

RecD Plays an Essential Function During Growth at Low Temperature in the Antarctic Bacterium *Pseudomonas syringae* Lz4W

K. Regha,^{1,2} Ajit K. Satapathy¹ and Malay K. Ray³

Centre for Cellular and Molecular Biology, Hyderabad 500007, India

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ABSTRACT

The Antarctic psychrotrophic bacterium *Pseudomonas syringae* Lz4W has been used as a model system to identify genes that are required for growth at low temperature. Transposon mutagenesis was carried out to isolate mutant(s) of the bacterium that are defective for growth at 4° but normal at 22°. In one such cold-sensitive mutant (CS1), the transposon-disrupted gene was identified to be a homolog of the *recD* gene of several bacteria. *Trans*-complementation and freshly targeted gene disruption studies reconfirmed that the inactivation of the *recD* gene leads to a cold-sensitive phenotype. We cloned, sequenced, and analyzed ~11.2 kbp of DNA from *recD* and its flanking region from the bacterium. *recD* was the last gene of a putative *recCBD* operon. The RecD ORF was 694 amino acids long and 40% identical (52% similar) to the *Escherichia coli* protein, and it could complement the *E. coli recD* mutation. The *recD* gene of *E. coli*, however, could not complement the cold-sensitive phenotype of the CS1 mutant. Interestingly, the CS1 strain showed greater sensitivity toward the DNA-damaging agents, mitomycin C and UV. The inactivation of *recD* in *P. syringae* also led to cell death and accumulation of DNA fragments of ~25–30 kbp in size at low temperature (4°). We propose that during growth at a very low temperature the Antarctic *P. syringae* is subjected to DNA damage, which requires direct participation of a unique RecD function. Additional results suggest that a truncated *recD* encoding the N-terminal segment of (1–576) amino acids is sufficient to support growth of *P. syringae* at low temperature.

BACTERIA growing at low temperature are generally grouped into two categories, psychrotrophs and psychrophiles, both having the ability to grow at zero and subzero temperatures (MORITA 1975). The psychrophiles have an optimal growth temperature below 15° and an upper limit of ~20°. The psychrotrophs on the other hand generally grow optimally at temperatures between 20° and 25° with an upper limit of 30°. These bacteria are of particular importance in the global ecology since >80% of the Earth's biosphere is permanently or seasonally subjected to temperatures <5°. Despite constituting the predominant bacterial population on our planet, the unique features that enable these bacteria to cope with such low temperatures are poorly understood (GOUNOT 1991; RAY *et al.* 1998; HEBRAUD and POTIER 1999). We are using an Antarctic psychrotrophic bacterium *Pseudomonas syringae* Lz4W as a model system to understand the molecular basis of cold adaptation (RAY *et al.* 1994a,b, 1998; KANNAN *et al.* 1998; UMA *et al.* 1999; JANİYANI and RAY 2002; SESHUKUMAR *et al.* 2002).

Little information exists at present regarding the

genes that are essential for growth of psychrotrophic bacteria at low temperature. A few genes that might be important for low-temperature growth have been identified mostly from mesophilic bacteria. These include *rbfA* (encoding ribosome-associated factor) and *csdA* (encoding DEAD-box helicase) and the genes for the CspA family of proteins (THIERINGER *et al.* 1998), *pnp* (encoding the polynucleotide phosphorylase that is involved in RNA degradation) (LUTTINGER *et al.* 1996), *hns* (encoding DNA-binding histone-like nucleoid protein) (DERSCH *et al.* 1994), and *bipA* (encoding ribosome-associated GTPase) (PFENNIG and FLOWER 2001). The disruption of the *pnp* gene was found to confer cold sensitivity both in the mesophilic *Escherichia coli* and *Bacillus subtilis* (LUTTINGER *et al.* 1996) and in the psychrotrophic *Yersinia enterocolitica* (GOVERDE *et al.* 1998). In the psychrotrophic *Listeria monocytogenes*, failure to produce the oligopeptide-binding protein, OppA, resulted in cold sensitivity (BOREZEE *et al.* 2000). Due to the lack of information from any highly cold-adapted bacteria, we took a genetic approach to identify gene(s) that are essential for growth at lower temperature in the Antarctic bacterium *P. syringae* Lz4W (SHIVAJI *et al.* 1989). We report here the isolation and characterization of a cold-sensitive mutant (CS1) of *P. syringae* obtained by transposon mutagenesis. The characterization of the mutant suggests that the *recD* gene (AMUNDSEN *et al.* 1986) is essential for low temperature (4°) growth in this bacte-

¹These authors contributed equally to this work.

²Present address: Center of Molecular Medicine, Institute of Microbiology and Genetics, University of Vienna, Vienna Biocenter, Dr. Bohr-gasse 9/4, A1030 Vienna, Austria.

³Corresponding author: Centre for Cellular and Molecular Biology, Uppal Rd., Hyderabad 500007, India. E-mail: malay@ccmb.res.in

TABLE 1
Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Sources/references
<i>Escherichia coli</i>		
MC4100	<i>F</i> ⁻ <i>araD139</i> Δ(<i>argF-lac</i>) <i>U139 rpsL150 thi-1 rbsR relA</i>	CASADABAN (1976)
CAG12135	<i>recD1901::Tn10</i>	BIEK and COHEN (1985)
V66	<i>argA21 hisG4 recF143 met rspL31 galK2 xyl-5 λ⁻ F⁻</i>	AMUNDSEN <i>et al.</i> (2002)
V68	As V66 plus <i>recC73</i>	AMUNDSEN <i>et al.</i> (2002)
V222	As V66 plus <i>recD1013 argA::Tn10</i>	AMUNDSEN <i>et al.</i> (2002)
S17-1	<i>F</i> ⁻ <i>pro recA1 (r⁻ m⁻)</i> RP4-2 integrated (<i>Tc::Mu</i>) (<i>Km::Tn7</i>) [<i>Sm^r T^p</i>] used as mobilizing strain	SIMON <i>et al.</i> (1983)
<i>Pseudomonas syringae</i>		
<i>P. syringae</i> Lz4W	Wild-type, Antarctic isolate	SHIVAJI <i>et al.</i> (1989)
R5	Spontaneous rifampicin-resistant mutant of <i>P. syringae</i> Lz4W	This study
CS1	Cold-sensitive mutant of R5, isolated by <i>Tn5</i> mutagenesis	This study
LDD11	<i>recD</i> -disrupted (at the 3' end) strain of <i>P. syringae</i> Lz4W	This study
LDD22	<i>recD</i> -disrupted (at the 5' end) strain of <i>P. syringae</i> Lz4W	This study
Plasmids		
pOT182	Suicide plasmid for <i>Tn5</i> mutagenesis <i>Tc^r, Ap^r, Cm^r, ColEI</i> replicon	MERRIMAN and LAMONT (1993)
pBluescript SK (+)	Cloning vector, <i>Ap^r</i>	Stratagene (La Jolla, CA)
pSS1	Plasmid obtained by the self cloning of the <i>SalI</i> -digested genomic DNA of CS1; <i>Ap^r</i>	This study
pCSC	Plasmid obtained by the self cloning of the <i>ClaI</i> digested genomic DNA of CS1; <i>Tc^r, Ap^r</i>	This study
pXC6.6	pBluescript SK containing a 6.6-kbp <i>XhoI-ClaI</i> fragment from pCSC	This study
pBB3	3-kbp <i>BamHI</i> DNA fragment from pSS1 containing the IS50L end of <i>Tn5</i> and <i>recD</i> 3'-end region, subcloned into pBluescript	This study
pRD2.4	pBluescript containing a 2.4-kbp <i>SacII-EcoRV</i> DNA fragment with the 3' end of <i>recB</i> and a major part of <i>recD</i>	This study
pRD4.4	pBluescript with a 4.4-kb fragment containing the 3' end of <i>recB</i> , <i>recD</i> , and its downstream region	This study
pGL10	Broad-host cloning vector, IncP replicon, <i>mob⁺ Km^r</i>	BIDLE and BARTLETT (1999)
pKB65	<i>E. coli recD</i> gene cloned in pGL10	BIDLE and BARTLETT (1999)
pGR5	<i>P. syringae recD</i> gene on a 3.8-kbp <i>KpnI</i> fragment cloned in pGL10	This study
pGOR2	pGL10 with the PCR amplified <i>recD</i> gene from <i>P. syringae</i>	This study
pSm08	A 837-bp <i>SmaI</i> fragment of <i>recD</i> in the <i>EcoRV</i> site of pBluescript	This study
pHC07	A 754-bp <i>HincII</i> fragment of <i>recD</i> in the <i>SmaI</i> site of pBluescript	This study
pTc28	2.4-kbp <i>tet</i> gene block from pOT182 cloned in pBluescript	This study
pDD1	pSm08 interrupted by Tc cassette at the <i>EcoRV</i> site	This study
pDD2	pHC07 interrupted by Tc cassette at the <i>SmaI</i> site	This study

rium. Unlike the mesophilic *E. coli*, the cold-sensitive *recD* mutants of *P. syringae* are sensitive to DNA-damaging agents, such as UV and mitomycin C, and the mutant cells accumulate DNA fragments at 4°, indicating that the RecD of this psychrotroph might have a vital role in DNA repair, especially during growth at low temperature.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions: The bacterial strains and plasmids used in this study are listed in Table 1. The psychrotrophic *P. syringae* Lz4W was isolated from a soil sample of Schirmacher Oasis, Antarctica (SHIVAJI *et al.* 1989) and routinely grown at 22° or 4° (for high and low temperatures, respectively) in Antarctic bacterial medium (ABM)

composed of 5 g liter⁻¹ peptone and 2.5 g liter⁻¹ yeast extract as described earlier (JANIYANI and RAY 2002). *E. coli* strains were cultured at 37° in Luria-Bertani (LB) medium, which contained 10 g liter⁻¹ tryptone, 5 g liter⁻¹ yeast extract, and 5 g liter⁻¹ NaCl. For solid media, 15 g liter⁻¹ bacto-agar (Hi-Media, India) was added to ABM or LB. When necessary, the LB medium was supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹), or tetracycline (20 µg ml⁻¹) for *E. coli*. For *P. syringae*, the ABM was supplemented with tetracycline (30 µg ml⁻¹), carbenicillin (1000 µg ml⁻¹), rifampicin (100 µg ml⁻¹), or kanamycin (50 µg ml⁻¹), when required.

