Identification of genes involved in the susceptibility of *Serratia marcescens* to polyquaternium-1

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Received 3 March 2004; returned 13 May 2004; revised 1 June 2004; accepted 6 June 2004

**Objectives:** Polyquaternium-1 (PQ-1) is a biocide used commercially in a contact lens disinfecting solution, ‘Opti-Free Express (Alcon) Multi-Purpose Disinfecting Solution’. The genetic basis for resistance of *Serratia marcescens* to PQ-1 was investigated using a random transposon-based mutagenesis approach.

**Methods:** *S. marcescens* was subjected to random transposon mutagenesis using a mini-Tn5Km2 transposon. Mutants with increased susceptibility to PQ-1 were selected and the disrupted genes were identified. Antibiotic susceptibility profiles were also determined for all of the mutants.

**Results:** A wide range of genes were found to be disrupted in the mutants. The most common were genes associated with the cell membranes, or involved in biosynthesis and metabolism.

**Conclusions:** This study shows that random transposon mutagenesis is an effective tool for the elucidation of mechanisms of action and resistance to biocides. The results support our previous findings that PQ-1 is active against the cytoplasmic membrane of *S. marcescens*.

Keywords: *S. marcescens*, biocides, antibiotics, transposon mutagenesis, gene identification, genetics

**Introduction**

The mechanisms of action and resistance to biocides have been well studied at the phenotypic level. In general, these studies have relied on methods which identify specific forms of cellular injury, for example leakage of intracellular materials, inhibition of metabolic processes, and observations of structural damage by electron microscopy.¹⁻⁷ Mutational approaches commonly used involve the selection of mutants which develop spontaneous resistance or have been trained to become more resistant to a biocide.⁸⁻⁹ Although these methods are useful, they suffer the disadvantage that limited information on the genetic basis for resistance is obtained. Modern molecular genetic approaches in combination with the recent revolution in bacterial genome sequencing offer enormous potential for the elucidation of biocide action and resistance mechanisms at the genetic level, but so far have not been widely exploited.

There have been few studies to date which have identified genes involved in the mode of action of, or resistance to, biocides. Enoyl-reductase, an essential enzyme in the bacterial fatty acid biosynthesis pathway, has been identified as one of the targets for the biocide triclosan in *Escherichia coli*.¹⁰ The gene *inhA*, encoding a mycobacterial enoyl-reductase has also been shown to be the genetic basis for resistance to triclosan in these pathogens.¹¹ Various bacterial multi-drug efflux pumps have also been shown to play a role in the resistance of bacteria to biocides.¹² However, beyond fatty acid biosynthesis and multi-drug efflux genes, very little is known about the molecular basis for biocide resistance and action, with the majority of studies on biocide action and resistance describing phenotypic changes at the cytoplasmic or outer membrane level.¹³

Transposon mutagenesis is an excellent strategy for defining the genetic basis of positively expressed bacterial traits such as antimicrobial resistance. For example, Tamayo *et al.*¹⁴ used transposon mutagenesis to identify genes involved in polymyxin resistance in *Salmonella enterica* serovar Typhimurium. Polymyxin resistance was also investigated in *Burkholderia pseudomallei* using this method.¹⁵

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JAC vol.54 no.2 © The British Society for Antimicrobial Chemotherapy 2004; all rights reserved.
Recently, the susceptibility of *Staphylococcus aureus* to the antimicrobial peptide CG 117–136 was investigated using a transposon mutagenesis strategy. It was found that a mutant which was more resistant to CG 117–136 had a transposon insertion in the *cspA* gene (encoding a cold-shock protein), suggesting that susceptibility to the peptide was associated with a stress-response mechanism. In this study, random transposon mutagenesis was used to elucidate the genetic basis for mechanisms of action of the contact lens biocide polyquaternium-1 (PQ-1) against *Serratia marcescens*.

