Characterisation of two new gene cassettes, \( \text{aadA5} \) and \( \text{dfrA17} \)

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Abstract

\textit{Escherichia coli} INS33 was isolated from the urinary tract of an infected patient. It was resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfafurazole, tetracycline and trimethoprim. PCR screening revealed the presence of a class 1 integron that harboured two new gene cassettes, designated \( \text{dfrA17} \) and \( \text{aadA5} \). The new \( \text{dfrA17} \) cassette was 91% identical to the known \( \text{dfrA7} \) cassette. The \( \text{aadA5} \) cassette was 95% identical over the first 830 bp to \( \text{aadA4} \), but lacked the \( \text{IS26} \) element found at the 3' end of this truncated cassette. Cloning and expression of the cassette region demonstrated that \( \text{dfrA17} \) conferred high level resistance to trimethoprim but \( \text{aadA5} \) conferred resistance to spectinomycin but not to streptomycin.

\( \text{dfrA17} \) and \( \text{aadA5} \). \( \text{dfrA17} \) confers resistance to trimethoprim and \( \text{aadA5} \) encodes an aminoglycoside-3'-adenylyl-transferase. The relationship of the encoded polypeptides to other related enzymes is discussed.

1. Introduction

Many of the antibiotic resistance genes found in clinical isolates of Gram-negative microorganisms are part of a gene cassette inserted into an integron [1], and the sequences of over 60 cassettes have been deposited in the GenBank/EMBL databases. Cassettes contain genes that confer resistance to a range of antibiotics, including aminoglycosides, \( \beta \)-lactams, chloramphenicol and trimethoprim, as well as genes that confer resistance to antiseptics and disinfectants [1,2]. Mobile gene cassettes consist of a gene together with one of a family of recombinase sites, known as 59-base elements (59-be). Gene cassettes can exist in the cell as discrete circular molecules [3], although the circular form does not replicate or express the associated gene. Alternatively, the integron encoded site-specific recombinase enzyme (IntI) mediates insertion of the cassette into the integron at the \( \text{attI} \) recombination site [4]. This insertion event places the gene within the cassette in the correct orientation to allow expression from an upstream promoter (\( \text{P}_c \)) in the integron [4]. Four classes of integron (1, 2, 3 and 4) have been identified which are distinguished by the sequence of their respective integrase proteins [1,5].

In this study we describe two new gene cassettes -

2. Materials and methods

2.1. Bacterial strains and culture conditions

\textit{Escherichia coli} INS33 was isolated in 1998 from the urinary tract of a patient attending The Prince of Wales Hospital, Sydney, N.S.W., Australia. Other strains used were \textit{E. coli} JM109 and \textit{E. coli} NCTC 10418. Bacteria were cultured in LB-broth supplemented with the appropriate antibiotics, and solid medium was LB containing 1.5% agar.

2.2. Antibiotic susceptibility testing

Resistance profiles were initially established using the calibrated dichotomous sensitivity (CDS) test [6]. The minimal inhibitory concentrations (MICs) were determined using agar dilution as described previously [7].

2.3. PCR detection of integrase genes and gene cassettes

Chromosomal DNA and plasmids were extracted as described previously [8]. Integrons were detected using PCR.
with degenerate primers designed to hybridise to conserved regions of integron encoded integrase genes \( \text{intI1, intI2 and intI3} \). Primers used were: hep35, 5' TGCG-GGTYAARGATBTKGATT3' and hep36, 5' CARCA-CATGCGRTRARAT3', where B = C or G or T, K = G or T, R = A or G and Y = C or T. Primers used to amplify the class 1 integron cassette region were: hep58, 5' TCA-TGGCTTGTATGACTG3', and hep59, 5' GTAGG-GCCTATTAGCAGC3'. PCR amplifications were carried out in 40-μl reaction mixtures containing 2 μl of DNA, 0.5 μM of each oligonucleotide primer, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), and 1.5 U Taq polymerase, or Pfu polymerase (Stratagene). PCR was performed for 30 cycles, each cycle consisted of 94°C for 30 s, 55°C for 30 s and extension at 72°C for 45 s for amplification of the integrase genes, or 4 min for amplification of the cassette region.