For determination of generation time, the bacterial cells from overnight culture were inoculated into fresh medium at a dilution of 1:100, and the optical density of the cultures at 600 nm (OD₆₀₀) was measured at various time intervals.

Recombinant DNA methods: General molecular biology techniques including isolation of genomic DNA, cloning, Southern

hybridization, polymerase chain reaction (PCR), etc., were performed as described by SAMBROOK *et al.* (1989). Plasmids were introduced into *P. syringae* either by electroporation or by conjugation. The electroporation was carried out with a 40 μ l cell suspension, using a BTX electroporator at 15 kV cm⁻¹ (25 μ F and 186 ohm). The conjugal transfer of plasmid into *P. syringae* was carried out by a biparental mating method using the *E. coli* strain S17-1, as described (SIMON *et al.* 1983). Transconjugants were selected by their resistance to appropriate antibiotics.

Pulsed-field gel electrophoresis: Conditions for pulsed-field gel electrophoresis (PFGE) were as described (THOMS and WACKERNAGEL 1998; HANDA and KOBAYASHI 2003) with modifications. Typically, bacterial cells were harvested when the cultures reached an OD₆₀₀ of ~0.6 and embedded in 1% LGT agarose (FMC Bioproducts, Rockland, ME) blocks, each block containing ~10⁷–10⁸ cells. Electrophoresis was performed with the CHEF-DRII System (Bio-Rad, Hercules, CA) at a constant voltage (180 V) and increasing pulse time of 5–35 sec over a period of 16 hr at 14°. Molecular size markers used were concatemers of λ DNA, yeast chromosomes, and λ DNA *Hind*III digests (New England Biolabs, Beverly, MA).

DNA sequence analysis: Sequencing reactions were carried out using double-stranded plasmid DNA as templates and the ABI PRISM Dye terminator cycle sequencing method (Perkin-Elmer, Norwalk, CT) and analyzed on an automated DNA sequencer (ABI model 377). Analyses of DNA sequences were performed with BLAST (ALTSCHUL *et al.* 1997) and other programs available on the web server (<http://www.ncbi.nlm.nih.gov/>). The nucleotide sequence of the *P. syringae recD* gene was deposited in the GenBank/EMBL database (accession no. AY078390).

Transposon mutagenesis and isolation of cold-sensitive mutants: Mutagenesis of *P. syringae* was carried out with a *Tn5*-transposon-based suicide plasmid vector (pOT182), as described earlier (MERRIMAN and LAMONT 1993). Briefly, a rifampicin-resistant mutant (R5) of *P. syringae* was used as a recipient for conjugation with the donor strain *E. coli* S17-1 (pOT182). The transconjugants were selected on ABM-agar supplemented with rifampicin (100 μ g ml⁻¹) and tetracycline (30 μ g ml⁻¹) as described (KANNAN *et al.* 1998). For isolation of the cold-sensitive mutants, the transconjugants were patched in duplicates on ABM-agar plus rifampicin; one set was incubated at 22° and the other at 4°. Cold-sensitive mutants were identified by their inability to grow on the plates incubated at 4° for 15 days. Wild type grows within 2–3 days on ABM-agar plates at 4°.

Cloning of the *Tn5* insertion site from CS1 and the *recD* region from *P. syringae*: The region flanking the *Tn5* insertion in CS1 (Figure 1) was cloned by plasmid rescue, utilizing the self-cloning property of *Tn5*-OT182 (MERRIMAN and LAMONT 1993). Briefly, genomic DNA from the CS1 strain was digested with *Sal*I and self-ligated to rescue the plasmid pSS1, which contained the IS50L proximal region of transposon on the genome. A similar self-cloning strategy using *Cla*I was employed to clone the IS50R proximal sequence of *Tn5* from the mutant on the plasmid pCSC. A *Tn5*-specific outwardly directed primer (5'-ACTTGTGTATAAGAGTCAG-3') was initially used to obtain the DNA sequence flanking IS50L, which led to identification of the transposon-disrupted *recD* gene. Subsequently, subclones were generated from the inserts of the above plasmids in pBluescript SK for various analyses. For example, a 3-kbp DNA fragment from the *Bam*HI-digested pSS1 was subcloned to generate pBB3 for characterization of the 3' end of the *recD* gene and its downstream sequence. The *Xho*I-*Cla*I fragment from pCSC was subcloned in pBluescript SK to generate the pXC6.6 for DNA sequence analysis.

To characterize the *recD* region from the wild-type strain of *P. syringae*, a 2.4-kbp DNA fragment, which was positive for

the probe from the 3' end of the *recD* gene (derived from the plasmid pBB3), was cloned in pBluescript SK (designated as pRD2.4). The *recD* sequence on insertion of the plasmid pRD2.4 overlapped with the sequences derived from pBB3 and pXC6.6 of CS1. Altogether, the DNA sequence of the *recD* region from *P. syringae* was initially determined and assembled from the plasmids pXC6.6, pRD2.4, pBB3, and pSS1, which together spanned a region of ~9 kbp of the DNA segment containing the incomplete *recC* gene. However, with the availability of genome sequences from different *Pseudomonas* sp., the 5' end upstream of *recC* was amplified from genomic DNA with the help of primers composing the 3' end of the flanking 5S rRNA gene (FP: 5'-GTAGGTCATCGTCAAGA-3') and the *recC*-specific primer (RP: 5'-GGCTTCACGTACTACGG-3'), cloned, and sequenced. Altogether, an ~11.2-kb DNA segment from the *recD* region of *P. syringae* Lz4W was characterized by a combination of PCR-based amplification and cloning of overlapping restriction fragments, which span the region (Figure 1A).

Genetic complementation analysis: For complementation analysis, the *recD* gene of *P. syringae* was cloned as a 3.8-kbp *Kpn*I fragment in a broad-host-range plasmid vector, pGL10 (BIDDLE and BARTLETT 1999). The resulting plasmid (pGR5) was transferred into the cold-sensitive strain CS1 by conjugation. Genetic complementation was also carried out with another plasmid, pGOR2, which contained the *recD* gene on a 2.0-kbp PCR-amplified DNA from *P. syringae*, without any downstream region.

Directed disruption of *recD* in wild-type *P. syringae*: Directed disruption of the *recD* gene in a wild-type background was carried out with the nonreplicative plasmid (pBluescript SK) containing DNA fragments internal to the *recD* gene of *P. syringae* and a tetracycline (Tc) resistance cassette within the DNA fragment. Two such disruption plasmid vectors, pDD1 and pDD2, were generated in the present study. For construction of pDD1, an 837-bp internal *Sma*I fragment from the 3' end of the *recD* gene was cut out from pRD2.4 and cloned into the *Eco*RV site of pBlueScript SK to generate pSm08. Then, a 2.4-kbp Tc cassette was cloned into the internal *Eco*RV site of pSm08 to generate pDD1. For construction of the disruption vector pDD2 a 754-bp *Hinc*II internal fragment from the 5' end of the *recD* gene was initially cloned into the *Sma*I site of pBluescript SK to create pHc07. This was followed by cloning of the Tc cassette into the *Sma*I site of the *Hinc*II fragment.

The plasmids, pDD1 and pDD2, were introduced separately into wild-type *P. syringae* by electroporation, and the mutants with chromosomal integrants were selected by plating them onto ABM-agar supplemented with tetracycline. The *recD*-disrupted strains generated by homologous recombination of pDD1 and pDD2 into the genomes (Figure 1C) were designated as LDD11 and LDD22, respectively. The site of integration of the tetracycline cassette was confirmed by Southern and PCR analyses. A ³²P-labeled 0.837-kbp *Sma*I DNA fragment from the *recD* gene of *P. syringae* was used as a probe for Southern hybridization. For PCR analyses of LDD11- and LDD22-related disruptions, primer sets were: 5'-CGTIT GCCGCTGGACGTGCTGGTG-3' and 5'-CACCGTTTCCACATCATTGAGGCG-3' and 5'-CGGGATCCGAGTCGTTTCATTTGCCGAG-3' and 5'-TTGCAGACTGATGGACTCG GTCAA-3', respectively.