### Materials and methods

**Chemicals, culture media and bacterial strains**

All chemicals were purchased from Sigma (Poole, UK) or Fisher (Loughborough, UK). Culture media were purchased from Oxoid (Basingstoke, UK). PQ-1 was supplied by Alcon Research Ltd (Texas, USA). Restriction and ligation enzymes and their buffers were purchased from Promega UK (Southampton, UK). Chemicals for Southern hybridization probe labelling and detection were purchased from Roche (Mannheim, Germany), and PCR reagents from Qiagen (Sussex, UK). E. coli strains were transformed to chloramphenicol-resistant (pUTKm2) as template DNA: NptIIF1, S17.1. The pUT suicide vector as described by de Lorenzo & Timmis. 17 The plasmid vector pUC18 as described previously. 17 Sequence analysis of DNA flanking the transposon was carried out using the transposon-specific primer 3 (5'-CGGATTACAGCCGGATCCG-3') and standard dye-deoxyterminator chemistry on an Applied Biosystems ABI3100 Automated Sequencer. Sequence flanking each transposon insertion was correlated to the *S. marcescens* strain Db11 genome project. Genome sequence was produced by the Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge and can be obtained from http://www.sanger.ac.uk/Projects/S_marcescens/.

**Random mutagenesis of *S. marcescens***

*S. marcescens* ATCC 13880 was subjected to random transposon mutagenesis using a mini-Tn5Km2 transposon, delivered on the pUT suicide vector as described by de Lorenzo & Timmis. The pUT plasmid carrying mini-Tn5Km2 was maintained in an *E. coli* S17.1pir donor strain and introduced to *S. marcescens* by conjugal transfer according to the method of Lewenza et al. Mutants were isolated on TSA containing kanamycin (Km) 30 mg/L, to select for the transposon, and polymyxin (60,000 U/L) to counter-select against the *E. coli* donor.

**Southern hybridization mapping of transposon insertions**

Southern hybridization was carried out using digoxigenin-labelled mini-Tn5Km2 specific probes. Probes against the neomycin phosphotransferase (*npt*) gene encoded on the transposon were prepared by PCR using the following primers under standard amplification conditions with the pUTKm2 plasmid as template DNA: NptIIIF1, 5'-CTTGCTCAGGCCGGATTTAATT-3'; and NptIIR1, 5'-TTC-CATAGGATGGCAAGATCCTGG-3'. During PCR, the probe was simultaneously labelled with digoxigenin as described by the manufacturer (Roche, Lewes, UK). Chromosomal DNA from each transposon mutant was digested separately with the restriction enzymes PstI and SalI under the conditions outlined by the manufacturer (Promega, Southampton, UK). Southern hybridization was carried out using standard methods under high stringency conditions. Probe hybridization was detected using an antibody-based chemiluminescent method as described (Roche, Lewes, UK).

**Identification of disrupted genes**

Genes disrupted in the mutants with increased susceptibility to PQ-1 were identified by sequence analysis of DNA flanking the site of transposon insertion. Briefly, *S. marcescens* chromosomal DNA flanking mini-Tn5Km2 inserts was initially sub-cloned into the plasmid vector pUC18 as described previously. Sequence analysis of DNA flanking the transposon was carried out using the transposon-specific primer 3 (5'-CGGATTACAGCCGGATCCG-3') and standard dye-deoxyterminator chemistry on an Applied Biosystems ABI3100 Automated Sequencer. Sequence flanking each transposon insertion was correlated to the *S. marcescens* strain Db11 genome project. Genome sequence was produced by the Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge and can be obtained from http://www.sanger.ac.uk/Projects/S_marcescens/.

**Antimicrobial susceptibility testing of mutants**

The minimal lethal concentrations (MLCs) of PQ-1 for the 21 mutants were evaluated as described previously. Antibiotic susceptibility profiles were determined for all of the mutants using Etest antibiotic strips, according to the manufacturer’s instructions. The antibiotics used were ampicillin, ciprofloxacin, chloramphenicol, gentamicin, meropenem, piperacillin, tetracycline, ticarcillin and trimethoprim. The stability of the mutants was tested by serial subculture without selection for 8 days. Samples were tested daily for changes in PQ-1 susceptibility by plating onto TSA containing PQ-1 at appropriate concentrations, and comparing growth with that of fresh cultures.

