Characterization of the recA gene from Pseudomonas fluorescens OE 28.3 and construction of a recA mutant

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The recA gene of Pseudomonas fluorescens OE 28.3 was isolated by complementation of the Fec- phenotype of recombinant lambda EMBL3 phages in a RecA- Escherichia coli strain. The subcloned recA restored resistance to UV and methyl methanesulphonate (MMS) exposure in recA mutants of E. coli. DNA sequence analysis showed that the coding region of the P. fluorescens gene, specifying a protein of 352 amino acid residues, was preceded by an SOS box highly similar to those of Pseudomonas aeruginosa and Azotobacter vinelandii. The deduced amino acid sequence displayed highest homology to the RecA proteins from P. aeruginosa (87.8% identity) and A. vinelandii (84.3% identity). In both the regulatory region and the structural gene, a relatively high degree of sequence divergence from the Pseudomonas cepacia gene was observed. A mutant of P. fluorescens was constructed by inserting a kanamycin resistance cassette into its recA gene. This mutant exhibited an increased sensitivity to UV irradiation and MMS, and was strongly impaired in homologous recombinational activity.

Introduction

Certain soil bacteria occurring in the rhizosphere of crop plants exert a beneficial effect on the development of their hosts by either a direct (hormone production) or an indirect (biocontrol of soil phytopathogens) mechanism (Lugtenberg et al., 1991). In view of the agronomic potential of inocula based on such plant-growth-promoting rhizobacteria (PGPR) for environmentally friendly cropping practices, interest in these bacteria has increased steadily during the last decade, providing new insights into the mechanisms of growth promotion and root colonization. In temperate soils, the fluorescent Pseudomonas species P. fluorescens and P. putida represent the major PGPR.

Recently, we identified a root adhesin in the wheat rhizobacterium P. fluorescens OE 28.3 (De Mot & Vanderleyden, 1991), and characterized the corresponding oprF-like gene, encoding a major outer-membrane protein (De Mot et al., 1992). Studying the role of this adhesin in root colonization by PGPR requires gene manipulation in P. fluorescens. For this purpose, the availability of a recombination-deficient mutant is essential.

The recA gene product plays a pivotal role in bacterial homologous recombination, as well as in other cellular processes such as DNA repair (Miller & Kokjohn, 1990; Roca & Cox, 1990). Within the Pseudomonas group, recA mutants have been characterized for the opportunistic human pathogen P. aeruginosa (Früh et al., 1983) and for the foliar pathogen P. syringae (Willis et al., 1988; Barbé et al., 1991), both belonging to the same rRNA cluster as P. fluorescens (group I). In addition, the recA genes of P. aeruginosa (Sano & Kageyama, 1987) and of the rRNA group II species P. cepacia (Nakazawa et al., 1990) have been sequenced.

In this paper we describe the molecular cloning and sequencing of the recA gene from P. fluorescens OE 28.3, and the construction and characterization of a RecA-deficient strain for this wheat isolate.

Methods

Bacterial strains, plasmids, phages, media and growth conditions. The bacterial strains, plasmids and phages used in this work are listed in Table 1. E. coli and P. fluorescens strains were grown in Luria broth.
Table 1. Bacterial strains, plasmids and phages

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<td>$P$. fluorescens</td>
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<td>OE 28.3</td>
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| (LB) medium (37 °C) and trypticase soy broth (TSB) medium (30 °C), respectively. For solid media, 15 g agar $l^{-1}$ was added. In conjugation experiments, M9 minimal medium was used for selection of $P$. fluorescens transconjugants. Antibiotics were used at the following concentrations: ampicillin 100 µg ml$^{-1}$, kanamycin 50 µg ml$^{-1}$, chloramphenicol 25 µg ml$^{-1}$, tetracycline 10 µg ml$^{-1}$. α-Complementation of lacZ was determined on LB plates supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl β-D-thiogalactopyranoside at 20 µg ml$^{-1}$. Phage-infected $E$. coli cells were plated as described by Frischauf et al. (1987). Cell doubling times were estimated from the increase in OD$_{560}$ during aerobic incubation in TSB medium on a rotary shaker (200 r.p.m.).