λ -phage plaque size assay: The ability of the *recD* homolog of *P. syringae* to complement the *recD* mutation of *E. coli* (MC4100 *recD::Tn10*) was determined by plaque size assay of the mutant λ -phage MMS805 (*rec⁻ gam⁻*) on an agar plate using a published method (CHAUSSEE *et al.* 1999). The *E. coli* strain MC4100 *recD::Tn10* was constructed by P1 transduction of the mutation from *E. coli* CAG12135 (JENSEN 1993). The diameters of plaques were measured using a microscope (Wild M3Z; Heerbrugg, Switzerland) fitted with an occludometer in the eyepiece. The data

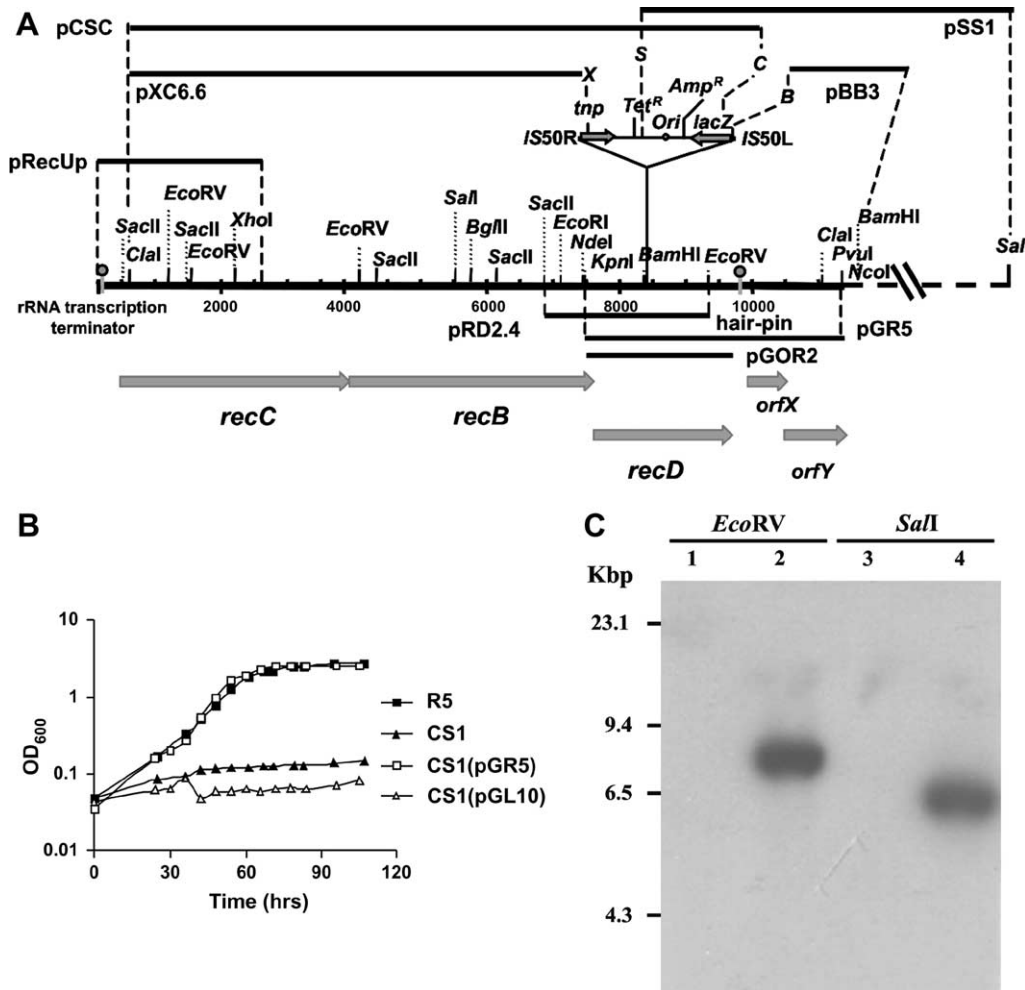


FIGURE 1.—Physical and genetic maps of the *recD* region from *P. syringae* and the effect of *recD* disruption on growth at low temperature (4°C). (A) The thick solid line represents a physical map of the ~11.2-kbp DNA fragment of the region. A few important restriction sites are shown on the map. The insertion site of the transposon Tn5-OT182 in the mutant CS1 is shown above the line. The locations of *recB*, *recC*, *recD*, and two hypothetical ORFs (*orfX* and *orfY*) are indicated as thick shaded arrows below the line. The direction of the arrows is the direction of transcription of the genes. The dashed portion of the line indicates that the DNA from the region has not been sequenced. Plasmids with different DNA fragments used for characterization of the *recD*-spanning region are indicated as bars above and below the physical map. The upstream rRNA transcription terminator and a hairpin structure downstream of *recD*, which might work as a transcription terminator, are shown. (B) The growth at 4°C was monitored by measuring turbidity

at OD₆₀₀. R5, rifampicin-resistant parent strain of *P. syringae*; CS1, *recD*-disrupted mutant of R5. The growth profiles of CS1 with pGR5 containing the *recD* gene of *P. syringae* and the empty plasmid vector (pGL10) are also indicated. (C) Southern analysis of the *recD*-disrupted strain CS1. The *EcoRV*- and *SalI*- (that cuts only once within the transposon) digested genomic DNAs from the parent strain R5 (lanes 1 and 3) and the mutant strain CS1 (lanes 2 and 4) were hybridized with a ³²P-labeled transposase gene (*tnp*)-specific probe and analyzed.

were presented as the mean diameter (millimeters) ± SEM. A minimum of 100 plaques from each plate were measured. MMS805 (*red⁻ gam⁻*) was kindly provided by Frank Stahl (University of Oregon).

Determination of efficiency of plating of T4 2⁻ phages: The efficiency of plating (EOP) of T4 2⁻ phages in *E. coli* strains V66 (*argA21 hisG4 recF143 met rspL31 galK2 xyl5 λ⁻ F⁻*) and V222 (V66 with *recD1013 argA::Tn10*) was determined as described (AMUNDSEN *et al.* 2002). The *E. coli* strains and phage stocks were received from Gerald Smith's laboratory (Fred Hutchinson Cancer Research Center, Seattle).

Assay for sensitivity to UV and mitomycin C: UV sensitivity was determined by spreading an appropriate dilution of the wild-type and CS1 strains onto ABM-agar plates and exposing them to UV (20 J m⁻²). The plates were incubated at 22°C in the dark by wrapping them with aluminum foil. The number of colonies developed on unirradiated plates was taken as 100% for calculation of survivability. Sensitivity to mitomycin C was determined by spreading appropriate dilutions of the cell culture in log phase (OD₆₀₀ ~ 0.5) onto ABM-agar plates supplemented with various concentrations of mitomycin C and counting the number of colonies (CFU) appearing on the plates. The viable count on plates containing no mitomycin C was taken as 100%.

Assays for cell death: Cell death upon transfer to low temperature was monitored by spreading appropriately diluted cultures of the *P. syringae* strains on ABM-agar plates and incubating the plates at 4°C for different periods. On different days, two to three plates were taken out from 4°C, incubated at 22°C for growth, and the colonies (CFU) on plates were counted to determine the number of surviving cells. The CFU at 22°C on day zero was taken as 100%. Cells were also microscopically examined by staining with DAPI (5 μg ml⁻¹), or with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR), using a fluorescent microscope (Carl Zeiss, Germany) with appropriate filters. The kit utilizes mixtures of SYTO 9 and propidium iodide as nucleic acid-binding fluorescent stains. When used alone, the SYTO 9 generally labels all bacteria in a population with intact or damaged membranes, like DAPI. In contrast, propidium iodide (PI) penetrates only bacteria with damaged membranes (dead cells), causing a reduction in the SYTO 9 fluorescence when both dyes are present. Thus, live and dead cells (green and red fluorescing, respectively) can be distinguished in a population. Cell size was measured from phase contrast images using an axiovision ver. 3.1 software provided with the Zeiss microscope (Axioplan 2 imaging). A minimum of 200 cells from ~10–20 visual fields were used for the calculation of cell size.

Other methods: SDS-PAGE and transfer of proteins onto PVDF membrane for Western analyses were carried out as described (SAMBROOK *et al.* 1989), using a semidry blotting apparatus (Bio-Rad). The protein blots were probed with *E. coli* RecD-specific monoclonal antibody (kindly carried out by Susan Amundsen, Fred Hutchinson Cancer Research Center).

RESULTS

Isolation and characterization of cold-sensitive mutant CS1 of *P. syringae*: To identify gene(s) that are essential for growth at a very low temperature, we set out to isolate and characterize a cold-sensitive mutant of the Antarctic psychrotrophic bacterium *P. syringae* Lz4W that is defective for growth at 4° but normal at 22°, the optimum growth temperature of the bacterium. From a library of ~1500 transconjugants, generated by random insertional mutagenesis of Tn5-OT182, 10 putative cold-sensitive mutants were initially obtained. On subsequent analysis, only one of the mutants exhibited a severe cold-sensitive phenotype, which was designated as CS1 and chosen for further studies (Figure 1). The growth rates of CS1 were compared to that of its isogenic parent R5 at 22° and 4° in liquid broth by measuring the OD₆₀₀ of the cultures. At 22°, the R5 and CS1 strains grew with a generation time of 2.0 and 2.1 hr, respectively. At 4°, R5 grew with an average generation time of 6.8 hr and reached stationary phase in ~110 hr. The growth of the CS1 at 4° was, however, severely inhibited (Figure 1B).