### Results

**Transposon mutagenesis screen**

The MIC of PQ-1 against *S. marcescens* ATCC 13880 was found to be 525 mg/L (in TSA). A total of 960 mutants were screened for increased susceptibility to PQ-1 by their inability to grow on TSA containing PQ-1 at 500 mg/L, a sub-lethal concentration of the biocide just below the strain’s MIC. Spontaneous mutation of *S. marcescens* to a PQ-1 susceptible phenotype was not observed when 1000 individual colonies of the wild-type strain were screened at this sub-lethal PQ-1 concentration. Of the 92 mutants with increased PQ-1 susceptibility, 19 random mutants were selected for further characterization (termed SM2, SM3, etc.). In addition, 2 control transposon mutants which showed no change in PQ-1 susceptibility were also included in all further studies (S7 and S8). All of the mutants were found to be stable under subculturing without selection for the transposon for 8 days. Southern hybridization showed that the mutants all had distinct, stable, single insertions of the transposon (Figure 1).

**Antimicrobial susceptibility of transposon mutants**

The MLCs for the mutants of PQ-1 were greatly reduced compared with the wild-type. At a PQ-1 concentration of 15 mg/L...
which hybridized to the npt gene probe. Each mutant contained a single transposon insertion and mutants SM2, SM3, SM4, SM5, SM6, SM8, SM14, SM15 and SM16, respectively. Each lane shown on the Southern hybridization is as follows: lane M, DNA from mutants SM2, SM3, SM4, SM5, SM6, SM8, SM14, SM15 and SM16, respectively. Each mutant contained a single transposon insertion which hybridized to the npt gene probe.

Figure 1. Localization of transposon insertion by Southern hybridization. The npt gene probe was used to detect the transposon insertion in SalI-digested chromosomal DNA from each S. marcescens PQ-1-susceptible mutant as described in the Materials and methods section. The content of each lane shown on the Southern hybridization is as follows: lane M, DNA molecular size standards (relevant size bands are indicated in bp); lanes 1 to 9, DNA from mutants SM2, SM3, SM4, SM5, SM6, SM8, SM14, SM15 and SM16, respectively. Each mutant contained a single transposon insertion which hybridized to the npt gene probe.

(10% of the wild-type MLC), the mutants all suffered a 3 log reduction in viability after 5 min of contact at 25°C (initial inoculum size approximately 10^6 cfu/mL). MLCs of PQ-1 for control transposon mutants S7 and S8 were the same as those for the wild-type parental strain ATCC 13880, demonstrating that insertion of the transposon alone did not alter susceptibility to the biocide. Table 1 shows MLC data for the mutants and wild-type strain.

The antibiotic susceptibility profiles of the PQ-1 mutants are presented in Table 2. The majority of the mutants (15/19) showed a decreased MIC to at least one antibiotic, with mutant SM25 being more susceptible to four antibiotics (ampicillin, chloramphenicol, meropenem and piperacillin). Less than half (7/19) showed an increased MIC to any antibiotic; all seven were more resistant to only a single antibiotic class, with CIP being the most common antibiotic to show a raised MIC. Three of the mutants showing antibiotic MIC increases (SM18, 22 and 27) also demonstrated a decrease in MIC to another antibiotic class. Four of the mutants did not show any decreases in antibiotic MICs (SM6, 8, 14 and 15), however, all of these demonstrated an increased MIC to one antibiotic class (Table 2). The most common antibiotics to have decreased MICs associated with the PQ-1 susceptibility of the mutants were ampicillin (10/19), chloramphenicol (7/19) and piperacillin (12/19). Six mutants (SM12, 18, 19, 22, 25 and 27) showed decreased susceptibility to all three of these antibiotics. S. marcescens ATCC 13880 and all of the mutants were resistant to tetracycline and none of the mutants demonstrated any alterations with regard to gentamicin MIC when compared with the parental strain.

**Genetic basis for PQ-1 susceptibility**

DNA flanking the transposon for all 19 PQ-1-susceptible mutants was successfully cloned and sequenced. Each flanking sequence was mapped to the S. marcescens genome sequence and the transposon-disrupted coding sequence identified. Analysis of mutant SM28 is shown in Figure 2 as an example of the bioinformatic investigation performed. The DNA sequence flanking each transposon was initially matched to the genome sequence (Figure 2). DNA surrounding this insertion point was then analysed using BLAST to detect sequences with homology to genes within the DNA databases. The putative identity of each transposon-disrupted gene, and relevant coding sequences in the same vicinity, were then mapped for each mutant (Figure 2).