Determination of sensitivity to UV irradiation and methyl methanesulphonate (MMS). $E$. coli or $P$. fluorescens cells were grown in LB (37 °C) or TSB (30 °C) to an OD$_{560}$ of about 1. The cells were washed and diluted to approximately 5 x 10$^7$ cells ml$^{-1}$. Aliquots (100 µl) of several tenfold dilutions were spread in triplicate on LB or TSB plates. When testing MMS sensitivity, the media contained 0-1, 0-5, 1 or 2 mm of this DNA-methylating agent. Different doses of UV radiation (312 nm), ranging from 50 to 200 J m$^{-2}$, were applied to the plated cells using a transilluminator. Plates were incubated overnight (or longer when delayed growth was observed in the presence of MMS) at 37 °C ($E$. coli) or 30 °C ($P$. fluorescens). The level of resistance was calculated from the relative number of surviving cells using MMS-free or non-irradiated plates as a control.

DNA methods and sequence analysis. $E$. coli plasmids were extracted using the alkaline lysis method, whereas total DNA from Pseudomonas strains was isolated with the cetyltrimethylammonium bromide procedure (Sambrook et al., 1989). Phage DNA was prepared according to Lockett (1990). Double-stranded DNA sequencing of pUC19 subclones was carried out using the AutoRead Sequencing Kit (Pharmacia-LKB) on an automated sequencer (A.L.F., Pharmacia-LKB). Sequence data were processed using the ASSEGEL program (PC Gene, IntelliGenetics). The PC Gene software was also used for sequence comparison of deduced proteins by pairwise (PALIGN) or multiple (CLUSTAL) alignments.

Southern hybridization. Genomic or plasmid DNA cut with appropriate restriction endonucleases was transferred by capillary blotting to Hybond-N membranes (Amersham) after agarose electrophoresis. The following probes were isolated by preparative electrophoresis and non-radioactively labelled with the random priming method (DIG chemiluminescent system, Boehringer Mannheim): the 1.2 kb EcoRI fragment from pKN3 carrying most of the $P$. cepacia recA gene; the 1.25 kb HindIII fragment containing the kanamycin resistance cassette from pUKA800; and the 1.8 kb EcoRI fragment from strain OE 28.3. pSUP102 with HindIII fragment from pSUP102. The non-radioactive hybridization was carried out as recommended by the manufacturer using a hybridization temperature of 68 °C and final washing in 0.1 x SSC plus 0.1 % SDS. SSC (1 x) contains 0.15 M-NaCl and 15 mm-sodium citrate at pH 7.0.

Homologous recombination. Following random cloning of genomic EcoRI fragments from $P$. fluorescens OE 28.3 in pSUP102, two clones with inserts of 4 kb (pFAJ2083) and 5 kb (pFAJ2084) were selected to assess the homologous recombination activity in strains OE 28.3 and FAJ2025 by the method of Selbitschka et al. (1991). The recombinant plasmids were introduced into these Pseudomonas strains by biparental conjugation using $E$. coli S17-1 as a donor. The frequency of appearance of tetracycline-resistant transconjugants per acceptor cell was determined.

Results and Discussion

Cloning of the recA gene

A previously constructed genomic library of $P$. fluorescens OE 28.3 in lambda EMBL3 (De Mot et al., 1992) was plated on $E$. coli HB101 cells. Ten plaque-
forming recombinant phages were purified by repeated plating and finally checked for lytic activity on other RecA- strains of *E. coli* (DH1, DH5az). The identical SalI restriction patterns of these clones indicated that all of them contained the same insert. The *P. cepacia* recA gene was used to probe a Southern blot of DNA from a selected phage (pFAJ2003) restricted with several endo-nucleases. For a 3.8 kb EcoRI and a 3 kb HindIII fragment strong signals were obtained. These fragments were subcloned in pUC19 to generate pFAJ2006 and pFAJ2009, respectively.

A recombinant phage, containing an unrelated DNA fragment (pFAJ2001; De Mot et al., 1992), was plated on *E. coli* DH5az cells containing pFAJ2006 or pFAJ2009. Whereas pFAJ2001 was incapable of lytic growth on strain DH5az (or other RecA- strains such as HB101 and DH1), its plaque-forming ability was restored by the presence of the recombinant plasmids, intermediate to the level obtained with RecA+ strains. These observations indicated that a functional recA gene had been cloned. This was further substantiated by the enhanced UV resistance and reduced MMS sensitivity of RecA- *E. coli* hosts carrying pFAJ2006 or pFAJ2009, approaching the levels attained by the RecA+ strain RR1 (results not shown).