By Southern hybridization analysis, using a part of the transposase gene (*tnp*) of Tn5-OT182 as probe, it was confirmed that CS1 has a single chromosomal integration of the transposon in the genome (Figure 1C). A segment of DNA flanking the Tn5 was then cloned from CS1 by rescuing it on the plasmid pSS1 (Figure 1A) and ~700 bp DNA sequence flanking the IS50L of the transposon was determined. A BLAST analysis revealed that the sequence had a high degree of homology to the *recD* gene of *E. coli* (FINCH *et al.* 1986) and several other bacteria, suggesting that the transposon-disrupted gene in CS1 is most probably a homolog of *recD*.

Cloning, sequencing, and analysis of the *recD* region from *P. syringae*: For an unambiguous identification of the disrupted gene in CS1, the full-length *recD* gene and its upstream and downstream regions were cloned and sequenced from *P. syringae*. A total of 11.199 kbp DNA sequence spanning the *recD* region of *P. syringae* (Figure 1A) was determined (GenBank accession no. AY078390). BLAST analysis of the sequence led to the identification of genes for three complete ORFs with homology to the RecD, RecB, and RecC proteins, respectively, from several bacteria. The corresponding genes (*recD*, *recB*, and *recC*) belonged to the same operon, with the direction of transcription being from *recC* through *recB* and *recD*. A potential hairpin structure with a predicted free energy (ΔG) of -13.2 kCal was located 107 nucleotides downstream of the translational stop codon of the putative *recD* gene (Figure 1A), which might work as a transcription termination signal for the

recCBD operon. At the upstream of *recC* was the gene for 5S rRNA of a putative rRNA operon, similar to the gene organization observed in the plant pathogen *P. syringae* pv. tomato (BUELL *et al.* 2003). The intergenic distance between the end of the transcription termination signal of the rRNA operon and the "ATG" start codon of the *recC* gene was 245 bp. A putative promoter with a -10 site (TGATAA) and a -35 site (AATACT) was identified within the intergenic region, 39 bp upstream of the start codon (ATG) of *recC*. The start codons of *recB* and *recD* overlapped with the stop codon "TAA" of *recC*, and the "TGA" of *recB* by one and four nucleotides, respectively. The three genes *recC*, *recB*, and *recD* transcribed together in a single transcript (A. K. SATAPATHY and M. K. RAY, unpublished results).

The observed gene organization (*recCBD*) of *P. syringae* Lz4W was similar to that of the Pseudomonads group of species (STOVER *et al.* 2000; BUELL *et al.* 2003; NELSON *et al.* 2003) but unlike that of *E. coli* (FINCH *et al.* 1986), where a protease III gene (*ptr*) occurs between *recB* and *recC* in the genome. The downstream region of *recD* was, however, variable among the Pseudomonas species. For example, two ORFs (*orfX* and *orfY*) with homology to the hypothetical proteins, PA1121 (59% identical) and PA1120 (50% identical) of *P. aeruginosa* (accession nos. E83505 and D83505, respectively) occurred downstream in both *P. syringae* Lz4W and *P. syringae* pv. tomato, but *P. aeruginosa* itself contained genes for a probable exonuclease (PA4282) and SbcD exonuclease (PA4281) on the complementary strand downstream.

The RecD homolog of *P. syringae* Lz4W is 694 amino acids long and displayed maximum similarity (65–78%) with homologs from other Pseudomonads [*P. syringae* pv. tomato (BUELL *et al.* 2003), *P. putida* (NELSON *et al.* 2003), and *P. aeruginosa* (STOVER *et al.* 2000)] but modest homology to others: *Yersinia pestis* (652 aa, 41%; acc. no. NP_404635.1), *E. coli* (608 aa, 40%), and *Photobacterium profundum*, a piezophilic deep sea bacterium (701 aa, 39%) (BIDDLE and BARTLETT 1999). While most of the similarity was observed in the C-terminal region containing conserved DNA helicase motifs (HALL and MATSON 1999), the N-terminal region (~200 aa) varied considerably among bacteria. Analysis also indicated that the putative RecB and RecC of *P. syringae* are 1226 and 1149 amino acids long, respectively. These two proteins are also highly similar to their putative counterparts (54–79% and 63–81% similar, respectively) from other Pseudomonad species.

Complementation of *recD* mutations by the cloned *recD* gene of *P. syringae*: To confirm that the cold-sensitive phenotype of CS1 is only due to inactivation of the *recD* gene and not due to the polar effect of Tn5 insertion on the downstream genes, a genetic *trans*-complementation analysis was carried out. On introduction of the plasmid pGR5 containing the wild-type *recD* gene, CS1 cells regained the colony-forming ability at 4°. The growth profiles of CS1 (pGR5) in liquid culture at 22° and 4° were similar to that of the parental R5 (Figure 1B).

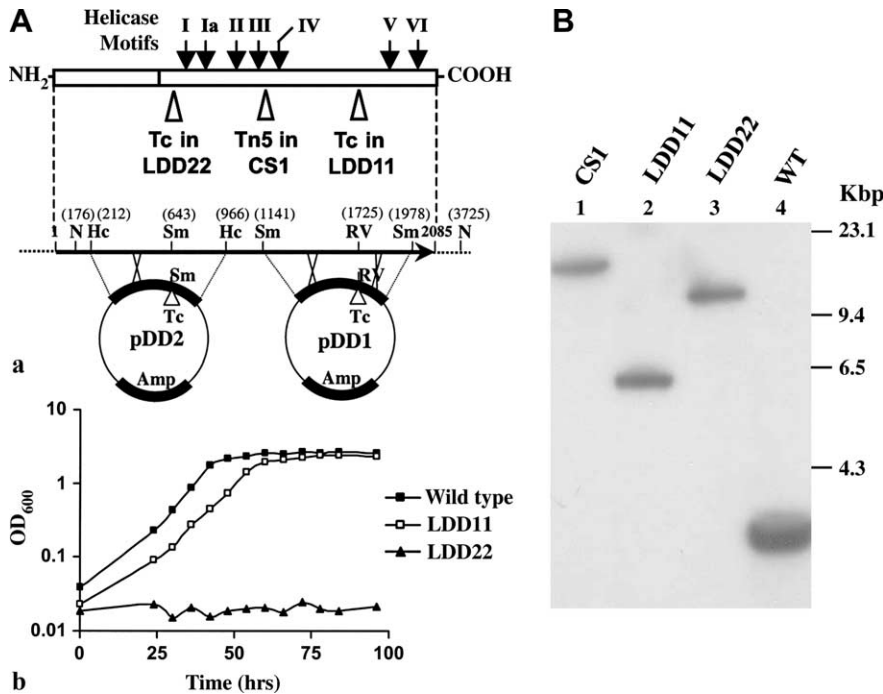


FIGURE 2.—(A) Targeted disruption of the *recD* gene by tetracycline cassette (Tc). (A, a) The plasmids (pDD2 and pDD1) used for disruption of RecD at N-terminal and C-terminal ends, respectively, are shown. The Tc cassette in the *Sma*I site of an internal fragment from the 5' end of *recD* in pDD2 recombined with the genomic *recD* to produce the mutant LDD22. Similarly, the Tc cassette in the *Eco*RV site of the 3' end fragment in pDD1 recombined with the genomic *recD* to generate LDD11. The restriction sites indicated are: Hc, *Hinc*II; N, *Nco*I; RV, *Eco*RV; and Sm, *Sma*I. The numbers on genomic *recD* refer to the nucleotides in the *recD* reading frame. The locations of helicase motifs on the RecD reading frame are indicated. (A, b) Growth profiles of *recD*-disrupted mutants (LDD11 and LDD22) and wild-type *P. syringae* at 4°. (B) Southern blot analysis of genomic DNA from LDD22 and LDD11 strains. Lanes 1–4 contain *Nco*I digests of genomic DNA from the indicated strains. The ³²P-labeled probe was a 0.837-kbp *Sma*I DNA fragment from the *recD* of *P. syringae*. An expected increase in the size of the *Nco*I fragment from the *recD* region in different strains is seen.

Although the above result suggested that the *recD* gene on plasmid pGR5 was possibly responsible for restoration of growth of CS1 at 4°, the plasmid contained an additional ~1.6-kbp DNA from the downstream of the *recD* gene containing two hypothetical genes (*orfX* and *orfY*). To examine whether complementation of the cold-sensitive phenotype is not due to these hypothetical genes it was necessary to complement the cold-sensitive phenotype with the *recD* gene alone. For this, the plasmid pGOR2 containing the *recD* gene without the downstream ORFs was introduced into CS1. The mutant regained the ability to grow at 4°, both on an ABM-agar plate (Figure 3a) and in liquid broth (data not shown), indicating that the *recD* gene is solely responsible for restoring the growth at low temperature.