Fourteen of the mutants demonstrated a transposon insertion in coding sequences with homology to known genes (Table 2). Five mutants (SM2, 4, 8, 16 and 18) possessed transposon insertions in hypothetical genes with no homology to sequences present in the databases (Table 2). Examination of the genes flanking the transposon insertions enabled categorization of the putative functions of the disrupted loci to be assigned into five major classes: membrane-associated (including efflux pumps and permeases), biosynthesis and metabolism, gene regulation, virulence, and unknown function (Table 3). The majority of genes associated with resistance to PQ-1 carried out biosynthetic or metabolic functions, or were membrane-associated (Table 3).

**Discussion**

Transposon mutants of S. marcescens susceptible to the biocide PQ-1 were isolated demonstrating that this strategy is an effective means of identifying the genetic basis for biocide resistance and action. The majority of PQ-1-susceptible mutants possessed co-susceptibility to at least one antibiotic demonstrating that biocide and antibiotic resistance mechanisms are often linked at the genetic level. Mutations associated with biocide resistance spanned many different functional pathways from cellular metabolism to virulence, demonstrating that the targets of PQ-1 are multifactorial and that a number of intrinsic resistance mechanisms are possessed by S. marcescens.

Previous studies have demonstrated that quaternary ammonium compounds (QACs) such as PQ-1 are active against the bacterial cytoplasmic membrane. Mutants SM6, 14, 22, 27 and 28 were all disrupted in membrane-associated proteins. This is significant because changes in the cytoplasmic membrane have been associated with antimicrobial susceptibility. For example, Winder et al. found that Pseudomonas aeruginosa exposed to isothiazolone biocides lost the outer membrane protein T-OMP. Guérin-Méchin et al. investigated the effects of QACs on the fatty acid composition of the membrane of P. aeruginosa. They found that P. aeruginosa trained to be more resistant to benzyl-28 were all disrupted in membrane-associated proteins. This is significant because changes in the cytoplasmic membrane have been associated with antimicrobial susceptibility. For example, Winder et al. found that Pseudomonas aeruginosa exposed to isothiazolone biocides lost the outer membrane protein T-OMP. Guérin-Méchin et al. investigated the effects of QACs on the fatty acid composition of the membrane of P. aeruginosa. They found that P. aeruginosa trained to be more resistant to benzyl-dimethyltetradecylammonium chloride (C14) and didecyl-di-methylammonium bromide (DDAB) had greater amounts of hydroxylated fatty acids and lauric acid in the membrane. It has been shown previously that PQ-1 damages the cytoplasmic membrane of S. marcescens, and the fact that several mutants

**Table 1. Summary of MLC results for S. marcescens wild-type and mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>PQ-1 concentration (mg/L)</th>
<th>Log reduction (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. marcescens wild-type</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>PQ-1-susceptible mutants</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Control mutants</td>
<td>150</td>
<td>3</td>
</tr>
</tbody>
</table>

*aAll mutants isolated demonstrated the same MLC results.*

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have been disrupted in genes associated with the membrane further supports these results.\(^2^1\)

Of the membrane-associated genes linked to PQ-1 susceptibility only one could be attributed to a putative efflux system, SM14. The SM14 transposon insertion was in a gene homologous to that encoding the microcin H47 secretion protein, MchE, of *E. coli*. Microcins are antimicrobial agents produced by bacteria and MchE is a membrane fusion protein that is part of an ATP-binding cassette (ABC) type efflux system which exports microcin H47 out of the cell.\(^2^6\) The increased susceptibility of mutant SM14 suggests that this export system may also remove PQ-1 from *S. marcescens*. Interestingly, one of the control transposon mutants (S7) was also disrupted in a putative AcrB/D/F family efflux system. However, this control mutant demonstrated no alteration in PQ-1 MIC when compared with the wild-type, suggesting the AcrB/D/F family of efflux systems is not associated with PQ-1 resistance in *S. marcescens*.