Thus, the recA gene from *P. fluorescens* was isolated from a phage library by interspecific complementation of the Fec- phenotype of the recombinant phage in *E. coli*. Recently, this approach proved equally successful for the recA gene from *Rhizobium phaseoli* (Michiels et al., 1991; Martinez-Salazar et al., 1991). Whereas most recA genes have been identified by the ability to confer resistance to DNA damaging agents when cloned in RecA- *E. coli* strains, the direct screening of a phage library on a RecA- host provides a straightforward alternative method for their identification.

**Nucleotide sequence of the recA gene**

Physical mapping of the inserts from pFAJ2006 and pFAJ2009 showed that the EcoRI fragment encompassed the HindIII fragment (Fig. 1). The approximate position of the recA gene within the latter fragment was established by Southern hybridization. For the region of interest, several overlapping pUC19 subclones were isolated and sequenced as shown in Fig. 1.

The region sequenced (1444 bp, G+C content 57%) revealed one large open reading frame spaced five nucleotides from a typical Shine–Dalgarno sequence. GAGGA (Fig. 2). Further upstream of the coding region (positions 100–119), the sequence TACTGTCTCAT-TTATACAGGT was identified as a putative SOS box, corresponding to the *E. coli* consensus sequence CTG-N6-CAG that enables binding of the LexA repressor protein (Walker, 1987). This *P. fluorescens* SOS box most closely resembles those reported for *P. aeruginosa* and *Azotobacter vinelandii* (Fig. 3). Only one single base out of 20 is different between these three species. Higher sequence divergence is seen for another *Pseudomonas* species, *P. cepacia*, five and six bases being different from *P. aeruginosa* and *P. fluorescens*, respectively. Surprisingly, the *P. cepacia* SOS box shows closer resemblance to the one from *Bordetella pertussis* (differences at four positions only).

Partially overlapping with the putative repressor-binding region of *P. fluorescens* recA, a putative σ70-type promoter sequence is found between positions 89–116 (Fig. 3). This TTTGTG-NG6-TATAC sequence is similar to known σ70-dependent *Pseudomonas* promoters (Deretic et al., 1989). In the regulatory region of the *recA* gene from *Pseudomonas aeruginosa*, the sequence CTGTGG-NG6-TATAA is present, differing only at the first position (Sano & Kageyama, 1987). Despite the high homology between their SOS boxes (covering the −10 promoter region in both fluorescent *Pseudomonas* species), no obvious *Pseudomonas*-like −35 region appears to occur in *A. vinelandii* (Venkatesh & Das, 1992). However, a related −35/−10 sequence (TTGCA-N6-TATAA) partially overlaps with the SOS box from *P. cepacia* (Nakazawa et al., 1990) and is reminiscent of the motif found in *B. pertussis*: TGCCAG-N6-AGTAC (Favre & Viret, 1990). A promoter region overlapping with a potential LexA binding site also exists in *Vibrio anguillarum* (Tolmasky et al., 1992).

These observations indicate that a LexA-like mediated SOS response is probably operative in several bacteria outside the *Enterobacteriaceae* (Zhao & McEntee, 1990), including fluorescent *Pseudomonas* species. Actually, this was recently shown by introducing a broad-host-range plasmid, carrying an *E. coli* recA-lacZ operon fusion, into various Gram-negative bacteria (Fernandez de Henestrosa et al., 1991). Repression and induction of recA transcription in, respectively, the absence and presence of the DNA damaging agent mitomycin C was demonstrated in, among other bacteria, *P. syringae*. *P.
Fig. 2. DNA sequence and deduced amino acid sequence of the *P. fluorescens* recA gene. The putative ribosome binding site and downstream potential stem-loop structures are underlined. In the 5' regulatory region, the SOS sequence is boxed and the -35/-10 regions of a putative σ70-type of promoter are printed in italic type. The arrow points out the unique BbrPI site (CACGTG) used for insertional inactivation of the gene.

