DNA homology between siderophore genes from fluorescent pseudomonads

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Many species of pseudomonads produce fluorescent siderophores involved in iron uptake. We have investigated the DNA homology between the siderophore synthesis genes of an opportunistic animal pathogen, Pseudomonas aeruginosa, and three plant-associated species Pseudomonas syringae, Pseudomonas putida and Pseudomonas sp. B10. There is extensive homology between the DNA from the different species, consistent with the suggestion that the different siderophore synthesis genes have evolved from the same ancestral set of genes. The existence of DNA homology allowed us to clone some of the siderophore synthesis genes from P. aeruginosa, and genetic mapping indicates that the cloned DNA lies in a locus previously identified as being involved in siderophore production.

Introduction

The fluorescent pseudomonads are a major group of Gram-negative bacteria which are found in a range of ecological niches. Most species are found in soil or fresh water, and some are plant pathogens (for example, strains of Pseudomonas syringae) or are beneficial to plant growth (for example, strains of the P. putida–P. fluorescens group) (Kloeper et al., 1980). One species, P. aeruginosa, is able to cause infection of immunocompromised patients or cystic fibrosis patients (Bodey et al., 1983), and infection can be fatal. As a group, these bacteria are characterized by the production of water-soluble fluorescent yellow-green pigments called pyoverdines or pseudobactins, which are siderophores involved in iron uptake. These pigments consist of a chromophore joined to a short peptide; the chromophore is conserved between species whereas the exact nature of the peptide is species-specific, with differences reflecting specificities of uptake (Hohnadel & Meyer, 1988). The very close similarities between pyoverdines from different species suggest that the siderophores (and by implication the genes involved in their synthesis) have evolved from a common ancestor.

The objective of the work described here was to investigate homologies between genes which are involved in synthesis of fluorescent siderophores by different species of Pseudomonas. Genes involved in siderophore synthesis have been cloned from the plant-associated species P. syringae, P. putida, Pseudomonas sp. B10 and Pseudomonas sp. M114 (Loper et al., 1984; Marugg et al., 1985; Moores et al., 1984; O’Sullivan et al., 1990). Our approach was to use DNA hybridization to investigate homology between siderophore genes from three plant-associated species and those of the animal pathogen P. aeruginosa.

Methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Strains of Escherichia coli were grown in LB medium (Miller, 1972) and strains of Pseudomonas aeruginosa in Brain Heart Infusion Broth (Oxoid; 18.5 g l−1) at 37 °C, with good aeration unless otherwise stated. Solid media used were nutrient agar (Oxoid) for E. coli and Brain Heart Infusion Broth solidified with agar (2% w/v; Difco) for P. aeruginosa. Production of pyoverdine by P. aeruginosa strains was detected using King’s B agar (King et al., 1954) supplemented as required with the iron-chelating agent ethylenediamine-dis(-hydroxyphenylacetic acid) (EDDA; 200 µg ml−1), and was quantitatively measured by growing the bacteria in succinate minimal medium (Meyer & Abdallah, 1978) and spectrophotometric analysis of the culture supernatant. Media were supplemented with antibiotics at the following concentrations (µg ml−1): ampicillin, 50 µg; carbenicillin, 300 µg; chloramphenicol, 30 µg for E. coli and 100 µg for P. aeruginosa; streptomycin, 1 mg; and tetracycline, 12.5 µg for E. coli and 20 µg for P. aeruginosa. Tetracycline was used at 100 µg ml−1 in the gene disruption experiment.

DNA isolation, manipulation and analysis. Chromosomal DNA was prepared using the method of Marmur (1961). Cosmid and plasmid DNA isolation was done by the method of Ish-Horowicz & Burke (1981). λ DNA preparations were carried out using the method of Grossberger (1987). Enzymic manipulation and sub-cloning of DNA, and Southern analysis, were carried out using standard methods (Maniatis et al., 1982); during Southern analysis filters were washed at

Abbreviation: EDDA, ethylenediamine-dis(-hydroxyphenylacetic acid).

