrade several regulatory proteins specifically (Gottesman & Maurizi, 1992). They are multi-enzymic complexes with an ATP-dependent unfolding activity that arranges the substrate for proteolysis by the catalytic components (Gottesman et al., 1997; Gottesman, 1999).

In Escherichia coli, the proteolytic subunit ClpP (21 kDa) forms two stacked heptameric rings. This proteolytic component can degrade peptides (Maurizi et al., 1990a) but its association with ATPase regulatory subunits is required to degrade larger substrates, and in particular denatured proteins or the enzyme’s specific substrates. A hexamer of ATPase subunits binds to one or both ends of the ClpP tetradecamer (Grimaud et al., 1998). This ATPase is involved in recognition, unfolding and translocation of the substrate to the proteolytic chamber (Lee et al., 2010b; Xia et al., 2004).

The ATP-dependent proteases of the Clp family are ubiquitous in both prokaryotic and eukaryotic cells (Maurizi et al., 1990b). However, their functions and targets differ substantially between different organisms.

Brötz-Oesterhelt et al. (2005) showed that ADEP, a molecule of the acyl depsipeptide family, had an antibiotic activity when bound to the Clp protease of Bacillus subtilis. In the current model, ADEP binding is believed to lead to a change of ClpP structure, facilitating access of unfolded protein to the proteolytic chamber in the absence of the regulatory Clp ATPases (Kirstein et al., 2009). This favours proteolysis and relieves regulation. Thus, ADEP treatment results in uncontrolled protein degradation and consequently cell death. This antibiotic could be valuable therapeutically, as it is active in vivo against various Gram-positive pathogenic bacteria including Streptococcus pneumoniae, Staphylococcus aureus and Enterococcus faecalis. Furthermore, because it has a unique mode of action, mechanisms of resistance currently expressed in multidrug-resistant pathogens were considered unlikely to confer resistance to ADEP (Brötz-Oesterhelt et al., 2005).

ADEP is produced by Streptomyces hawaiensis. Streptomyces species are filamentous, soil-dwelling bacteria which undergo a complex differentiation cycle. They produce various secondary metabolites, including several clinically valuable antibiotics.

Supplementary material is available with the online version of this paper.
Most genomes of sequenced *Streptomyces* species contain five clpP genes. It has hitherto been unknown whether the various *Streptomyces lividans* Clp proteases are all sensitive to ADEP.

The regulation of clpP gene expression in *Streptomyces* is complex: the clpP1clpP2 operon belongs to a large regulon (including *lon, clpC, SCO5169* and other genes), which is controlled by the activator ClgR, whereas the transcription of clpP3clpP4 is not regulated by ClgR but is strictly dependent on its activator PopR, a ClgR paralogue. ClpP1 degrades the PopR activator of the clpP5 operon. Therefore, clpP3clpP4 is not expressed in the wild-type, but is expressed in the clpP1 mutant so that ClpP3 is produced (Bellier & Mazodier, 2004; Bellier et al., 2006; Viala et al., 2000; Viala & Mazodier, 2002, 2003). ClpP3 does not fully perform the role of ClpP1; this is clearly shown by the ‘bald’ phenotype of the clpP1 mutant, which fails to fully differentiate on various media (de Crécy-Lagard et al., 1999).

clpP1clpP2 expression is also under control of σR. This stress sigma factor senses the thiol–disulphide status of the cell via its cognate anti-sigma factor, RsrA (Paget et al., 1998). ClpP1ClpP2 degrade σRk, an unstable isoform of σR with an N-terminal extension of 55 amino acids (Kim et al., 2009). It is remarkable that ClpP1 degrades its two regulators, ClgR and σRk (Bellier et al., 2006; Kim et al., 2009), thereby establishing negative feedback loops by degrading two very different targets. Nevertheless, the stress-dependent transcription involving σR and a secondary promoter p2 makes only a minor contribution to the transcription rate of clpP1clpP2; it is mostly transcribed from the promoter p1 under the control of the activator ClgR.