![DNA sequence and deduced amino acid sequence of the *P. fluorescens* recA gene.](image)

Fig. 3. Alignment of the LexA operator sequence, preceding the *Escherichia coli* recA gene (*Ec*), with the putative SOS regulatory elements from *Azotobacter vinelandii* (*Az*), *Borderella pertussis* (*Bp*), *Erwinia carotovora* (*Er*), *Proteus mirabilis* (*Pm*), *Pseudomonas aeruginosa* (*Pa*), *P. cepacia* (*Pc*), *P. fluorescens* (*Pf*), *Rhizobium meliloti* (*Rm*), *Serratia marcescens* (*Sm*) and *Vibrio anguillarum* (*Vu*). The corresponding sequences of *Shigella flexneri* (identical to *E. coli*) and of *Proteus vulgaris* (identical to *P. mirabilis*) are not shown. Boxed regions indicate perfect base conservation throughout currently recognized SOS sequences (Favre & Viret, 1990; Selbitschka et al., 1991; Tolmasky et al., 1992; Venkatesh & Das, 1992).

![Alignment of the LexA operator sequence](image)
aeruginosa and P. putida. From the latter two species, lexA-like genes have now been cloned (Calero et al., 1991). In *Bacillus subtilis*, the putative repressor of the SOS-like SOB regulon recognizes an operator sequence different from the LexA box of *E. coli* (Cheo et al., 1991).

**Deduced amino acid sequence of RecA**

The open reading frame in Fig. 2 encodes a protein of 352 amino acids with a calculated molecular mass of 37634 Da. Since the comparison of RecA sequences by Roca & Cox (1990), several new recA genes have been sequenced, including those from some species belonging to quite distant genera such as *Bacteroides* and *Acholeplasma*. For a detailed comparison of *P. fluorescens* RecA with the currently known corresponding proteins from other Eubacteria, a multiple sequence alignment was carried out for 24 sequences in order to construct a RecA-based dendrogram (Fig. 4). The actual sequence alignment is shown in Fig. 5. In these alignments, the amino acid sequence deduced from the *Mycobacterium tuberculosis* recA locus is not included since it contains a large central non-homologous region of 440 residues (Davis et al., 1991).

Apparently, the extent of sequence homology among RecA proteins reflects well the phylogenetic relationship of the respective bacteria. Indeed, nicely separated clusters were generated for enterobacteria, rhizobia and cyanobacteria. The representatives of Gram-positive bacteria and the related mycoplasmas, as well as of the bacteroides are also well separated (Fig. 4).

The *P. fluorescens* RecA is most similar to the *P. aeruginosa* protein: 87.8% identical residues as compared to 69.5% with *E. coli* RecA (Fig. 4). Surprisingly, RecA from *Azotobacter vinelandii* shares 84.3% of its residues with the *P. fluorescens* protein. As can be deduced from their clustering in Fig. 4, homology between the *Azotobacter* species and *P. aeruginosa* is even higher (89.2% identical amino acids). As mentioned above, this high level of sequence conservation is extended to the upstream regulatory region of these three genes, namely their putative SOS boxes. A substantially lower degree of similarity exists between the *P. fluorescens* and *P. cepacia* RecA proteins (69.8% identity). Conversely, *P. cepacia* RecA shows much better alignment with the *Bordetella pertussis* protein (Fig. 4). The RecA proteins from these species possess as much as 81.2% identical amino acids. As discussed earlier, the rather unexpected clustering of these *Bordetella* and *Pseudomonas* species is again reflected in a high degree of similarity of the respective regulatory regions. These observations add to the uncertainty about the current taxonomic position of *P. cepacia* within the large *Pseudomonas* group.

As more recA genes are sequenced, the extremely high degree of sequence conservation among their gene products (23% overall identity) becomes more striking. For instance, *P. fluorescens* RecA has over 56% residues
in common with distant species such as *Synechococcus* sp. and *Acholeplasma laidlawii* (Fig. 4). In view of these data, it is unlikely that the sequence reported for *Vibrio cholerae* (Ghosh et al., 1992) represents its RecA protein, sharing only 13% of its amino acids with the protein from the rather closely related *E. coli*. Also, no significant alignment with *V. anguillarum* RecA could be achieved. Therefore, the *V. cholerae* sequence was excluded from the multiple alignment and dendrogram building in this paper.