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low (50 °C), medium (55 or 60 °C) or high (65 °C) stringency, as appropriate. Restriction enzymes were purchased from Amersham, T4 DNA ligase from Promega, and calf-intestinal phosphatase from Boehringer-Mannheim. A library of DNA was prepared in the cloning vector AEMBL3 (Frischauf, 1987). DNA probes were labelled with [32P]dATP using the oligonucleotide priming method (Feinberg & Vogelstein, 1983) and were used to screen genomic DNA prepared from P. aeruginosa DNA. For the Southern analysis was carried out using the cloned DNA as a probe. Hybridization of P. aeruginosa DNA with cloned siderophore genes from Pseudomonas sp. strain Mi14 (Loper et al., 1984) was shown to hybridize to several bands on the gel. DNA was prepared from P. putida, Pseudomonas sp. sp. strain M114 (Loper et al., 1984; O'Sullivan et al., 1984; Marugg et al., 1985; Moores et al., 1984; O'Sullivan et al., 1990). DNA was prepared from P. aeruginosa and Southern analysis was carried out using the cloned siderophore genes from P. putida and Pseudomonas sp. B10 as probes. The results of one such experiment are shown in Fig. 1. They clearly show that the probes hybridized to P. aeruginosa DNA. The two probes gave very similar results, with each hybridizing to several fragments of P. aeruginosa DNA. For the P. putida clone pMA3, it has been shown that all of the cloned DNA is involved in siderophore metabolism (Marugg et al., 1989) so that hybridization is not due to genes encoding other functions which are present in the clone.

### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant genotype and phenotype*</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
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<tr>
<td>OT11</td>
<td>lew-1 pro-1 Pvd+</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>OT373</td>
<td>lew-1 lys-1 (RP1) Pvd+</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>OT2021</td>
<td>lew-1 pro-1 pvd::pSUP202 Tc&lt;sup&gt;A&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>OT2021(RP1)</td>
<td>lew-1 pro-1 pvd::pSUP202 (RP1) Tc&lt;sup&gt;A&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt; Cb&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PAO307</td>
<td>argC54 Pvd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Haas et al. (1977)</td>
</tr>
<tr>
<td>PAO4032</td>
<td>catA1 mzu-9002 tyu-9030 met-9020 Pvd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Matsumoto et al. (1981)</td>
</tr>
<tr>
<td>PAO6232</td>
<td>catA1 mzu-9002 tyu-9030 Pvd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hohndel et al. (1986)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
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</tr>
<tr>
<td>JM83</td>
<td>Δ(lac-proAB) lacZ ΔM15</td>
<td>Yanish-Perron et al. (1985)</td>
</tr>
<tr>
<td>S17-1</td>
<td>hsdR HsdM&lt;sup&gt;+&lt;/sup&gt; recA integrated RP4-2-Tc&lt;sup&gt;R&lt;/sup&gt; :Mu Km&lt;sup&gt;R&lt;/sup&gt; : : Tn7</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>NM539</td>
<td>supF hsdR (P2 cox&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>Frischau et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pSUP202</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;A&lt;/sup&gt; Ap&lt;sup&gt;Cb&lt;/sup&gt;</td>
<td>Simon et al. (1986)</td>
</tr>
<tr>
<td>pMA1</td>
<td>P. putida siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Marugg et al. (1985)</td>
</tr>
<tr>
<td>pMA3</td>
<td>P. putida siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Marugg et al. (1985)</td>
</tr>
<tr>
<td>pJLM3A</td>
<td>Pseudomonas sp. B10 siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Moores et al. (1984)</td>
</tr>
<tr>
<td>pJLM300</td>
<td>Pseudomonas sp. B10 siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Magazin et al. (1986)</td>
</tr>
<tr>
<td>pSFL10</td>
<td>P. syringae siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Loper et al. (1984)</td>
</tr>
<tr>
<td>pSFL11</td>
<td>P. syringae siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Loper et al. (1984)</td>
</tr>
<tr>
<td>pSFL12</td>
<td>P. syringae siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Loper et al. (1984)</td>
</tr>
<tr>
<td>pSFL14</td>
<td>P. syringae siderophore genes in pLAFR1; Tc&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Loper et al. (1984)</td>
</tr>
</tbody>
</table>

* Abbreviations: Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Pvd, pyoverdine; Tc, tetracycline.

**Results**

**Hybridization of P. aeruginosa DNA with cloned siderophore genes**

We first investigated the possibility that P. aeruginosa contains DNA which is homologous to siderophore-synthesizing genes of other fluorescent pseudomonads. Genes involved in siderophore synthesis have been cloned from P. syringae, P. putida, Pseudomonas sp. B10 and Pseudomonas sp. strain M114 (Loper et al., 1984; Marugg et al., 1985; Moores et al., 1984; O'Sullivan et al., 1990). DNA was prepared from P. aeruginosa and Southern analysis was carried out using the cloned siderophore genes from P. putida and Pseudomonas sp. B10 as probes. The results of one such experiment are shown in Fig. 1. They clearly show that the probes hybridized to P. aeruginosa DNA. The two probes gave very similar results, with each hybridizing to several fragments of P. aeruginosa DNA. For the P. putida clone pMA3, it has been shown that all of the cloned DNA is involved in siderophore metabolism (Marugg et al., 1989) so that hybridization is not due to genes encoding other functions which are present in the clone.
Cloning of hybridizing P. aeruginosa DNA