2.4. Cloning and DNA sequencing

All PCR products were purified by PEG precipitation [9] and were sequenced directly on an ABI 377 DNA sequence using a Prism DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer). PCR products were cloned into pGEM-T (Promega, USA) for further analysis.

2.5. Computer analysis of sequence data

Database searches were conducted using BLAST and multiple alignments carried out using CLUSTAL W [10]. Evolutionary distances between sequences were determined using the DNA DIST program (Kimura two-parameter method) of the PHYLIP package (version 3.57) [11]. The computed distances were used for the construction of phylogenetic trees using the program FITCH. Trees were plotted using the program TREEVIEW (version 1.5) [12]. To gain an internal estimate of how well the data supported the phylogenetic tree, bootstrap resampling (100 data sets) of the multi-sequence alignments were carried out with the programs SEQBOOT, DNADIST and FITCH. The consensus tree was calculated with CONSENSE.

The GenBank accession number of the \( \text{dfrA17 and aadA5} \) gene sequences determined in this study is AF169041.

3. Results and discussion

3.1. Characterisation of \( \text{E. coli INS33} \)

\( \text{E. coli INS33} \) was resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfafurazole, tetracycline and trimethoprim, and susceptible to gentamicin, kanamycin, tobramycin, amikacin, netilmicin, augmentin, cefotaxime, cephalaxin, cefotetan, impenem, norfloxacain and nitrofurantoin. The presence of an integron within \( \text{E. coli INS33} \) was ascertained by PCR detection of \( \text{intI genes} \). The nucleotide (nt) sequence of the resultant 491-bp PCR product demonstrated 100% identity with the integron associated \( \text{intI1} \) gene. A number of experiments suggested that the \( \text{E. coli INS33} \) integron was not located on a plasmid. Plasmid extraction followed by either agarose gel electrophoresis or transformation with subsequent selection on trimethoprim did not reveal the presence of a plasmid. Furthermore, conjugation experiments failed to demonstrate transfer of trimethoprim resistance to a recipient strain. Therefore, the cassettes were most likely part of the \( \text{E. coli} \) chromosome.

3.2. Characterisation of the cassette region and flanking sequences

Amplification of the cassette region revealed a product of 1.7 kb. Sequences were identified on either end of the PCR product that corresponded to portions of the 5'-conserved segment (CS) and 3'-CS, which flank cassettes in class 1 integrons. The 3'-CS is known to contain \( \text{qacEa1} \) and the \( \text{sul} \) gene that confers sulfafurazole resistance. The cassette region contained two open reading figures.
frames (ORF) of 474 bp and 789 bp. Analysis of the sequence flanking the two ORFs revealed several features characteristic of gene cassettes. These included imperfect repeat 59-be sequences [13] downstream of the genes, sequence indicative of a core site (GTTRRRY) at the 5' end of the genes, an inverse core site (RYYYAAC) close to the stop codon, and internal 2L and 2R core sites within the 59-be [14]. The 59-be were 133 bp (nts 630^762) and 57 bp (nts 1601^1657) in length.

The first cassette contained a 474-bp ORF, designated dfrA17, with a TTG initiation codon, (nts 162^164) preceded by a putative ribosome-binding site (RBS) (nts 153^156). The 616-bp dfrA17 gene cassette sequence was 91% identical to the dfrA7 cassette. dfrA7 confers resistance to

<table>
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<tr>
<th>Strain</th>
<th>MIC (mg l^-1)a</th>
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<tr>
<td></td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>E. coli NCTC 10418</td>
<td>0.25</td>
</tr>
<tr>
<td>E. coli INS33</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>1</td>
</tr>
<tr>
<td>E. coli JM109/pVRL43</td>
<td>&gt; 32</td>
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</tbody>
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*Range of MICs for susceptible strains: trimethoprim, 0.01–1.0 mg l^-1; streptomycin, 2–4 mg l^-1; and spectinomycin, 8–16 mg l^-1 [29].
AAD, but also termed ANT(3–26 residue protein of 29,042 Da that is related to other the protein alignment of the five members of the AADA family.