Due to the particular mechanism of action of ADEP, which requires ClpP proteolytic activity, one plausible mechanism of ADEP resistance would be ClpP inactivation. Brötz-Oesterhelt et al. (2005) readily obtained such ADEP-resistant isolates due to clpP mutations in *B. subtilis*. However, clpP is inessential in *B. subtilis* and several other micro-organisms, whereas the ClpP proteolytic system seems to be essential to actinomycetes. Indeed, no mutation totally abolishing ClpP proteolytic activity could be selected in *Mycobacterium* (Sassetti et al., 2003) or *Corynebacterium* and we could not construct *Streptomyces* strains carrying deletions in both clpP1clpP2 and clpP3clpP4 (data not shown). This raises the issue of the role of the clpP3clpP4 operon, which is generally present in *Streptomyces*, but appears not to be expressed, at least in *Streptomyces coelicolor* and *S. lividans*. Here, we show that the clpP3clpP4 operon provides an ADEP-insensitive ClpP protease activity, which could contribute resistance by target substitution (ClpP3 substituting for ClpP1).

Little is known about the clpP5 gene. It is monocistronic and present in all sequenced *Streptomyces* genomes. It is not present in either the closely related *Saccharopolyspora erythraea* or the distantly related actinomycetes (*Mycobacterium, Corynebacterium*). clpP5 is transcribed constitutively; it is not induced by heat shock and is not controlled by PopR or ClgR. A clpP5 mutant and a mutant with both clpP5 and clpP1clpP2 mutations are viable (A. Bellier and others, unpublished results). clpP5 does not complement clpP1clpP2 and clpP3clpP4: a double deletion strain was impossible to construct even with an intact copy of clpP5. The ClpP5 protein has atypical spacing of the catalytic triad (serine, histidine and aspartate). It is shorter by one amino acid between the active serine and the histidine residues, and we cannot therefore be certain that ClpP5 is indeed a functional protease. ClpP4 also lacks a canonical catalytic triad. Non-functional ClpP proteins have been described in cyanobacteria and are predicted to have regulatory roles (Stanne et al., 2007). In view of these observations, we focused on clpP1clpP2 and clpP3clpP4 rather than clpP5.

### METHODS

#### Bacterial strains, plasmids and media.

*Streptomyces lividans* 1326 was obtained from the John Innes Culture Collection. *Streptococcus pneumoniae* and *S. pneumoniae* clpP mutants were gifts from Patrick Trieu-Cuot, Institut Pasteur, Paris. *Streptomyces hawaiiensis* NRRL 15010 was obtained from the NRRL Culture Collection. The various mutants used are described in Table 1. Plasmids used in this study are shown in Table 2. *Streptomyces* strains were grown on NE (Murakami et al., 1989), R5 or R2YE solid media and on YEME liquid medium (Hopwood et al., 1985). SFM medium was used for conjugation and sporulation (Floriano & Bibb, 1996). The antibiotics apramycin and tinostrptom were added to final concentrations of 25 μg ml⁻¹ to solid media and 20 and 10 μg ml⁻¹, respectively, to liquid media. *E. coli* strains were grown in Luria–Bertani (LB) medium, supplemented when appropriate with ampicillin (100 μg ml⁻¹), kanamycin (25 μg ml⁻¹) or apramycin (50 μg ml⁻¹).

#### DNA manipulation and transformation procedures.

Plasmid DNA was extracted from *E. coli* using a Qiagen kit. DNA fragments were purified from agarose gels with Ultrabase-DA (Amicon-Millipore). Restriction enzymes were used as recommended by the manufacturers. DNA fragments were amplified by a PCR technique (Mulhis & Falona, 1987; Saiki et al., 1988). The oligonucleotides used are listed in Table 3. Standard electroporation procedures were used for *E. coli* transformation.

Intergeneric conjugation from *E. coli* to *S. lividans* was performed essentially as described previously, although SFM plates were used instead of LB plates (Mazodier et al., 1989).

#### ADEP production.

Spores of *S. hawaiiensis* were spread on NE medium in a line 0.5 cm wide at the centre of the plate and the plates were incubated at 30 °C for 3 days. Spore suspensions of indicator strains were diluted to obtain roughly equal densities (10⁶ ml⁻¹) and were spread on the same plates. Up to 12 indicator strains were streaked perpendicular to the producer growth, taking care not to touch it (six strains on each side of the producer). The indicator strains included various controls, one of which was the producer itself.