For further confirmation of the essential role of the *recD* gene during growth at low temperature, two more *recD*-disrupted strains of *P. syringae* were constructed in a wild-type genetic background. This would also rule out, in principle, any possible role of the parental *Rif*^r allele in cold sensitivity of the CS1 strain. Two *recD*-disrupted strains, LDD11 and LDD22, were generated by homologous recombination between genomic DNA and the suicide plasmids pDD1 and pDD2, respectively (Figure 2A). The integration of the Tc in LDD11 and LDD22 was confirmed by Southern hybridization (Figure 2B) and PCR amplification analyses, using appropriate probes and primers (data not shown). The analyses suggested that LDD22 and LDD11 were generated by a single and a double crossover, respectively, as shown

in Figure 2A. The tetracycline cassette in pDD1 and pDD2 was placed immediately after the codons for the 576th and 214th amino acids, respectively, which would produce upon translation 82 and 28% of the full-length RecD (henceforth referred to as *recD*^{1–576} and *recD*^{1–214}). It is, however, to be noted that the integration of pDD2, which occurred by a single crossover into the genome of LDD22 (Figure 2), could also lead to the production of an N-terminally truncated protein of almost full-length RecD (shorter by ~71 amino acids) if expressed from any plasmid-encoded promoter. Such a possibility was thought unlikely due to the lack of Shine-Dalgarno sequence for translation initiation and a chance in-frame fusion with any upstream reading frame of the plasmid.

The growth profiles of the LDD11 and LDD22 strains were then examined at low temperature (4°). It was observed that LDD11 with *recD*^{1–576} could grow at 4°, but not LDD22 with *recD*^{1–214} (Figure 2A). In the CS1 mutant, the transposon was inserted in the 373rd amino acid codon, keeping the N-terminal 1–373 amino acids of RecD undisturbed. It, therefore, appears that the C-terminal 577–694 amino acids of RecD might not be crucial for growth of *P. syringae* at 4°. It can also be inferred that the *Rif*^r allele of the CS1 strain does not have any role in the cold-sensitive phenotype.

Complementation of the *recD* mutation of *E. coli* with the *P. syringae recD* gene: To examine whether the *recD* homolog of *P. syringae* could complement the RecD function in *E. coli*, two independent assay methods, the

TABLE 2
Plaque diameters formed by the λ -phage MMS805 on *E. coli* strains

Strains infected with MMS805	Plaque diameter (mm \pm SEM)
MC4100 (<i>recD</i> ⁺)	0.656 \pm 0.044
MC4100 <i>recD</i> ::Tn10	1.310 \pm 0.061
MC4100 (pGL10, empty)	0.646 \pm 0.042
MC4100 (pKB65 with <i>E. coli recD</i>)	0.630 \pm 0.047
MC4100 (pGR5 with Lz4W <i>recD</i>)	0.755 \pm 0.061
MC4100 <i>recD</i> ::Tn10 (pGL10, empty)	1.394 \pm 0.052
MC4100 <i>recD</i> ::Tn10 (pKB65 with <i>E. coli recD</i>)	0.707 \pm 0.042
MC4100 <i>recD</i> ::Tn10 (pGR5 with Lz4W <i>recD</i>)	0.762 \pm 0.025

plaque size assay with λ MMS805 (*recD*⁻ *gam*⁻) (CHAUSSEE *et al.* 1999; THALER *et al.* 1989) and the T4 2⁻ phage multiplication assay (AMUNDSEN *et al.* 2002) were employed. The results of the first method showed that when *E. coli* strain MC4100 (*recD*::Tn10) was transformed with the *recD* genes from *P. syringae* (on pGR5) and *E. coli* (on pKB65), the transformants produced smaller λ -plaques, similar to *E. coli* with the *recD*⁺ gene (Table 2). Similarly in the second method, *recD* of both *P. syringae* and *E. coli* when expressed in *trans* from pGR5 and pKB65 reduced the EOP of T4 2⁻ phage on the *E. coli* V222 strain (*recD*⁻) (Table 3). Thus, the RecD homolog of *P. syringae* had the ability to complement exonuclease deficiency of the *recD* mutants of *E. coli*.

***E. coli recD* fails to complement the cold-sensitive phenotype of CS1:** In a reciprocal experiment when the *E. coli recD* gene was introduced into CS1, the gene could not complement the cold-sensitive phenotype of the mutant *P. syringae*. The CS1 (pKB65) failed to grow at 4° (Figure 3a). This lack of complementation was not due to the inability of the *E. coli recD* gene to express in the heterologous *P. syringae* strain, as evidenced by

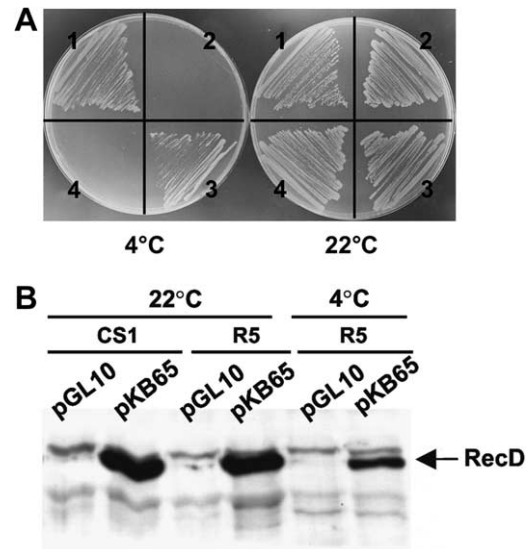


FIGURE 3.—(a) Genetic *trans*-complementation of cold-sensitive growth of CS1. The complementation was carried out with the following plasmids: (1) pGOR2, with only the *recD* gene of *P. syringae*; (2) pGL10, a basic vector without insert; (3) pGR5, with a 3.8-kbp insert containing *recD* and its downstream region; and (4) pKB65, with the *recD* gene from *E. coli*. The growth of the strains on ABM-agar plates incubated at 22° (2 days) and 4° (7 days) is shown. (b) Expression of the *E. coli* RecD protein in *P. syringae* at low (4°) and high (22°) temperatures. Whole-cell lysates (30 μ g of protein) from the indicated strains were separated by SDS-PAGE and analyzed by Western blot hybridization, using a monoclonal antibody against *E. coli* RecD. Samples from 22°- and 4°-grown cells of R5 and CS1 with indicated plasmids are shown.

Western analyses of the proteins of CS1 (pKB65), using a monoclonal antibody (mAb) against *E. coli* RecD (Figure 3b). Therefore, the lack of complementation could be due to a functional requirement of RecD, which is missing in the *E. coli* protein. The possibilities of improper folding of the *E. coli* RecD and/or assembly defect with RecBC proteins of *P. syringae* also could not be ruled out in this study. In another experiment, how-

TABLE 3

Complementation of the *E. coli recD* mutant by *P. syringae recD* using a T4 2⁻ phage titration

<i>E. coli</i> strain	Plating efficiency of phage T4 2 ^{-a}	Plating efficiency of phage T4 ^b
V66 (<i>recD</i> ⁺)	9.1 \times 10 ⁻³	1.43
V66 (pGL10, empty)	9.0 \times 10 ⁻³	0.9
V66 (pKB65 with <i>E. coli recD</i>)	1.0 \times 10 ⁻²	1.43
V66 (pGR5 with Lz4W <i>recD</i>)	1.1 \times 10 ⁻²	1.6
V222 (<i>recD</i> 1013 <i>argA</i> ::Tn10)	1.0	1.0
V222 (pGL10, empty)	0.68	1.1
V222 (pKB65 with <i>E. coli recD</i>)	6.9 \times 10 ⁻³	0.66
V222 (pGR5 with Lz4W <i>recD</i>)	8.5 \times 10 ⁻³	1.36

^a T4 2⁻ phage titer on the indicated strain divided by the titer on V222.

^b T4 phage titer on the indicated strain divided by the titer on V222.

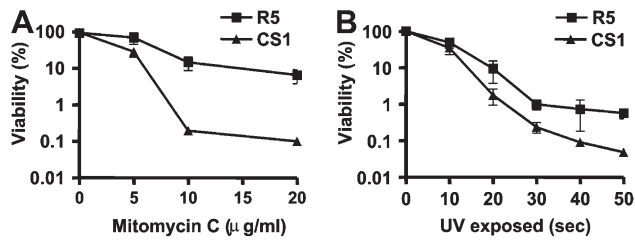


FIGURE 4.—Sensitivity *P. syringae* cells to DNA-damaging agents mitomycin C (a) and UV (b). Assays were carried out at 22° as described in MATERIALS AND METHODS with the cold-sensitive strain CS1 and isogenic parent R5.

ever, to find out whether *E. coli* RecD functions at all in *P. syringae*, we examined the UV sensitivity of CS1 in the presence of *E. coli* *recD* (on pKB65) at 22°. We did not observe any UV resistance conferred by pKB65 as seen with *P. syringae* *recD* on pGR5 (our unpublished observation). Thus, absence of the cognate subunits of RecBCD enzyme, rather than the low temperature *per se*, might also be responsible for the lack of complementation in *P. syringae*.