Various biosynthetic pathway genes appear to play a role in the resistance of *S. marcescens* to PQ-1. Where genes involved in biosynthesis and metabolism were disrupted, the drop in PQ-1

### Table 2. Characteristics of PQ-1-susceptible transposon mutants of *S. marcescens*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Disrupted gene</th>
<th>Decreased MICs (^a)</th>
<th>Increased MICs (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM2</td>
<td>hypothetical unknown</td>
<td>PIP (0.56) (\times 0.38)</td>
<td></td>
</tr>
<tr>
<td>SM3</td>
<td>UDP-galactose-4-epimerase</td>
<td>PIP (0.61)</td>
<td></td>
</tr>
<tr>
<td>SM4</td>
<td>hypothetical unknown</td>
<td>AMP (0.54) PIP (0.47)</td>
<td></td>
</tr>
<tr>
<td>SM5</td>
<td>transcriptional regulator</td>
<td>PIP (0.56)</td>
<td></td>
</tr>
<tr>
<td>SM6</td>
<td>membrane protein</td>
<td></td>
<td>CIP (2.89)</td>
</tr>
<tr>
<td>SM8</td>
<td>hypothetical unknown</td>
<td></td>
<td>TIC (69.75)</td>
</tr>
<tr>
<td>SM10</td>
<td>sugar binding or transport</td>
<td>AMP (0.44) PIP (0.56) TMP (0.63)</td>
<td></td>
</tr>
<tr>
<td>SM12</td>
<td>ATP-binding component of amino acid transport system</td>
<td>AMP (0.22) CHL (0.63) PIP (0.62)</td>
<td></td>
</tr>
<tr>
<td>SM14</td>
<td>microcin H47 secretion protein</td>
<td></td>
<td>CIP (2.24)</td>
</tr>
<tr>
<td>SM15</td>
<td>transcriptional regulator</td>
<td></td>
<td>MEM (2)</td>
</tr>
<tr>
<td>SM16</td>
<td>hypothetical unknown</td>
<td>PIP (0.61)</td>
<td></td>
</tr>
<tr>
<td>SM18</td>
<td>hypothetical unknown</td>
<td>AMP (0.22) CHL (0.54) PIP (0.56)</td>
<td>TMP (1.83)</td>
</tr>
<tr>
<td>SM19</td>
<td>serralysin (metalloprotease)</td>
<td>AMP (0.25) CHL (0.42) PIP (0.56)</td>
<td></td>
</tr>
<tr>
<td>SM20</td>
<td>ArgD (aminotransferase)</td>
<td>AMP (0.5)</td>
<td></td>
</tr>
<tr>
<td>SM22</td>
<td>periplasmic murein peptide-binding protein</td>
<td>AMP (0.31) CHL (0.5) PIP (0.56)</td>
<td>CIP (1.86)</td>
</tr>
<tr>
<td>SM24</td>
<td>arylsulphatase</td>
<td>AMP (0.5) CHL (0.58)</td>
<td></td>
</tr>
<tr>
<td>SM25</td>
<td>long chain fatty acid CoA ligase</td>
<td>AMP (0.44) CHL (0.5) MEM (0.62) PIP (0.62)</td>
<td></td>
</tr>
<tr>
<td>SM27</td>
<td>transporter transmembrane protein</td>
<td>AMP (0.5) CHL (0.54) PIP (0.56)</td>
<td>CIP (3.85)</td>
</tr>
<tr>
<td>SM28</td>
<td>inner membrane protein, tolerance to colicin E2</td>
<td></td>
<td>TMP (0.5)</td>
</tr>
</tbody>
</table>

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; MEM, meropenem; PIP, pipercillin; TIC, ticarcillin; TMP, trimethoprim.

\(^a\) MICs are presented as ratios of the wild-type MIC. Only those of 0.65 or less, and 1.5 or higher are shown (ratios indicated in parentheses). Ratios of 1 indicate that the MIC for the mutant and wild-type were the same, greater than 1 indicates that the MIC was higher in the mutant than the wild-type, and ratios less than 1 show that the MIC was lower in the mutant.