Zones of growth inhibition of the indicator strains were observed for the next 3 days.

#### Protein extraction and Western blotting experiments.

Cultures of *S. hawaiiensis*, *S. lividans* 1326 and various derivative strains were grown in YEME and harvested after 24 and 48 h by centrifugation. Pellets were resuspended in the sonication buffer (20 mM Tris, 5 mM
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>General cloning host strain</td>
<td>Promega</td>
</tr>
<tr>
<td>E. coli ET12567</td>
<td>Methyltransferase-deficient E. coli host</td>
<td>MacNeil (1988)</td>
</tr>
<tr>
<td>E. coli ET12567(pUZ8002)</td>
<td>Contains the non-transmissible plasmid pUZ8002 which allows mobilization in trans of vectors containing oriT</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>S. lividans 1326</td>
<td>Wild-type</td>
<td>John Innes Culture Collection</td>
</tr>
<tr>
<td>S. lividans clpP1</td>
<td>Apramycin cassette in clpP1</td>
<td>de Crécy-Lagard et al. (1999)</td>
</tr>
<tr>
<td>S. lividans clpP3</td>
<td>Neomycin cassette in clpP3</td>
<td>Viala et al. (2000)</td>
</tr>
<tr>
<td>S. hawaiiensis NRRL 15010</td>
<td>ADEP producer</td>
<td>NRRL collection</td>
</tr>
<tr>
<td>OS 45.6</td>
<td>S. lividans lrm mgt</td>
<td>Pernodet et al. (1996)</td>
</tr>
<tr>
<td>OS 47.83</td>
<td>S. lividans OS 45.6 sclR (apramycin cassette in sclR)</td>
<td>Murad (2003)</td>
</tr>
<tr>
<td>OS 47.84</td>
<td>S. lividans OS 45.6 sclA (apramycin cassette in sclA)</td>
<td>Murad (2003)</td>
</tr>
<tr>
<td>OS 47.85</td>
<td>S. lividans OS 45.6 sclA sclR (apramycin cassette in sclR and sclA) promoters and coding region of both genes are deleted and replaced with the cassette</td>
<td>Murad (2003)</td>
</tr>
<tr>
<td>OS 47.86</td>
<td>S. lividans OS 45.6 sclA sclR (apramycin cassette excised, apramycin-sensitive)</td>
<td>Murad (2003)</td>
</tr>
</tbody>
</table>

EDTA, 1 mM β-mercaptoethanol, 0.5 mM PMSF) and lysed by sonication. The resulting suspension was centrifuged for 15 min at 4 °C and 20,800 g, and the supernatant was treated with 0.3 % SDS for 5 min at 85 °C. The sample was centrifuged for 15 min at 4 °C and the protein concentration in the supernatant was determined by the method of Bradford (1976). A 20 μg aliquot of protein extract was subjected to SDS-PAGE as described by Laemmli (1970). The proteins were transferred to a nitrocellulose membrane (Hybond C), which was subjected to SDS-PAGE as described by Laemmli (1970). The proteins were transferred to a nitrocellulose membrane (Hybond C), which was subsequently probed with rabbit polyclonal anti-ClpP1 (1 : 1000) antibodies. Signals were detected with the ECL Western blotting Detection kit (Amersham Biosciences).

RESULTS

Assessment of the sensitivity of different Streptomyces strains to ADEP

Purified ADEP is not presently available. However, Streptomyces hawaiiensis strain NRRL 15010, the only known ADEP producer, is available from the NRRL collection. S. hawaiiensis culture supernatants inhibited cultures of wild-type Streptococcus pneumoniae, but not of a clpP mutant, consistent with the production of ADEP (results not shown). We attempted purification from S. hawaiiensis culture supernatants; we obtained enough ADEP for mass spectrometry authentication but failed to obtain larger-scale amounts of stable material. Thus, the sensitivity of Streptomyces lividans to ADEP was assayed on NE solid medium; growth of wild-type S. lividans was clearly inhibited in the vicinity of S. hawaiiensis, whereas growth of the clpP1 mutant was not inhibited (Fig. 1a).