***recD* mutants of *P. syringae* are sensitive to DNA-damaging agents and accumulate DNA fragments in the cell:** RecD functions as a part of the RecBCD complex or exonuclease V that is involved in homologous recombination and repair of double-strand DNA breakage (DSB) in bacteria (KUZMINOV 1999; KOWALCZYKOWSKI 2000; Cox 2001). However, the inactivation of *recD* in *E. coli* does not confer sensitivity to DNA-damaging agents as do the mutations in *recB* and *recC* genes (AMUNDSEN *et al.* 1986; KUZMINOV 1999), possibly due to the functioning of the RecBC RecJ-dependent pathway (LOVETT *et al.* 1988). Surprisingly, a *recD* mutant of *P. syringae* (CS1) was found to be modestly sensitive to DNA-damaging agents, such as UV and mitomycin C (Figure 4, a and b). Among the Tc-cassette-disrupted *recD* mutants, LDD11 and LDD22, which were examined for UV sensitivity, only LDD22 was sensitive (data not shown). Altogether, the data indicated that RecD might play a role in the DNA repair process, especially during growth of *P. syringae* at low temperature. To confirm this, PFGE was carried out to examine the status of chromosomal DNA in *P. syringae* cells. Remarkably, the cold-sensitive strains CS1 and LDD22 were found to accumulate a size class of DNA fragments (~25–30 kbp) upon transfer to 4° (Figure 5, a and b). These strains on complementation with pGR5 grew well and did not accumulate this DNA in cells at 4°. DNA fragments were also not detected in the cells growing at 22°. It was also noted that the accumulation of this DNA of chromosomal origin decreases at ~144 hr in CS1 cells (Figure 5b), when the suppressors start growing in the culture (see below). The cold-sensitive LDD22 also exhibited a similar pattern of cold sensitivity, accumulation of DNA fragments,

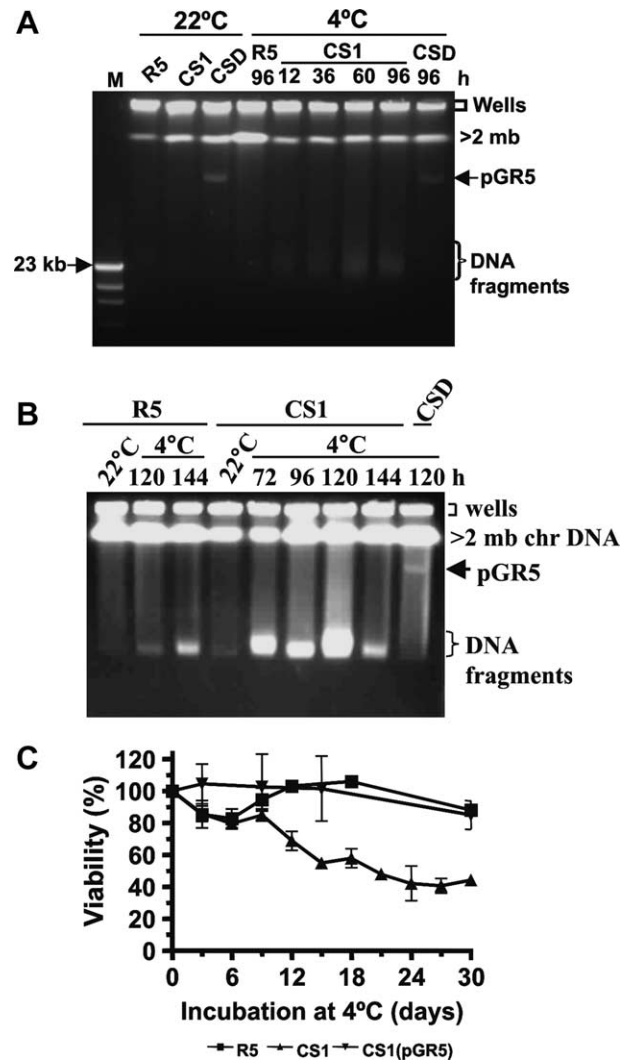


FIGURE 5.—Analyses of DNA damage and cell viability of *P. syringae* strains. (a and b) PFGE analyses of DNA were carried out with the cold-sensitive strain CS1, isogenic parent R5, and CS1 complemented with pGR5 (labeled as CSD). The cells were grown at 22° and subsequently shifted to 4° for the indicated periods of time. Wells containing DNA, noncircular chromosomal DNA (labeled as >2 Mb) that enters the gel, the damaged genomic DNA fragments, and the 23-kb λ -*Hind*III fragment (lane M) are indicated. Note the anomalous mobility of circular plasmid pGR5 (12.3 kb) in PFGE (HANDA and KOBAYASHI 2003). (a) Analysis with cells that were incubated for 0, 12, 36, and 96 hr at 4°. Each lane in this experiment contained DNA equivalent to $\sim 10^7$ cells. (b) Analysis with cells that were incubated for 72, 96, 120, and 144 hr at 4°. Each lane in this experiment contained DNA equivalent to $\sim 5 \times 10^7$ cells. (c) The cell viability as assessed by colony-forming ability (CFU) of the strains CS1, R5, and CSD following transfer to 4° is shown.

and generation of suppressors in the culture (data not shown).

Cell death of *recD*-inactivated mutants at low temperature: Parallel monitoring of viable cells in the cultures of *recD*-inactivated strains (CS1 and LDD22) was carried out by measuring colony formation ability (Figure 5c)

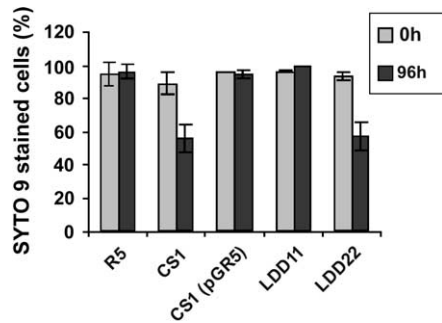


FIGURE 6.—Estimation of viable cells in the cultures of *recD* mutants at 4°, using the LIVE/DEAD BacLight staining kit. The bars represent the percentage of SYTO 9-stained live cells in the cultures following their shift to 4° for 96 hr. Data are shown for cold-sensitive *P. syringae* strain CS1, isogenic parent R5, CS1 complemented with *recD* on pGR5, and LDD22 and LDD11 containing *recD*¹⁻²¹⁴ and *recD*¹⁻⁵⁷⁶, respectively.

and live/dead staining of cells (Figure 6). It was observed that the cell viability decreases in CS1 and LDD22 following transfer of the cultures from 22° to 4°. By comparing the time of first visible appearance of DNA fragments in cells by PFGE analysis (Figure 5a) and the drop in colony-forming units (~3 days) it would appear that the accumulation of this DNA precedes cell death in the mutants at low temperature. Live/dead staining indicated that the number of dead cells stained by propidium iodide in CS1 and LDD22 started increasing after ~12 hr of the temperature shift (data not shown).

One striking feature of the data on cell survival at 4° is that the number of survivors in the culture was large (~50%) in most experiments (Figures 5c and 6). This is remarkable for the fact that most of the decrease in cell number takes place within 24–36 hr of the shift to the low temperature. These nongrowing cells in the liquid culture start growing only after 5–6 days, probably due to accumulation of suppressor mutations. PFGE analysis of these growing cells from a 144-hr postshift culture showed a remarkably dramatic decrease in the accumulation of DNA fragments inside the cells (Figure 5b). These putative suppressor mutants upon fresh culturing grew like wild-type isogenic parents, both at low (4°) and high (22°) temperatures (data not shown). Southern analysis of six independent clones isolated from CS1-derived suppressor mutants indicated that the mutants have retained a transposon in the original site of insertion. The experiments suggest that there is a very high rate of accumulation of suppressor mutations in the cold-sensitive *recD* mutants during their growth at low temperature.

We also examined the morphological change associated with the *recD* mutation in *P. syringae* (Figure 7). Although the cell sizes were variable among different strains, the average cell size of CS1 and LDD22 was larger when compared with the cell size of wild type, R5, or the complemented CS1 (pGR5) strains at 4°. But

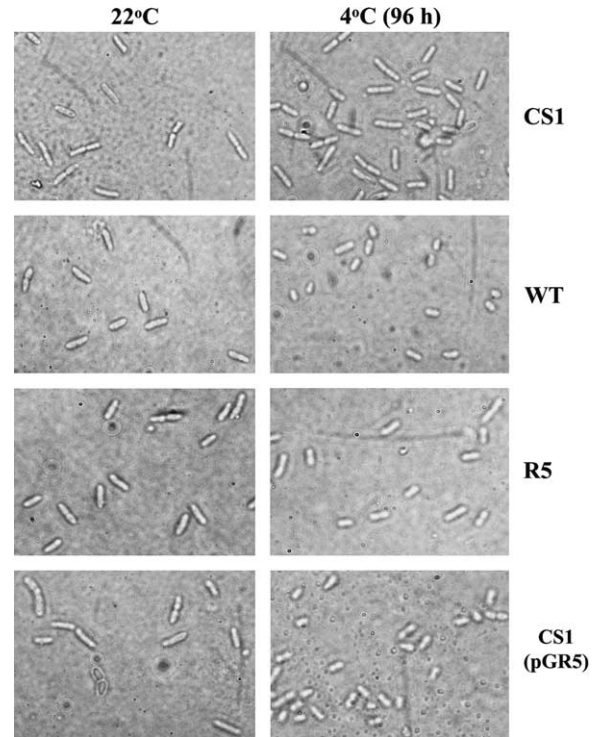


FIGURE 7.—Change in cell size of *P. syringae* at lower temperature. Cultures were shifted from 22° to 4° and the cells were examined before and after 96 hr of shift under a phase contrast microscope. Note the lack of reduction in cell size of CS1 at the lower temperature.