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**Figure 2.** Mapping of the site of transposon insertion to the *S. marcescens* genome sequence. Genes disrupted in each transposon mutant were identified by matching the DNA sequence flanking each insertion point to the *S. marcescens* genome as described in the Materials and methods section. Analysis of mutant SM28 using Artemis software\(^2^0\) is shown. The forward DNA strand and each of the three forward reading frames are shown above the numerical DNA sequence scale; the reverse DNA strand and corresponding reverse reading frames are shown below. Vertical lines within each reading frame indicate the position of a stop codon. Coding sequences with homology to known genes are indicated by the labelled arrow bars. The site of the transposon insertion in mutant SM28, within a coding sequence homologous to an inner membrane protein conferring tolerance to colicin, is also shown.
MLC could be because the mutants may have been nutrient-deficient, so were more susceptible to the biocide.\textsuperscript{23} Mutant SM25 possessed a transposon insertion in a long chain fatty acid CoA ligase, indicating that like the biocide triclosan,\textsuperscript{10} PQ-1 also targets lipid metabolism.

Three of the mutants (SM5, SM15 and S8) were disrupted in genes associated with regulation, and therefore several different processes are likely to have been affected. The gene identified to be disrupted in SM15 is a cation transport regulator homologue, implicating cation transport systems in PQ-1 action. The transposon insertion in the control mutant S8 was adjacent to that of SM15, but located on the complementary DNA strand. The fact that PQ-1 susceptibility was unaltered in the control mutant SM8 demonstrates the specificity of the SM15 transposon mutation.

Greater than 75% of the \textit{S. marcescens} PQ-1-susceptible mutants examined showed a corresponding decrease in MIC to at least one antibiotic class (Table 2), suggesting that if mechanisms of resistance to a biocide are disrupted, these can also lead to increased antibiotic susceptibility. In contrast to this logical expectation was the observation that seven PQ-1-susceptible mutants (SM6, 8, 14, 15, 18, 22 and 27) actually demonstrated increased resistance to at least one antibiotic (ciprofloxacin, ticarcillin, meropenem or trimethoprim; Table 2). There may be several explanations as to why antibiotic resistance may have been elevated in these mutants. The genetic basis of mutants SM6, 14, 22 and 27, were all linked to genes encoding membrane-associated proteins (putatively for transport, secretion or just membrane located; Table 2). If these membrane factors normally functioned as potential mechanisms by which antibiotics permeate bacterial cells, then their absence after transposon disruption may cause increased antibiotic resistance. Notably, all four mutants with an increased MIC of ciprofloxacin (SM6, 14, 22 and 27) were disrupted in genes encoding membrane-associated factors (Table 2). The site of action of quinolone antibiotics is intracellular, inhibiting DNA gyrase, therefore mutations which alter the cell membrane and reduce the permeability of this barrier to ciprofloxacin may lead to an increased resistance to this antibiotic. Mutant SM15 was disrupted in a transcriptional regulator gene which may be linked to the control of several pathways (such as membrane synthesis or efflux) that could have caused the increased meropenem resistance observed. The last two mutants with elevated antibiotic MICs, SM8 and 18, both contained mutations in hypothetical genes, hence the basis for their resistance remains unknown at this time.

The results clearly show that a wide range of transposon mutants was generated using this approach to define biocide resistance/susceptibility genes in \textit{S. marcescens}. Just under 10% of the 960 mutants screened demonstrated alterations in susceptibility to PQ-1. The 19 mutants we genetically characterized demonstrated putative functions associated with a wide range of cellular activities from membrane-associated, to metabolism and also a significant number of hypothetical genes of no current known function. Simple screens were used to identify changes in biocide activity and antibiotic MICs, proving that random transposon mutagenesis is an ideal method for the elucidation of biocide action and resistance, together with any association with antibiotic susceptibility or action. Major advantages of this method are that the mutants generated are random, generally single mutations are created, and the identification of genes is facilitated. No prior knowledge of genes involved is required, so that genes can be identified which may not have previously been considered to be important in, or associated with biocide resistance or activity. However, an important limitation of transposon mutagenesis screens is that essential genes involved in resistance to PQ-1 cannot be identified; such mutations would be lethal and lead to the death of the resulting transposon mutant.

Acknowledgements

We would like to thank Alcon Research (Fort Worth, TX, USA) for a research studentship (to C.E.C.).

References


S. marcescens biocide resistance genes