This result was not expected because the clpP1 mutant still shows functional Clp protease activity. Indeed, in this strain as ClpP1 does not degrade PopR, clpP3clpP4 is transcribed and ClpP3 is produced (Viala & Mazodier, 2002). Resistance of the clpP1 mutant to ADEP indicates that presence of ClpP3 does not lead to ADEP sensitivity.

To confirm that the resistance of the S. lividans clpP1 mutant to ADEP was a consequence of the clpP1 mutation, we transformed the strain with pVDC742 carrying the wild-type clpP1clpP2 operon; this complementation fully restored ADEP sensitivity (Fig. 1a).

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVDC742</td>
<td>2.5 kb fragment of S. lividans clpP1clpP2 operon inserted into pUWL219</td>
<td>de Crécy-Lagard et al. (1999)</td>
</tr>
<tr>
<td>pHM11a</td>
<td>E. coli–Streptomyces shuttle vector, integrative vector for gene expression in Streptomyces under control of the permE promoter</td>
<td>Motamedi et al. (1995)</td>
</tr>
<tr>
<td>pJV41</td>
<td>1465 bp fragment of S. lividans clpP3clpP4 operon inserted into pHM11a</td>
<td>Viala &amp; Mazodier (2002)</td>
</tr>
<tr>
<td>pJV50</td>
<td>1470 bp fragment of S. lividans clpP1clpP2 operon inserted into pHM11a</td>
<td>Viala &amp; Mazodier (2002)</td>
</tr>
<tr>
<td>pJV51</td>
<td>620 bp fragment of S. lividans clpP1 gene using the first start codon (corresponding to the peptide sequence VTNLM) inserted into pHM11a</td>
<td>Viala &amp; Mazodier (2002)</td>
</tr>
<tr>
<td>pJV52</td>
<td>810 bp fragment of S. lividans clpP2 gene using the first clpP2 possible translation initiation codon inserted into pHM11a</td>
<td>Viala &amp; Mazodier (2002)</td>
</tr>
<tr>
<td>pJV53</td>
<td>700 bp fragment of S. lividans clpP2 gene (short); the second start codon of clpP2 (corresponding to the peptide sequence VIPRF), inserted into pHM11a</td>
<td>Viala &amp; Mazodier (2002)</td>
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</table>
Analysis of spontaneous ADEP-resistant mutants

Abolition of the expression of clpP1clpP2 may result in a strain producing ClpP3ClpP4, allowing the survival of the bacteria and ADEP resistance by a mechanism of target substitution.

To test this possibility, we first analysed three strains of S. lividans which appeared and grew in the inhibition zone in the vicinity of the ADEP-producing S. hawaiiensis. Stocks of spores of these spontaneous resistant strains were prepared on SFM plates, and their ability to grow in the vicinity of S. hawaiiensis was tested. The three strains continued to exhibit ADEP resistance, indicating that it was not a transient epigenetic character.

The three strains were analysed by Western blotting using anti-ClpP1 and anti-ClpP3 antibodies. Such Western blot experiments are very informative because in addition to detection of the proteins, the presence of ClpP1 and absence of ClpP3 also indicates that ClpP1 prevents ClpP3 production through PopR degradation. This provides evidence of ClpP1 proteolytic activity. The absence of ClpP1 and presence of ClpP3 indicates clpP1 mutation.

ClpP1 but no ClpP3 was detected in two strains. In the third strain ClpP3 but no ClpP1 was detected and the strain was therefore named AS1S (ADEP Selected ClpP1 Stifle) (Fig. 2). Absence of ClpP1ClpP2 may thus be responsible for resistance to ADEP in S. lividans, although it is not the only mode of resistance. The two ADEP-resistant clones with no clpP3 expression appear to have functional ClpP1ClpP2 and therefore express a different resistance mechanism. These strains also showed slight increases in resistance to erythromycin, lincomycin and tetracycline, consistent with a mechanism involving for example altered influx or increased secretion (results not shown).

The AS1S strain producing ClpP3 and no ClpP1 protein was further analysed. The clpP1clpP2 operon together with its upstream region was sequenced. Relative to the S. lividans wild-type sequence, there is a single nucleotide insertion in the AS1S clpP1 gene (at position 243). This frame-shift mutation in the 5’ part of the structural gene clpP1 explains the absence of the ClpP1 protein (Table 4).