the cell size of all these strains at 22° was similar, except for that of LDD11 and LDD22, which was marginally bigger (Table 4). It appears that a general reduction of cell size that is observed in *P. syringae* at low temperature does not occur in the cold-sensitive *recD* mutants (CS1 and LDD22), which accumulate DNA fragments. A relatively larger cell size of the *recD*-disrupted LDD11 (com-

TABLE 4
Change in cell size of wild-type and *recD* mutants of *P. syringae* at low temperature (4°)

Strain	Length × width (μm)	
	0 hr	96 hr
Lz4W (WT, <i>recD</i> ⁺)	2.8 ± 0.22	1.36 ± 0.24
R5 (<i>Rif</i> ^r <i>recD</i> ⁺)	1.01 ± 0.10	0.65 ± 0.09
CS1 (<i>Rif</i> ^r <i>recD</i> ::Tn5)	2.8 ± 0.3	1.7 ± 0.22
CS1 (pGR5 with <i>recD</i> ⁺)	1.09 ± 0.23	0.65 ± 0.08
LDD11 (<i>recD</i> ¹⁻⁵⁷⁶)	0.9 ± 0.16	0.93 ± 0.11
LDD22 (<i>recD</i> ¹⁻²¹⁵)	2.9 ± 0.30	1.43 ± 0.06
	1.01 ± 0.15	0.64 ± 0.06
	3.56 ± 0.35	2.0 ± 0.32
	0.9 ± 0.10	0.99 ± 0.12
	3.5 ± 0.26	3.24 ± 0.30
	1.0 ± 0.09	1.1 ± 0.19

pared to the wild type), which was not cold sensitive, could not be correlated to any specific phenotype in this study. Remarkably, however, the cells in CS1 and LDD22 cultures after 5–6 days at 4°, when the suppressor cells start growing, show the reduction in size (data not shown).

DISCUSSION

This study was undertaken to identify gene(s) that are essential for growth of the Antarctic *P. syringae* at 4°. The study identifies unequivocally the essentiality of the *recD* gene for growth at low temperature. The results also suggest that *recD* probably participates in a crucial step of the DNA damage repair process during growth at 4°. It is believed that DNA lesions occur in normally growing cells due to oxidative damage caused by the action of hydroxyl radicals and other reactive oxygen species (KEYER *et al.* 1995). The primary lesions (nicks) in DNA turn into DSBs at the replication forks, which are normally repaired by RecBCD-dependent RecA-mediated homologous recombination. The RecBCD-dependent reestablishment of the replication fork plays a major role in the origin-independent initiation of chromosomal replication and cell viability (KOWALCZYKOWSKI 2000; COX 2001; LOVETT 2003). A RecA-independent role for RecBCD was also suggested by suppression of chromosomal lesions via degradation of linear DNA at reversed replication forks, which affects cell viability (MIRANDA and KUZMINOV 2003).

Enzymatically, the RecBCD complex processes DNA breaks for repair by its coordinated ATP-dependent helicase and nuclease activities, in association with the regulatory Chi sequence and RecA recombinase (KUZMINOV 1999). Although all the subunits of RecBCD contribute toward activities of the complex, RecD in *E. coli* does not exhibit any nuclease activity *in vitro*; it shows only ssDNA-dependent ATPase activity (CHEN *et al.* 1998) and 5' → 3' helicase activity (DILLINGHAM *et al.* 2003; TAYLOR and SMITH 2003). The essentiality of RecD helicase activity during growth of bacteria is not clear at present. However, it is interesting to note that the *recD* mutant, LDD11, has the ability to grow at 4°, but contains a Tc insertion in *recD*, which would prevent translation of the C-terminal 576–694 amino acids containing the helicase motifs V and VI of RecD (Figure 2). These two conserved motifs were shown earlier to have a role in communication between the DNA-binding and ATP-binding sites of the helicases for translocation along DNA (HALL and MATSON 1999). From the present study, it appears that these two motifs might not be important for RecD function in *P. syringae* during growth at low temperature.

The exonuclease activity of RecD *in vivo*, however, is important in cellular physiology as shown by the ability of *recJ*-encoding exonuclease to functionally complement the *recD* mutants of *E. coli* and *Salmonella* sp. for survival

in their hosts (CANO *et al.* 2002). It is believed that most of the DNA damage generates single-stranded tails of a length that prevents loading of RecBCD (THOMS and WACKERNAGEL 1998). These tails are effectively removed by single-strand-specific DNases (*e.g.*, exonuclease I, RecJ, sbcCD nuclease, etc.), and the processed tailed ends work as the entry sites for RecBCD enzyme (THOMS and WACKERNAGEL 1998; BURDETT *et al.* 2001). In this context, our observation that the *recD* inactivation of cold-adapted *P. syringae* leads to accumulation of a class of short DNA species is significant. Although further studies are needed to know the nature and origin of these DNA, we hypothesize that this might be related to a defect in the DNA damage/repair process. This is consistent with the data that *recD* mutants of *P. syringae* are sensitive to DNA-damaging agents, such as UV and mitomycin C. It is possible that the exposure of *P. syringae* cells to low temperature causes an increase in the amount of DNA damage, probably due to more reactive oxygen species generated by a slower rate of respiration (KEYER *et al.* 1995; SMIRNOVA *et al.* 2001). Since the CS1 lacks functional *recD*, the damaged DNA are not processed or efficiently repaired, thus resulting in cold sensitivity. At higher temperature (22°) another gene with overlapping function might complement the *recD* deficiency, as exemplified by the temperature-dependent expression of *rad52* and *rad55* in the yeast *Saccharomyces cerevisiae* (GASIOR *et al.* 2001). In this context, it is important to note that *recD* inactivation leads to a pressure-sensitive phenotype in the piezophilic bacterium, *Ph. profundum* (BIDLE and BARTLETT 1999). Whether the physical factor such as pressure, like low temperature, leads to DNA damage needs investigation.

Three important aspects of our observations need to be addressed in our future studies. First, Is the RecD requirement of the Antarctic *P. syringae* during growth at low temperature a function of the RecBCD complex, or the function of an as yet unknown complex of RecD, or a novel function of the RecD alone? Studies on *recB* and *recC* mutants of *P. syringae* have been hindered so far by our inability to get the mutations by a gene disruption method. We are at present trying to generate conditional mutations of these two genes. Simultaneously, we are using a recombinant 6× His-tagged RecD of *P. syringae* to find out its interactive partner proteins by Ni²⁺-agarose pull-down assay. A second issue concerns the size class of ~25- to 30-kbp DNA fragments, which is observed in PFGE analysis of the *recD* mutants at low temperature. Our analysis so far suggests that it is not due to mechanical shearing of DNA during processing of cells for PFGE. In addition, we noted that, although the major intensity of the DNA fragments centers around the ~25- to 30-kbp region on agarose gels, a DNA smear of lower fluorescence intensity also extends to a larger molecular size region in the gels. Third, the suppressors that accumulate in the cultures at 4° need investigation, which is likely to shed new light on the *recD* requirement

for growth at low temperature. Additionally, the explanation that the gene of overlapping function with *recD* fulfills the requirement of RecD during growth at higher temperature (22°), but does not express or becomes ineffective at lower temperature (4°), requires experimental proof. Toward this, we are attempting to analyze the role of candidate genes, such as *recJ* and *xonA* homologs of *P. syringae*, which are known to complement *recD* mutation in other bacteria. We are also investigating the biochemical activities of purified RecD from the Antarctic bacterium to search for a novel property, if any. Thus, this study has thrown up a plethora of questions, especially on the nature of the stress that causes DNA damage in cells at low temperature and on the activity of RecD that is required for alleviation of such stress in cold-adapted bacteria.

To conclude, this study for the first time identifies *recD* as an essential gene for growth at low temperature in a highly cold-adapted bacterium, *P. syringae*, and establishes that the low-temperature requirement of RecD in the Antarctic psychrotroph is related to its direct role in damaged-DNA processing.

