Detection of the prodigiosin biosynthesis protein (pigC) from *Serratia marcescens*: development of a novel PCR assay

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Serratia marcescens has become an important pathogen of nosocomial infection.[1] Taxonomically, it represents one species within the genus *Serratia*, which consists of 14 others species, including *S. entomophila*, *S. ficaria*, *S. fonticola*, *S. glossinae*, *S. grimesii*, *S. liquefaciens*, *S. marinorubra*, *S. nematodiphila*, *S. odorifera*, *S. plymuthica*, *S. proteamaculans*, *S. quinivorans*, *S. rubidaea* and *S. ureilytica*.

Previously, there have been clinical reports of several molecular methods and targets being used to identify this species, including RAPD-PCR[2] and PCR ribosomal DNA spacer polymorphisms.[3] *S. marcescens* is unique among the other species within this genus, in that it produces the red pigmentation prodigiostin (4-methoxy-5-[(Z)-(5-methyl-4-pentyl-2H-pyrrol-2-ylidene)methyl]-1H,1′H-2,2′-bipyrrole), as shown in Figure 1.[4] To date, there has been no description of a specific prodigiosin polymerase chain reaction (PCR) assay for employment to aid in the detection/identification of *S. marcescens* in the clinical microbiology laboratory and hence it is the aim of this study to develop a novel and simple PCR assay for the molecular detection of prodigiosin, which could act as a molecular biomarker for the presence of *S. marcescens*.

DNA sequence data of the prodigiosin biosynthesis protein C (pigC) operons were obtained from GenBank (www.ncbi.nlm.nih.gov/entrez) and conserved, and variable regions were subsequently identified by the Clustal alignment method employing the sequence alignment software package CLUSTALW2 (www.ebi.ac.uk/Tools/services/web_clustalw2). The novel primer pair, forward – SMf (5′- CGC TGG GCA TTC TCA GCC TGG TGG AGA CGG – 3′) (30-mer; %G+C= 67; $Temp_{melt}$ = 69.8°C) and reverse – SMr (5′– GGC CGG GTC GCT TCG CGG CGT TCG GCC -3′) (27-mer; %G+C=81.0; $Temp_{melt}$ = 73.4°C) was constructed *in silico*, as detailed in Figure 2.

All DNA isolation procedures were carried out in a Class II biological safety cabinet (MicroFlow, England) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room in accordance with the Good Molecular Diagnostic Procedure (GMDP) guidelines of Millar *et al*.,[5] in order to minimise contamination and hence the possibility of false-positive results. Genomic DNA was extracted from a well-characterised wild-type strain of *S. marscescens* (SM1-BC/08/230), which had previously been obtained from a positive blood culture from a patient. The isolate had been originally identified as *S. marcescens* by the API 20E Identification System (bioMérieux, UK) and subsequently by sequencing of the 16S ribosomal RNA (rRNA) gene, as previously described.[6] Genomic DNA isolated from the isolate was extracted using the High Purity PCR Template Preparation Kit (Roche, England), in accordance with the manufacturer's instructions. Extracted DNA was stored at –80°C prior to PCR amplification. For each batch of extractions, a negative extraction control containing all reagents minus organism was performed, as well as an extraction positive control with *S. marcescens*.

Amplification reactions were set-up in accordance with GMDP guidelines.[5] All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and 'post-PCR' room, in order to minimise contamination. Initially, PCR amplification conditions were optimised by separately varying magnesium chloride concentration, annealing temperature, primer concentration and DNA template concentration. Following optimisation, reaction mixes (25 µL) were set up as follows:10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.7 mmol/L $MgCl_2$, 200 µmol/L (each) dATP, dCTP, dGTP and dTTP; 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (GeneAmp, Applied Biosystems), 20 pmol (each) primer (SMf and SMr) and 4 µL DNA template. The reaction mixtures following a 'hot start' were subjected to the following empirically optimised thermal cycling parameters in a GeneAmp PCR system 9700 thermocycler: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec, followed by a final extension at 72°C for 7 min. Positive (*S. marcescens* SM1-BC/08/230 DNA) and multiple negative (water; LAL Grade, BioWhittaker, UK) amplification controls were included in every set of PCR reactions. Following amplification, aliquots (10 µL) were removed from each reaction mixture and examined by electrophoresis (100 V, 45 min) in gels composed of 1.5% (w/v) agarose (Gibco, UK) in TAE buffer

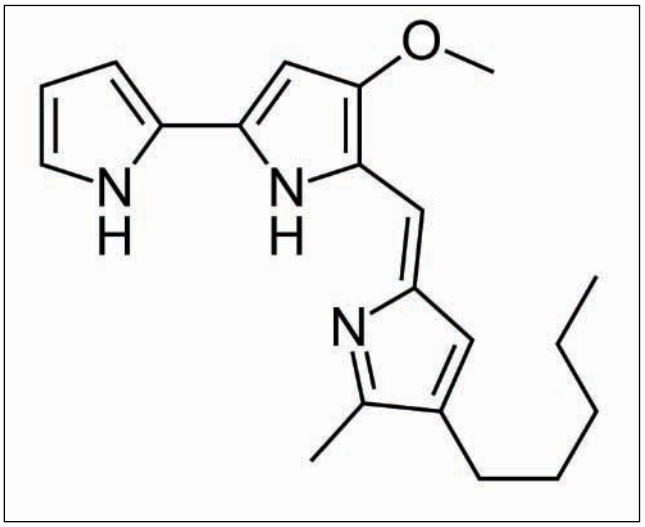

Fig. 1. Molecular structure of prodigiosin [4-methoxy-5-[(Z)-(5-methyl-4-pentyl-2*H*-pyrrol-2-ylidene)methyl]-1*H*,1′*H*-2,2′-bipyrrole] produced by *Serratia marcescens*.