Table 3. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tr>
<td>NS1clpP1 up For</td>
<td>CCGTCCCCGACGGATGTGAAAC</td>
<td>293 bp upstream from the clpP1 start codon</td>
</tr>
<tr>
<td>NS2clpP2 down Rev</td>
<td>ACTCTCTGGCTGCTGCCCTATAG</td>
<td>118 bp downstream from the clpP2 stop codon</td>
</tr>
<tr>
<td>NS3clpP1 Rev</td>
<td>AGTGTTCACTAGTCTCTCT</td>
<td>719 bp downstream from the clpP1 start codon</td>
</tr>
<tr>
<td>NS4clpP2 For</td>
<td>ATGGACGAGCTCATGCCAC</td>
<td>110 bp upstream from the clpP2 start codon</td>
</tr>
<tr>
<td>NS5popR For</td>
<td>AAGAATGGCCGGACACACTG</td>
<td>350 bp upstream from the popR start codon</td>
</tr>
<tr>
<td>NS6popR Rev</td>
<td>ATGGACGGGGAACACTG</td>
<td>141 bp downstream from the popR stop codon</td>
</tr>
<tr>
<td>NS13clpP3 up For</td>
<td>TTGAGGCCCCAGCAACTTCTG</td>
<td>419 bp upstream from the clpP3 start codon</td>
</tr>
<tr>
<td>NS14clpP3 Rev</td>
<td>AGCACGATCCGCTGTGGAG</td>
<td>124 bp downstream from the clpP3 stop codon</td>
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</tbody>
</table>

Fig. 1. ADEP resistance in S. lividans mutants. Spores of S. hawaiiensis were spread on NE medium in a line across the centre of the plate and incubated at 30 °C for 3 days. Then S. lividans indicator strains were streaked perpendicularly. Spore suspensions were diluted to obtain roughly equal densities. Plates were photographed after 36 or 48 h. (The genes present on each plasmid are indicated in parentheses.) (a) 1, Wild-type; 2, clpP1 mutant; 3, clpP1 mutant/pUWL219 (empty vector); 4, clpP1 mutant/pVDC742 (clpP1clpP2). (b) 1, clpP1 mutant; 2, clpP1/pJV50 (clpP1clpP2); 3, clpP1/pJV51 (clpP1); 4, clpP1/pJV52 (clpP2 long); 5, clpP1/pJV53 (clpP2 short). (c) Analysis of sclAB: 1, OS 45.6; 2, OS 47.83; 3, OS 47.84; 4, OS 47.85; 5, OS 47.86. (d) 1, Wild-type; 2, clpP1 mutant; 3, wild-type/pJV41 (clpP3clpP4); 4, clpP1/pJV41 (clpP3clpP4).
ADEP insensitivity of the ClpP3 protease

The ADEP resistance of the clpP1 mutant cannot be explained by weak ClpP3 production at much lower levels than required for ADEP sensitivity, but in sufficient amount to provide ClpP essential functions: microarray and QRT-PCR analysis of the clpP1 mutant indicated substantial transcription of clpP3 (Viala & Mazodier, 2002; A. Guyet & P. Mazodier, unpublished data). Furthermore, Western blots detected large amounts of ClpP3 (Figs 2 and 3), and analysis of S. lividans clpP1 bearing pJV41 (Viala & Mazodier, 2002), a plasmid which overexpresses clpP3clpP4 per se does not lead to ADEP sensitivity (Fig. 1d). Conversely, expression of clpP3clpP4 per se does not provide ADEP resistance; this is evidenced by the fact that ADEP sensitivity of ClpP is a dominant character over ClpP ADEP insensitivity. In theory it could be possible that ClpP4, which does not have the catalytic site, could provide resistance by titration of the antibiotic (by binding ADEP). Although the experiment of coexpression of clpP3 with clpP4 does not support this hypothesis, we investigated whether uneven unbalanced overproduction of ClpP4 could provide ADEP resistance. When overexpressed under control of the strong constitutive ermE promoter, clpP4 failed to confer ADEP resistance in S. lividans (result not shown).