Specific parts of the RecA proteins are most highly conserved (Fig. 5). For the ATP-binding domain proposed by Higgins et al. (1986), the consensus sequence \([I/V][I/V][I/V][Y/F]GPS[S]S/Y[GI/T/L/V]\), corresponding to amino acids 60-74 of *P. JIuorescens* RecA can now be deduced. Another contiguous, conserved portion is found between residues 202 and 222, reflecting the consensus \([F/Y][G/N][A/S][N/S][G/A][N/S][V/I][L/M][K/R]\) which is located in a spacer between two core elements of the proposed three-dimensional structure of *E. coli* RecA (Miller & Kokjohn, 1990). The present compilation of data favours the continued use of part of this sequence (AL...VR) as a RecA signature (Bairoch, 1991). High sequence conservation is also evident in the N-terminal domain with the consensus sequence \([Q/K][I/D/E][K/N][F/Y][G/K/T/S][L/M][R/H]\) (residues 15-27 of *P. fluorescens* RecA), which is essential for interaction with single-stranded DNA and for filament formation (Miller & Kokjohn, 1990). On the other hand, the C-terminal region of *P. fluorescens* RecA shows little sequence homology with its counterparts from other bacteria (Fig. 5). However, it is enriched in acidic residues (7 in a stretch of 13 amino acids), as are most other RecA proteins (Roca & Cox, 1990).

In the deduced consensus sequences, the corresponding regions of the anomalously long RecA from *Mycobacterium tuberculosis* have been included (Davis et al., 1991). Recently, short internal recA fragments from several Gram-positive bacteria and mycoplasmas were isolated by using the polymerase chain reaction and sequenced (Duwat et al., 1992; Dybvig et al., 1992).
Construction and characterization of a recA mutant

The following strategy was used to inactivate the recA gene of P. fluorescens OE 28.3. The 3 kb HindIII fragment was cloned in the unique HindIII site of PBS102. The resulting tetracycline-sensitive clone (pFAJ2058) contained two BbrPI sites, one of which was in the Pseudomonas DNA (Fig. 2). Therefore, the kanamycin resistance cassette, excised from pUKA800 as a 1.25 kb HindIII fragment, could be ligated to partially BbrPI-digested pFAJ2058. The recombinant suicide plasmid (pFAJ2059), with the cassette interrupting the red gene at its unique BbrPI site, was mobilized from E. coli S17-1 into P. fluorescens OE 28.3. Kanamycin-resistant transconjugants were screened for loss of vector-mediated chloramphenicol resistance prior to selecting mutants that had exchanged their wild-type gene with the inactivated red by a double crossover event. For one such strain (FAJ2025), this was unequivocally demonstrated by Southern hybridization experiments with specific probes for PCLB, for the kanamycin cassette, and for the suicide vector used (results not shown).

The recA mutant of P. fluorescens OE 28.3 produced somewhat smaller colonies on solid medium and had a slightly increased doubling time in liquid TSB medium (102 min as compared to 89 min for the wild-type strain).

However, in both reports the aforementioned conserved stretches are not covered by the enzymically amplified regions.

**Fig. 5.** Multiple alignment of RecA proteins from eubacterial species (codes as in Fig. 4). Perfectly conserved positions are marked by asterisks and positions with similar residues (S-T-A; L-V-I-M; K-R; D-E; Q-N; T-S; F-Y-W) occurring in all sequences are indicated by dashes. Boxed consensus sequences are discussed in the text.
mutants generated for *Rhizobium meliloti* and *R. leguminosarum* (Selbitschka et al., 1991).

Strain FAJ2025 was distinctly more sensitive to MMS and substantially less resistant to UV irradiation as compared to the parent strain (Fig. 6), which is typically observed for *recA* mutants (Miller & Kokjohn, 1990).

The relative recombinational activity of the *recA* mutant was estimated by determining the integration frequency of the tetracycline resistance marker from the suicide plasmid pSUP102 carrying a large *Pseudomonas* insert. Frequencies were reduced from $9 \times 10^{-4}$ in the wild type strain to $5 \times 10^{-9}$ in the mutant in case of pFAJ2083 (4 kb insert), and from $5 \times 10^{-4}$ to $4 \times 10^{-9}$ when using pFAJ2084 (5 kb insert). These data clearly demonstrate that the *recA* mutant FAJ2025 is severely impaired in performing homologous recombination.

The recombination-deficient *P. fluorescens* mutant will be useful for further genetic experiments that require a stable maintenance of introduced, plasmid-borne genes to study their involvement in processes such as root adhesion, antibiotic-mediated antagonism of phytopathogens or direct plant growth promotion.


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**References**