The probes used in the Southern analysis of genomic P. aeruginosa DNA contained genes involved in synthesis of fluorescent siderophores. We expected that the P. aeruginosa DNA hybridizing with the probes contained genes involved in the synthesis of pyoverdine, the fluorescent siderophore produced by P. aeruginosa. To test this possibility, and to allow us to further investigate hybridization between DNA from the different species, we cloned a portion of the hybridizing P. aeruginosa DNA. A library of P. aeruginosa DNA was prepared in a λ-based cloning vector, EMBL3 (Frischauf et al., 1983). Approximately 5000 clones were transferred onto nylon membrane and probed with radio-labelled pMA1 DNA, which contains siderophore synthesis genes from P. putida (Marugg et al., 1985). One clone which hybridized strongly with the probe was isolated and analysed further. This clone was named AOT1. A physical map of this clone is shown in Fig. 2.

Fig. 1. Southern analysis of genomic DNA from P. aeruginosa. Genomic DNA from P. aeruginosa was digested with EcoRI (E) or PstI (P), electrophoresed and transferred to a nylon membrane. The DNA was then probed with (a) pMA3 which contains siderophore synthesis genes from P. putida, or (b) plJM300 which contains siderophore synthesis genes from Pseudomonas sp. B10. The filters were washed under conditions of medium stringency [60 °C, 0.1 × SSC (1 × SSC is 150 mM-NaCl, 15 mM-sodium citrate)]. The positions of marker bands are shown.

Fig. 2. Physical map of AOT1. AOT1 is a clone of DNA from P. aeruginosa which hybridizes with cloned siderophore genes from other fluorescent pseudomonads. Sites for BamH1 (B), EcoRI (E), KpnI (K), PstI (P) and SalI (S) were mapped. The cloned DNA does not have restriction sites for BglII, HindIII, SacI or XbaI. The SalI sites at the ends of the DNA are in the cloning site of the EMBL3 vector and represent the boundaries of the cloned DNA. The 3.0 kb PstI fragment used in the chromosomal disruption experiment (see text) is indicated.

The six SalI fragments present in AOT1 were sub-cloned into pUC9 (Vieira & Messing, 1982). They were then used as probes in Southern analysis of the cloned genes from P. syringae, P. putida and Pseudomonas sp. B10, with filters being washed in 0.2 × SSC at 55 °C following hybridization. Similar results were obtained with all three species. The two central SalI fragments and the smallest right-most SalI fragment in AOT1 (Fig. 2) did not hybridize to the cloned DNA from the other species; the other SalI fragments did hybridize, with the largest (leftmost) fragment hybridizing with several fragments from each species (results not shown). These results confirmed that cloned P. aeruginosa DNA present in AOT1 hybridized with siderophore-synthesizing genes from the other three species.

Involvement of the cloned DNA in pyoverdine synthesis

DNA present in clone AOT1 hybridized with DNA from P. putida, Pseudomonas sp. B10 and P. syringae which is involved in the synthesis of siderophores by these species. We therefore expected the P. aeruginosa DNA in AOT1 to be involved in synthesis of pyoverdine, which is chemically similar to the siderophores made by the plant-associated species (Hohnadel & Meyer, 1988). The strategy used to test this involved disruption in P. aeruginosa of the chromosomal locus corresponding to the cloned DNA, followed by examination of the phenotypes of the resulting bacteria. The 3.0 kb PstI fragment present in AOT1 (Fig. 2) was sub-cloned into the PstI site of plasmid pSUP202 (Simon et al., 1983), which was then mobilized from E. coli S17-1 into P. aeruginosa OT11. As the plasmid cannot replicate in P. aeruginosa, selection for the CmR and TcR markers carried by the plasmid gave rise to strain OT2021 in which the plasmid had become integrated into the P. aeruginosa chromosome by homologous recombination between the cloned PstI fragment and the chromosome.
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Chromosome (ii)

Fig. 3. Disruption of the cloned locus in P. aeruginosa. (a) Homologous recombination between the chromosome of P. aeruginosa and a 3.0 kb fragment of cloned DNA in plasmid pSUP202 (i) gave rise to strain OT2021 in which the pSUP202 DNA is integrated into the chromosome at the site of recombination and the 3.0 kb fragment has been duplicated (ii). Relevant restriction sites were identified from the restriction maps of JOT1 (Fig. 2) and pSUP202 (Simon et al., 1983).