The ADEP-binding site in the ClpP protease of B. subtilis has been characterized very recently: several residues interacting with ADEP are located in the N-terminal region of the protein (Lee et al., 2010a). Alignment with B. subtilis ClpP shows a good conservation of most ADEP-binding residues in S. lividans ClpP1, whereas there is more divergence at these sites in ClpP3 (Fig. 4). In particular there is an R23Q change (B. subtilis ClpP coordinate), which affects a residue interacting with ADEP, and an F82L change, which affects the ADEP-core binding site at a position shown to be critical (Lee et al., 2010a).

All these data support the notion that ClpP3 is intrinsically an ADEP-insensitive protein.

### Resistance to ADEP due to the SclAB pump

We investigated whether an ABC transporter known to confer multidrug resistance in S. lividans was involved in resistance to ADEP.

In S. lividans, the lrm (23S rRNA methyltransferase) and mgt (macrolide glycosyltransferase) genes can confer resistance to macrolides by altering the ribosomal target (methylation) or the antibiotic molecule (glycosylation) (Jenkins & Cundliffe, 1991). Perndorf et al. (1996) constructed a S. lividans mutant strain, OS45.6, from which both genes are deleted. This strain has then been used to show that S. lividans can acquire spiramycin resistance through spontaneous activation of efflux pumps (Murad, 2003). One system was extensively characterized: slrAB, composed of slr, encoding a TetR-type regulator (SCO4358 orthologue), and the regulated genes sclAB, encoding an ABC transporter (SCO4359 and SCO4360 orthologues). Slr prevents the expression of sclAB. When

### Table 4. Mutations affecting clpP1clpP2 in spontaneous ADEP-resistant mutants of S. lividans

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Parent</th>
<th>Mutation type</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>A51S</td>
<td>1326</td>
<td>Frameshift</td>
<td>Single nucleotide insertion (C) in clpP1 (position 243)</td>
</tr>
<tr>
<td>85A</td>
<td>OS 47.85</td>
<td>Nonsense</td>
<td>C (289) to T substitution in clpP. CAG—TAG resulting in a 96 aa truncated peptide</td>
</tr>
<tr>
<td>85F</td>
<td>OS 47.85</td>
<td>Deletion in clpP1</td>
<td>240 bp deletion from clpP1 (47→287)</td>
</tr>
<tr>
<td>86A</td>
<td>OS 47.86</td>
<td>Deletion in clpP1clpP2</td>
<td>824 bp deletion starting —51 bp upstream from the clpP1 start codon and extending into clpP2</td>
</tr>
<tr>
<td>86C</td>
<td>OS 47.86</td>
<td>Nonsense</td>
<td>C (346) to T substitution in clpP. CAG—TAG resulting in a 115 aa truncated peptide</td>
</tr>
<tr>
<td>86E</td>
<td>OS 47.86</td>
<td>Missense</td>
<td>Single nucleotide modification in clpP, resulting in an M165R change in the ClpP1 protein</td>
</tr>
</tbody>
</table>
derepressed, the transporter provides specific resistance to some macrolides, including spiramycin, and also to several unrelated antibiotics such as daunorubicin and to chemical compounds such as ethidium bromide.

Using different \textit{S. lividans} sclABR mutants (a gift from J. L. Pernodet, Université Paris-Sud XI), we investigated the effect of the SclAB efflux pump on ADEP resistance.

In strain OS 47.83, the \textit{sclR} gene encoding the TetR-like repressor is disrupted and consequently the efflux system encoded by \textit{sclAB} is constitutively activated. We found that OS 47.83 was ADEP resistant (Fig. 1c). Two OS 47.83 \textit{sclA} deleted derivatives, OS 47.85 and OS 47.86, were ADEP sensitive. Thus, the ADEP resistance of OS 47.83 is mediated through functional SclAB transport.

\textbf{Evidence for other ADEP-resistance systems}

We used OS 47.85 or OS 47.86, strains unable to utilize the efflux pump SclAB, to isolate spontaneous ADEP-resistant mutants and look for other resistance mechanisms. Eleven ADEP-resistant clones were obtained and investigated by Western blotting with anti-ClpP1 and anti-ClpP3 antibodies.