Currently, 19 transferable DHFR genes have been identified in Gram-negative bacteria, and the sequences of all except those encoding DHFRIV and DHFRIIIb have been determined (this study, [18,19]). Many of the DHFR enzymes can be grouped into one of three families (1, 2 and 3) based on amino acid sequence [18–20]. Phylogenetic analysis of transmissible DHFRs and bootstrap resampling of the data demonstrated robust partitioning of the three families and revealed that only gene cassette encoded enzymes belong to one of the three families (Fig. 1). Family 2 consists of three enzymes DHFRIIa, IIb and Ic [21,22], which are different from all other proteins as they have significantly shorter amino acid sequences (78 residues). Family 3 members comprise DHFRXII and XIII ([20], GenBank/EMBL accession number Z50802) (Fig. 1). The four remaining DHFR proteins are unrelated and their genes are not found as part of a gene cassette (Fig. 1).

The second cassette analysed was 895 bp in length and included a putative streptomycin/spectinomycin resistance gene, designated aadA5. Streptomycin is an aminoglycoside widely used between 1950 and 1970, which is now only used in humans to treat tuberculosis, although it is used extensively in animal husbandry. aadA5 was 95% identical over the first 830 bp to the truncated aadA4 cassette (GenBank/EMBL accession number Z50802) but diverges from nt 1582. At this point, the aadA4 sequence contains an IS26 element which may have led to the deletion of the cassette-associated 59-be. The aadA5 cassette was 60% identical to the aadA1 cassette found in many integrons [17,23,24]. The 789-bp ORF of aadA5 encodes a 262 residue protein of 29,042 Da that is related to other aminoglycoside-3′-adenyllytransferases (referred to here as AAD, but also termed ANT). The AADA enzymes adenylylate streptomycin and spectinomycin, thereby inactivating these antibiotics. The polypeptide is most closely related to AADA4, with 91% amino acid identity. AADA5 also shares 57% identity to AADA1, 55% identity with AADA2 [25] and 54% identity with AADA3 (GenBank/EMBL accession number AF047479).

3.3. Expression of resistance genes dfrA17 and aadA5

The amplified cassette region, containing dfrA17 and aadA5, was cloned into pGEM-T and designated pVRL43. Amplification of the cassette region did not include the P promoter from the integron, thus expression relied on the lacZ promoter (Plac) of the cloning vector. Fragments cloned in the correct orientation for expression from Plac were recovered by patching 20 of the resultant transformants on to LB agar containing trimethoprim. Seven colonies were found to be resistant to trimethoprim.

The MICs of trimethoprim, streptomycin and spectinomycin for E. coli NCTC 10418, INS33, JM109 and JM109/pVRL43 were measured (Table 1). E. coli NCTC 10418 and JM109 were susceptible to trimethoprim, streptomycin and spectinomycin. Conversely, E. coli INS33 was resistant to trimethoprim, streptomycin and spectinomycin with MICs > 32 mg 1−1, > 64 mg 1−1 and 32 mg 1−1, respectively (Table 1). E. coli JM109/pVRL43 was resistant to trimethoprim and spectinomycin, but susceptible to streptomycin (Table 1). The dfrA17 and aadA5 cassettes account for the resistance levels of E. coli INS33 to trimethoprim and spectinomycin. However, the high level of streptomycin resistance in E. coli INS33 does not appear to be mediated by aadA5. Therefore resistance could be conferred by an alternative mechanism such as mutational modification of the S12 ribosomal protein, which provides resistance to streptomycin but not spectinomycin [26]. The lack of resistance to streptomycin exhibited by AADA5 contrasts markedly to AADA1, 2 and 3, this could be due to differences in the amino acid sequences, where AADA5 shares only 50–57% identity with AADA1, 2 and 3 (Fig. 2). AADA4 which is 91% identical to AADA5, has not been tested for resistance to streptomycin or spectinomycin. It would therefore be interesting to determine if this enzyme, like AADA5, confers resistance to spectinomycin but not streptomycin.

The identification of the aadA5 and dfrA17 cassettes, in addition to many other recently described cassettes that confer antibiotic resistance, highlight the capacity of bacteria to evolve resistance mechanisms that could have further negative implications for future therapeutic use of antibiotics.

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References

region of integrons are excised as covalently closed circles. Mol. Microbiol. 6, 2875–2885.