(b) Southern analysis of DNA from strain OT2021. Genomic DNA from strain OT2021 was digested with restriction enzymes EcoRI (E), BamHI (B), PstI (P) or Sall (S) and Southern analysis was carried out using pSUP202 as a probe. The sizes of the hybridizing fragments are 8.3 and 18.7 kb (EcoRI), 9.9 and 25.5 kb (BamHI), 7.7 kb (PstI), and 10.7 and 17.3 kb (Sall). These sizes are in agreement with the predicted sizes shown in (a).

Southern analysis of genomic DNA from strain OT2021 confirmed that the expected recombination event had taken place (Fig. 3).

Unlike the parental strain OT11, strain OT2021 was unable to synthesize pyoverdine as determined by several criteria. It failed to form the fluorescent pigment when grown on King's B agar; it failed to grow when the iron-chelating agent EDDA was added to the agar; and it did not give rise to any detectable pyoverdine when grown in succinate medium. We concluded that integration of pSUP202 into the chromosome in strain OT2021 prevented synthesis of pyoverdine, and as integration had occurred by homologous recombination between the cloned PstI fragment and the chromosome, the cloned DNA must also normally be involved in pyoverdine synthesis.

Genetic mapping of the cloned locus

The results described in the previous section showed that the segment of P. aeruginosa DNA present in JOT1 is normally involved in synthesis of pyoverdine. Genes required for pyoverdine synthesis are located at 23 and 47 min on the chromosomal map of P. aeruginosa (Holloway & Zhang, 1990) and are linked to argC, and to mtu and catA, respectively. We wished to determine which, if either, locus corresponded to the DNA in JOT1. This was done by taking advantage of the mob site present in pSUP202 and which was integrated into the chromosome at the cloned locus in strain OT2021. In the presence of the tra gene products of plasmid RP1 this site could act as an origin of chromosomal transfer during conjugation of OT2021 with recipient strains. The use of chromosomally integrated origins of transfer for gene mapping is well established in P. aeruginosa (O'Hoy & Krishnapillai, 1987). Plasmid RP1 was introduced into strain OT2021 from strain OT373 by conjugation. Strain OT2021(RP1) was then used as a donor in crosses with appropriate recipient strains, selecting for transfer of the argC, met-9011, catA and mtu markers. The results are summarized in Table 2. The catA marker was transferred at a high frequency and argC at a low frequency. This result shows that the integrated mob site in the chromosome of strain OT2021 is much more highly linked to the catA marker than to the argC marker. As the mob site is present in the cloned locus, this indicates that the cloned DNA present in JOT1 comes from the previously identified pyoverdine locus (pvd), at 47 min on the P. aeruginosa chromosome which is close to the catA locus at 46 min (Hohnadel et al., 1986; Ankenbauer et al., 1986). The mtu locus also lies in this region of the chromosome, at 48 min, but is on the opposite side of the pvd locus to catA (Hohnadel et al., 1986; Ankenbauer et al., 1986) so that failure to detect transfer of mtu was because the orientation of mob in strain OT2021 resulted in mtu being the last locus to be transferred during conjugation. The met-9011 marker was transferred at a frequency between those of catA and argC and this is consistent with its location between catA and argC on...
Table 2. Genetic mapping of the cloned locus.

Strain OT2021 (RP1) was used as a donor in conjugations with the recipients shown in the Table. The donor strain contained an origin-of-transfer site in the cloned locus. Selection for transconjugants was carried out on minimal medium, thereby selecting against donor and recipient bacteria. The frequency of transfer is expressed as the number of bacteria acquiring the selected marker divided by the number acquiring plasmid RP1 from the donor strain; expressing the results in this way allows results from different crosses to be compared. The total number of transconjugants receiving RP1 was between $1 \times 10^6$ and $4 \times 10^6$ in all three crosses.

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Selected marker*</th>
<th>Frequency of transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO307 argC</td>
<td>Arg*</td>
<td>$5.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>PAO4032 cat mtu tyu met</td>
<td>Met*</td>
<td>$2.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>PAO6232 cat mtu tyu</td>
<td>Cat*</td>
<td>$2.0 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>Mtu*</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>Tyu*</td>
<td>$&lt;10^{-6}$</td>
</tr>
</tbody>
</table>

* Abbreviations: Arg, arginine utilization; Cat, catechol utilization; Met, methionine utilization; Mtu, mannitol utilization; Tyu, tyrosine utilization.

the genetic map (Holloway & Zhang, 1990) and with the assignment of the cloned DNA to the pyoverdine locus near catA.