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LITERATURE CITED

- ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHAFER, J. ZHANG, Z. JIANG *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389–3402.
- AMUNDSEN, S. K., A. F. TAYLOR, A. M. CHAUDHURY and G. R. SMITH, 1986 *recD*: the gene for an essential third subunit of exonuclease V. *Proc. Natl. Acad. Sci. USA* **83**: 5558–5562.
- AMUNDSEN, S. K., A. F. TAYLOR and G. R. SMITH, 2002 A domain of RecC required for assembly of the regulatory RecD subunit into the *Escherichia coli* RecBCD holoenzyme. *Genetics* **161**: 483–492.
- BIDLE, K. A., and D. H. BARTLETT, 1999 RecD function is required for high-pressure growth of a deep-sea bacterium. *J. Bacteriol.* **181**: 2330–2337.
- BIEK, D. P., and S. N. COHEN, 1985 Identification and characterization of *recD*, a gene affecting plasmid maintenance and recombination in *Escherichia coli*. *J. Bacteriol.* **167**: 594–603.
- BOREZEE, E., E. PELLEGRINI and P. BERCHE, 2000 OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. *Infect. Immun.* **68**: 7069–7077.
- BUELL, C. R., V. JOARDAR, M. LINDBERG, J. SELENGUT, I. T. PAULSEN *et al.*, 2003 The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* **100**: 10181–10186.
- BURDETT, V., C. BAITINGER, M. VISWANATHAN, S. T. LOVETT and P. MODRICH, 2001 *In vivo* requirement for RecJ, ExoVII, ExoI, and ExoX in methyl-directed mismatch repair. *Proc. Natl. Acad. Sci. USA* **98**: 6765–6770.
- CANO, D. A., M. G. PUCCIARELLI, F. G. PORTILLO and J. CASADESUS, 2002 Role of RecBCD recombination pathway in *Salmonella* virulence. *J. Bacteriol.* **184**: 592–595.
- CASADABAN, M. J., 1976 Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* **104**: 541–555.
- CHAUSSEE, M. S., J. WILSON and S. A. HILL, 1999 Characterization of the *recD* gene of *Neisseria gonorrhoeae* MS11 and the effect of *recD* inactivation on pilin variation and DNA transformation. *Microbiology* **145**: 389–400.
- CHEN, H. W., D. E. RANDLE, M. GABBIDON and D. A. JULIN, 1998 Functions of the ATP hydrolysis subunits (RecB and RecD) in the nuclease reactions catalyzed by the RecBCD enzyme from *Escherichia coli*. *J. Mol. Biol.* **278**: 89–104.
- COX, M. M., 2001 Recombinational DNA repair of damaged replication forks in *Escherichia coli*: questions. *Annu. Rev. Genet.* **35**: 53–82.
- DERSCH, P., S. KNEIP and E. BREMMER, 1994 The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol. Gen. Genet.* **245**: 255–259.
- DILLINGHAM, M. S., M. SPIES and S. C. KOWALCZYKOWSKI, 2003 RecBCD enzyme is a bipolar DNA helicase. *Nature* **423**: 893–897.
- FINCH, P. W., A. STOREY, K. BROWN, E. D. HICKSON and P. T. EMMERSON, 1986 Complete nucleotide sequence of *recD*, the structural gene for the alpha subunit of exonuclease V of *Escherichia coli*. *Nucleic Acids Res.* **14**: 8583–8594.
- GASIOR, S. L., H. OLIVARES, U. EAR, D. M. HARI, R. WEICHELBAUM *et al.*, 2001 Assembly of RecA-like recombinase: distinct roles for mediator proteins in mitosis and meiosis. *Proc. Natl. Acad. Sci. USA* **98**: 8411–8418.
- GOUNOT, A. M., 1991 Bacterial life at low temperature: physiological aspects and biotechnological implications. *J. Appl. Bacteriol.* **71**: 386–397.
- GOVERDE, R. L. J., J. H. J. VELD, J. G. KUSTERS and F. R. MOOI, 1998 The psychrotrophic bacterium *Yersinia enterocolitica* requires expression of *pnp*, the gene for polynucleotide phosphorylase, for growth at low temperature (5° C). *Mol. Microbiol.* **28**: 555–569.
- HALL, M. C., and S. W. MATSON, 1999 Helicase motifs: the engine that powers DNA unwinding. *Mol. Microbiol.* **34**: 867–877.
- HANDA, N., and I. KOBAYASHI, 2003 Accumulation of large non-circular forms of the chromosome in recombination-defective mutants of *Escherichia coli*. *BMC Mol. Biol.* **4**: 5.
- HEBRAUD, M., and P. POTIER, 1999 Cold shock response and low temperature adaptation in psychrotrophic bacteria. *J. Mol. Microbiol. Biotechnol.* **1**: 211–219.
- JANIYANI, K. L., and M. K. RAY, 2002 Cloning, sequencing, and expression of the cold-inducible *hutU* gene from the Antarctic psychrotrophic bacterium *Pseudomonas syringae*. *Appl. Environ. Microbiol.* **68**: 1–10.
- JENSEN, K. F., 1993 The *Escherichia coli* 'wild types' W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *ppvE* expression levels. *J. Bacteriol.* **175**: 3401–3407.
- KANNAN, K., K. L. JANIYANI, S. SHIVAJI and M. K. RAY, 1998 Histidine utilisation operon (*hut*) is upregulated at low temperature in the Antarctic psychrotrophic bacterium *Pseudomonas syringae*. *FEMS Microbiol. Lett.* **161**: 7–14.
- KEYER, K., A. S. GORT and J. A. IMLAY, 1995 Superoxide and the production of oxidative DNA damage. *J. Bacteriol.* **177**: 6782–6790.
- KOWALCZYKOWSKI, S. C., 2000 Initiation of genetic recombination and recombination dependent replication. *Trends Biochem. Sci.* **25**: 156–165.
- KUZMINOV, A., 1999 Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage λ . *Microbiol. Mol. Biol. Rev.* **63**: 751–813.
- LOVETT, S. T., 2003 Connecting replication and recombination. *Mol. Cell* **11**: 554–556.
- LOVETT, S. T., C. LUISI-DELUCA and R. D. KOLODNER, 1988 The genetic dependence of recombination in *recD* mutants of *Escherichia coli*. *Genetics* **120**: 37–45.
- LUTTINGER, A., J. HAHN and D. DUBNAU, 1996 Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Mol. Microbiol.* **19**: 343–356.
- MERRIMAN, T. R., and I. L. LAMONT, 1993 Construction and use of a self-cloning promoter probe vector for Gram-negative bacteria. *Gene* **126**: 17–23.

- MIRANDA, A., and A. KUZMINOV, 2003 Chromosomal lesion suppression and removal in *Escherichia coli* via linear DNA degradation. *Genetics* **163**: 1255–1271.
- MORITA, R. Y., 1975 Psychrotrophic bacteria. *Bacteriol. Rev.* **39**: 144–167.
- NELSON, K. E., C. WEINEL, I. T. PAULSEN, R. J. DODSON, H. HILBERT *et al.*, 2003 Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **41**: 799–808.
- PFENNIG, P. L., and A. M. FLOWER, 2001 BipA is required for growth of *Escherichia coli* K12 at low temperature. *Mol. Genet. Genomics* **266**: 313–317.
- RAY, M. K., G. SESHU KUMAR and S. SHIVAJI, 1994a Phosphorylation of lipopolysaccharides in the Antarctic psychrotroph *Pseudomonas syringae*: a possible role in temperature adaptation. *J. Bacteriol.* **176**: 4243–4249.
- RAY, M. K., G. SESHU KUMAR and S. SHIVAJI, 1994b Phosphorylation of membrane proteins in response to temperature in an Antarctic *Pseudomonas syringae*. *Microbiology* **140**: 3217–3223.
- RAY, M. K., G. SESHU KUMAR, K. JANİYANI, K. KANNAN, P. JAGATAP *et al.*, 1998 Adaptation to low temperature and regulation of gene expression in Antarctic psychrotrophic bacteria. *J. Biosci.* **23**: 423–435.
- SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SESHU KUMAR, G., M. V. JAGANNADHAM and M. K. RAY, 2002 Low temperature-induced changes in the composition and fluidity of lipopolysaccharides in the Antarctic psychrotrophic bacterium *Pseudomonas syringae*. *J. Bacteriol.* **184**: 6746–6749.
- SHIVAJI, S., N. SHYAMALA, L. RAO, L. SAISREE, V. SHETH *et al.*, 1989 Isolation and identification of *Pseudomonas* sp. from Schirmacher Oasis, Antarctica. *Appl. Environ. Microbiol.* **55**: 767–770.
- SIMON, R., U. B. PRIEFER and A. PUHLER, 1983 A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**: 784–791.
- SMIRNOVA, G. V., O. N. ZAKIROVA and O. N. OKTIABRSKII, 2001 Role of antioxidant systems in the cold stress response of *Escherichia coli*. *Mikrobiologiya* **70**: 55–60.
- STOVER, C. K., X. Q. PHAM, A. L. ERWIN, S. D. MIZOGUCHI, P. WARRNER *et al.*, 2000 Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959–964.
- TAYLOR, A. F., and G. R. SMITH, 2003 RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* **423**: 889–893.
- THALER, D. S., E. SAMPSON, I. SIDDIQI, S. M. ROSENBERG, L. C. THOMASON *et al.*, 1989 Recombination of bacteriophage λ in *recD* mutants of *Escherichia coli*. *Genome* **31**: 53–67.
- THIERINGER, H. A., P. G. JONES and M. INOUE, 1998 Cold shock and adaptation. *BioEssays* **20**: 49–57.
- THOMS, B., and W. WACKERNAGEL, 1998 Interaction of RecBCD enzyme with DNA at double-strand breaks produced in UV-irradiated *Escherichia coli*: requirement for DNA end processing. *J. Bacteriol.* **180**: 5639–5645.
- UMA, S., R. S. JADHAV, G. SESHU KUAMR, S. SHIVAJI and M. K. RAY, 1999 A RNA polymerase with transcriptional activity at 0°C from the Antarctic bacterium *Pseudomonas syringae*. *FEBS Lett.* **453**: 313–317.

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