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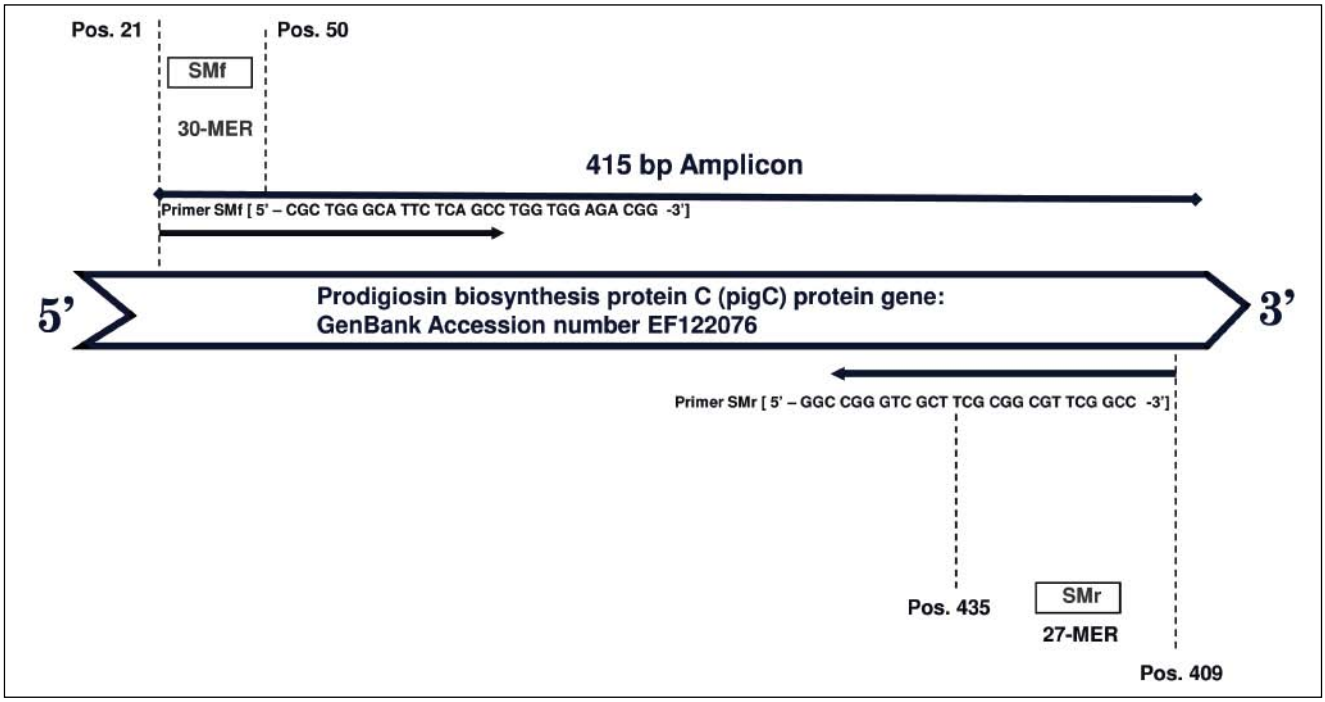

Fig. 2. Description and location of SMf and SMr primers in relation to prodigiosin biosynthesis protein C (pigC) gene in *Serratia marcescens* with GenBank Accession Number EF122076.

(40 mmolL Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]), stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, England) and all images were archived as digital graphic files (*.bmp).

The specificity of the newly designed primers was examined by challenging the primers to non-*S. marcescens* genomic DNA, isolated individually from pure cultures of the following organisms: *Escherichia coli*, *Enterobacter aerogenes*, *Mycobacterium abscessus*, *Pseudomonas aeruginosa*, *Streptococcus anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. salivarius* and *S. sanguinis*. An additional wild-type *Serratia marcescens* (SM-4; BC/08/94) isolate was examined as a positive control.

In silico sequence alignment analysis permitted the identification of two novel primer regions (SMf and SMr) on the prodigiostin biosynthesis protein (pigC) gene, as detailed (Fig. 1), which had 100% homology with all pigC DNA sequences, which had been deposited in GenBank. BLAST searches of these primers demonstrated, *in silico*, that *S. marcescens* containing this molecular marker was able to be amplified using this primer pair, but which would not allow the amplification of other bacterial species not containing the prodigiostin biosynthesis (pigC) protein. Subsequent laboratory PCR amplification of the 415 bp fragment was successful for the *S. marcescens* isolates tested, giving a PCR fragment of the expected size (approximately 415 bp), whereas none of the non-*S. marcescens* organisms tested were able to produce any PCR amplicons of the correct size.

In conclusion, this study developed a simple and novel PCR assay for the detection of the prodigiosin biosynthesis protein (pigC) gene in *S. marcescens* and this assay may be useful in clinical microbiology laboratories routinely culturing/identifying *S. marcescens*, as no such assay has been reported to date. At present, the authors recommend the adoption of this method solely for the identification of *S. marcescens* from primary culture plates where sensitivity is not an issue, as the presence of various biological matrices (e.g., blood, faeces and sputum) may adversely affect the sensitivity of such a PCR assay. Employment of these primers in such a primary diagnostic setting directly from clinical specimens would therefore require additional optimisation/calibration for the specimen type being examined. Overall, employment of this novel assay may help in the better understanding of the occurrence, aetiology and epidemiology of *S. marcescens* infections in a diverse range of patient populations.

References

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