Homology of λOT1 DNA with siderophore-synthesizing genes

The results described above showed that clone λOT1 contains DNA from *P. aeruginosa* which is involved in siderophore synthesis. We next wished to investigate the degree of homology of the cloned *P. aeruginosa* DNA with cloned siderophore genes from other species. The approach used was to carry out Southern analysis of the cloned DNA from three plant-associated species using the cloned DNA in λOT1 as a probe. Hybridization was initially carried out under conditions of low stringency, so that DNA fragments with relatively low homology were detected; the stringency was then gradually increased in order to identify the fragments which had most homology with the probe DNA. Typical results obtained under conditions of medium and high stringency are shown in Fig. 4. DNA from all three plant-associated species gave similar results, in that several DNA fragments hybridized with λOT1 DNA under conditions of low stringency and so must have some degree of homology to the *P. aeruginosa* DNA; at higher stringencies, hybridization only occurred for one or two fragments with each species, and these must have more extensive DNA homology. Some of the fragments did not hybridize with the probe even under conditions of low stringency indicating that homologous DNA was not present in λOT1, or that the homology was too low to give detectable hybridization.

Discussion

*P. aeruginosa*, an opportunistic animal pathogen, makes a fluorescent siderophore called pyoverdine which is chemically very similar to siderophores made by soil- and plant-associated pseudomonads. The experiments described here investigated the possibility that this similarity extends to the DNA level. The results show that there is extensive and strong homology of *P. aeruginosa* DNA with siderophore synthesis genes from at least three other pseudomonads, the plant pathogen *P. syringae* and the soil species *P. putida* and *Pseudomonas* sp. B10. This indicates that at least some of the DNA involved in siderophore synthesis has been conserved during the evolution of the fluorescent pseudomonads.
Homology had previously been reported between \textit{P. aeruginosa} DNA and the cloned \textit{P. syringae} DNA used here (Lawson et al., 1986). Lawson et al. did not demonstrate that the hybridizing \textit{P. aeruginosa} DNA contained siderophore synthesis genes, but the results presented here indicate that this is the case. We are not aware of other reports describing homology amongst siderophore synthesis genes from fluorescent pseudomonads. We confirmed that at least some of the hybridizing \textit{P. aeruginosa} DNA is involved in synthesis of the fluorescent siderophore pyoverdine by cloning a fragment of hybridizing DNA and then disrupting the chromosomal locus from which it was derived. The resulting strain, OT202, failed to synthesize pyoverdine. Furthermore, genetic mapping experiments showed that the cloned DNA is derived from a region of the chromosome which had previously been shown to contain genes involved in pyoverdine synthesis. These results showed that at least some of the cloned DNA is from a locus involved in synthesis of pyoverdine and not from another locus which coincidentally hybridized with the probes from other species.

The cloned \textit{P. aeruginosa} DNA was also used to investigate the degrees of homology of different fragments of DNA. The results (Fig. 3) showed that homology is not evenly distributed amongst the hybridizing fragments. A small number of fragments from each species were detected under hybridization conditions of high stringency, indicating a high degree of DNA sequence homology. Under less stringent conditions, more fragments were detected, and these must have lower (though still significant) sequence homology. The fact that different fragments show different degrees of homology presumably indicates that some genes involved in siderophore synthesis have been more highly conserved than others during the evolution of the fluorescent pseudomonads. This may be related to the functions of the genes. The chromophore moieties of the siderophores have been well conserved between the different species, so that it is likely that the relevant genes have also been highly conserved, whereas the small peptides which are attached to the chromophores show considerable evolutionary divergence (Hohnadel & Meyer, 1988) which is likely to be reflected in divergence at the level of DNA sequence. Biochemical functions have not been assigned to cloned genes involved in siderophore synthesis from any of the species used in this study.

In summary, our results show that there is extensive homology between siderophore genes of \textit{P. aeruginosa} and of plant-associated pseudomonads, and that different regions of DNA exhibit homology to differing extents, presumably reflecting the evolution of the DNA as the genes diverged from a common ancestor.

We are very grateful to Dieter Haas, John Leong, Joyce Loper, Reinhardt Simon and Peter Weisbeek for sending us strains and clones. We would also like to thank Andrew Sheiling for his assistance in constructing the library of \textit{P. aeruginosa} DNA. I. R. is a New Zealand Health Research Council Post-Graduate Scholar. This work was supported in part by a grant from the Otago Medical Research Foundation.

References