In five strains, ClpP3 but no ClpP1 was detected, indicating that in these mutants, ADEP resistance might be due to the absence of ClpP1 production. The \textit{clpP1clpP2} genes in these strains were sequenced: mutations of the \textit{clpP1} gene were found in all five. These nonsense mutations and partial deletions readily explain the observed phenotype (Table 4).

In one strain (86E) both ClpP1 and ClpP3 were detected. The ADEP resistance is therefore likely to be due to the production of a non-functional ClpP1. There is a missense mutation in the \textit{clpP1clpP2} genes in this strain, a single

\begin{figure}
\centering
\includegraphics[width=\textwidth]{clpP_alignment}
\caption{Alignment of ClpP proteins. ClpP1, ClpP2, ClpP3 and ClpP4 of \textit{S. lividans} are aligned with ClpP from \textit{B. subtilis}. Numbering is based on \textit{S. lividans} ClpP1. Amino acids of \textit{B. subtilis} ClpP known to interact with ADEP (Lee et al., 2010a) are indicated by oval dots. Diamonds (numbers 41 and 101) indicate amino acids shown to be critical for ADEP interaction in \textit{B. subtilis} ClpP (i.e. R23 and F82 in \textit{B. subtilis} ClpP). The proteolytic catalytic triad (Ser, His, Asp) is indicated by stars.}
\end{figure}
nucleotide change leading to an M165R substitution (S. lividans ClpP1 coordinate).

In five strains, ClpP1 but not ClpP3 was detected, suggesting that mechanisms unrelated to ClpP1 inactivation and distinct from the SclAB efflux pump were responsible for the ADEP resistance.

**Interplay between ClpP1 and ClpP2: study of the effects of ADEP on S. lividans strains expressing various forms of the proteases ClpP1 and ClpP2**

As clpP1 and clpP2 form an operon, a number of mutations in clpP1 will have a polar affect on clpP2 expression. This is the case with the clpP1::apraR mutant, where the insertion of the apramycin resistance cassette prevents the transcription of clpP2 by polar effect. In addition, we had previously shown that in *Streptomyces* the presence of ClpP2 is needed to obtain a processed ClpP1 and vice versa (Viala & Mazodier, 2002). The intimate interplay between ClpP1 and ClpP2 led us initially to consider the whole operon as a global genetic entity, as it is difficult to differentiate the intrinsic effect of clpP1 and clpP2 mutations. Nevertheless, we found that all the mutations in the clpP1clpP2 operon (six out of six) leading to ADEP resistance affect clpP1. The absence of mutations affecting only clpP2 among ADEP-resistant clones suggests that ClpP1 processing by ClpP2 is not required to produce an ADEP-sensitive phenotype.

In addition, insensitivity to ADEP of strains with clpP1 mutations that do not affect clpP2 expression (such as the missense mutation in 86E) indicates that ClpP2 might be ADEP insensitive. To determine if ClpP2 might lead to ADEP sensitivity when overproduced, we used a set of genetic constructions available in our laboratory (Viala & Mazodier, 2002). The clpP1::apraR mutant was complemented by introduction of plasmids expressing various forms of clpP2 and/or clpP1. pJV52 and pJV53, harbouring two forms of clpP2 (long and short, respectively) under the control of the strong heterologous ermE promoter, did not restore ADEP sensitivity, whereas the control plasmid pJV51 expressing the complete clpP1 under the control of the ermE promoter restored ADEP sensitivity (Fig. 1b). These results provide a further indication that ClpP2 is ADEP insensitive and that ADEP toxicity only requires processed or unprocessed ClpP1.

**Evolution of the ClpP paralogues and orthologues in Streptomyces**

A dozen *Streptomyces* genomes have been sequenced and there is no clpP3clpP4 operon in four of them: *S. griseus*, *S. pristinaespiralis*, *S. clavuligerus* and *S. roseosporus*. This indicates that these genes are not essential in *Streptomyces* as long as ClpP1clpP2 are functional. There is also no clpP3clpP4 operon in other actinobacterial strains, such as *Saccharopolyspora erythraea*, a close relative of *Streptomyces*. This implies that clpP3clpP4 acquisition or loss was recent. However, the five ClpP1–ClpP5 subtypes are similar as regards the non-ambiguous branches (see Fig. S1 in the supplementary material available with the online version of this paper), and the overall tree clearly rules out a recent acquisition of the clpP3clpP4 operon by horizontal transfer and subsequent spreading among *Streptomyces* species. The tree is consistent with an ancient duplication of the clpP operons and subsequent co-evolution [as observed for the clpP1clpP2 operon by Ventura et al. (2005)]. Clearly, the four sequenced *Streptomyces* species lacking clpP3clpP4 genes are clustered; presumably a common ancestor lost the clpP3clpP4 operon, an event, according to our hypothesis, that would have been detrimental in the presence of ADEP.

**DISCUSSION**

Following its discovery in 2005, it was suggested that ADEP should show no cross-resistance with any class of antibiotic on the market because it has a very different target; this was predicted to make it suitable for the treatment of infections involving multidrug-resistant bacteria (Brötz-Oesterhelt et al., 2005). In this report we show that resistance mechanisms to this antibiotic with its new mode of action are present even before the therapeutic use of the antibiotic. Obviously, such mechanisms have been well documented in antibiotic-producing strains, where production and resistance are linked. However, in *S. lividans*, which does not produce ADEP, we show here that selection for spontaneous spiramycin resistance can lead to a multi-resistant strain in which the activation of an ABC transporter (SclAB) provides ADEP resistance. Moreover, we provide evidence of other, still uncharacterized, systems allowing efflux-mediated ADEP resistance in *S. lividans*. Antibigrams were used to assess whether the ADEP resistance acquired by these derivative strains with a functional ClpP1 is due to the activation of one or various resistance mechanisms. The resistance patterns differed between the ADEP-resistant isolates, suggesting that several different mechanisms, which remain to be characterized, contributed to ADEP resistance. Loci showing a sclABR-like gene organization are present in *S. coelicolor* and *S. lividans* and are therefore candidates as sources of resistance. Indeed, there are 81 typical ABC permeases and 141 ATP-binding proteins encoded by the *S. coelicolor* genome (Bentley et al., 2002), and some of these may provide resistance to ADEP.

We have shown that ClpP3 might also provide a resistance mechanism by substitution for ClpP1. If the duplication of clpP operons and maintenance of clpP3clpP4 in several *Streptomyces* species is linked to ADEP resistance, the large proportion of strains showing this duplication (70%) would suggest that ADEP or compounds similar to ADEP are prevalent in nature. Therefore the strain collections of pharmaceutical companies must include producers of ADEP-like molecules. We suggest that it would be valuable to screen these banks to find more such natural molecules, potentially including some that target a different spectrum of ClpP proteins. We have investigated the ADEP resistance mechanism of *S. hawaiensis*. Sequencing revealed the
presence of both clpP1clpP2 and clpP3clpP4 operons, and Western blotting detected ClpP1 but not ClpP3. ClpP1 of *S. lividans* and ClpP1 of *S. hawaiiensis* are 98% identical, with no modification of catalytic residues or ADEP-binding sites. This indicates that ADEP resistance of the producer is not due to ClpP1 alteration, but more likely to another mechanism such as active export.

In *Streptomyces*, there are two genes for tryptophanyl-tRNA synthetase, a situation similar to the duplication of clpP operons. One gene codes for TrpRS, an enzyme sensitive to indomycin (an antibiotic produced by *S. griseus*); the other gene, which is normally not expressed, codes for an enzyme insensitive to indomycin (Vecchione & Sello, 2009). The presence of a family of paralogues in *Streptomyces* genomes has often been explained as allowing expression during different phases of the life cycle. However, *Streptomyces* might also use these paralogues through a mechanism of target substitution as a defence against the presence of antibiotics targeting particular enzymes.

Access to the new highly improved synthetic ADEP congeners obtained by Hinzen et al. (2006) in the pharmaceutical lead-structure optimization programme would be interesting. Indeed, this would allow evaluation of the relevance of the resistances described if ADEP or its derivatives were to be used therapeutically. It would also allow identification of ADEP-resistance gene(s) and potentially the biosynthetic genes in *S. hawaiiensis*.

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