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Degenerate Oligonucleotide Primers for Enzymatic Amplification of *recA* Sequences from Gram-Positive Bacteria and Mycoplasmas

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RecA protein in gram-negative bacteria, especially in *Escherichia coli*, has been extensively studied, but little is known about this key enzyme in other procaryotes. Described here are degenerate oligonucleotide primers that have been used to amplify by the polymerase chain reaction (PCR) *recA* sequences from several gram-positive bacteria and mycoplasmas. The DNA sequences of *recA* PCR products from *Streptococcus pyogenes*, *Streptococcus mutans*, *Enterococcus faecalis*, and *Mycoplasma pulmonis* were determined and compared. These data indicate that the *M. pulmonis recA* gene has diverged significantly from *recA* genes of other eubacteria. It should be possible to use cloned *recA* PCR products to construct *recA* mutants, thereby providing the means of elucidating homologous genetic recombination and DNA repair activities in these organisms.

The *recA* gene product is a key protein involved in DNA recombination and repair. In *Escherichia coli*, it is required for all pathways of homologous recombination and is essential for the initiation of the SOS response after DNA damage (3, 12, 21, 26). The *recA* gene from a variety of gram-negative bacteria has been studied (18), but little is known about *recA* in gram-positive bacteria other than *Bacillus subtilis*. In *B. subtilis*, RecA protein apparently regulates a global network analogous to the SOS response of *E. coli* (13, 14). The *B. subtilis recA* gene (formerly referred to as *recE*) has been cloned, and its sequence has extensive homology with those of gram-negative bacterial *recA* genes (25).

Using the *B. subtilis recA* gene as a hybridization probe, we have recently cloned the *recA* gene from the mycoplasma *Acholeplasma laidlawii* (8). Although mycoplasmas (class *Mollicutes*) lack cell walls, they are phylogenetically related to gram-positive bacteria (16, 27). The predicted amino acid sequence of the entire *A. laidlawii recA* gene product has 68% identity with the corresponding amino acids in the *B. subtilis* RecA protein, suggesting that these proteins are functionally very similar.

The initial goal of the present study was to isolate the *recA* gene from the murine pathogen *Mycoplasma pulmonis*. It has previously been shown that this organism is capable of incorporating DNA into its chromosome by homologous recombination (15). However, attempts in our laboratory to detect *recA*-like sequences in *M. pulmonis* by using the *B. subtilis* and *A. laidlawii recA* genes as hybridization probes were not successful. Therefore, an alternative approach for isolation of *recA* sequences, involving the use of the polymerase chain reaction (PCR), was explored. Because of the relative ease of this approach, the goal of this study was expanded to include *recA* sequences from several gram-positive bacteria.

The organisms used in this study were *A. laidlawii* 8195 (24), *M. pulmonis* KD735 (7), *Mycoplasma mycoides* subsp. *mycoides* GM9 (6), *Streptococcus mutans* UA96 (1), *Streptococcus pyogenes* D471 (23), *Streptococcus pneumoniae* 851 (obtained from S. Lacks), *Lactococcus lactis* subsp. *lactis* MG1363 (10), *Enterococcus faecalis* OGIX (11), *E. faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213. All organisms were grown at 37°C. *A. laidlawii* and *M. pulmonis* were grown in mycoplasma medium (5), and gram-positive bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Mycoplasmal DNA was isolated by lysing cells with sodium dodecyl sulfate (SDS) and extracting DNA with phenol as described elsewhere (4). For gram-positive bacteria, cells (10 ml) were centrifuged and resuspended in 200 μl of buffer consisting of 10 mM Tris (pH 8.0) and 50 mM EDTA. A 200-μl volume of nonionic detergent solution (0.45% Tween 20, 0.45% Nonidet P-40) containing 24 μg of proteinase K was added, and the mixture was incubated at 56°C for 1 h. Lysis was completed by the addition of 40 μl of a 10% (wt/vol) solution of SDS, and DNA was isolated by following the protocol used for mycoplasmal DNA.

The *recA* genes from *B. subtilis* (25), *A. laidlawii* (8), and *E. coli* (22) were aligned to identify conserved regions that could serve as candidate sites for binding of PCR primers. Conserved regions chosen for study encoded amino acid residues 14 to 20, 90 to 96, 117 to 123, 187 to 192, and 199 to

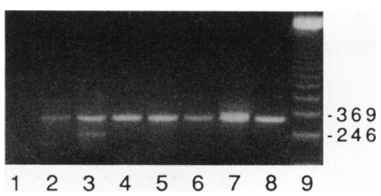

FIG. 1. Ethidium bromide-stained 2% agarose gel of DNAs amplified with *recA* primers. Lanes: 1, no template DNA; 2, *M. pulmonis*; 3, *E. faecalis*; 4, *S. pneumoniae*; 5, *S. aureus*; 6, *L. lactis*; 7, *S. mutans*; 8, *A. laidlawii*. Lane 9 contains as a marker the 123-bp molecular weight ladder available from GIBCO/BRL Life Technologies Inc., Gaithersburg, Md. The sizes of two of the marker DNAs (in base pairs) are shown in the right margin.

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FIG. 2. Alignment of gram-positive bacterial and mycoplasmal *recA* sequences. The top line contains nucleotides 268 through 576 of the *B. subtilis* (Bs) *recA* gene (the A nucleotide in the ATG initiation codon was assigned the number 1). Sequences which served as primer-binding sites for PCR amplification are underlined. Aligned to the *B. subtilis* sequence are *recA* sequences from *E. faecalis* (Ef), *S. pyogenes* (Sp), *S. mutans* (Sm), *A. laidlawii* (Al), *M. pulmonis* (Mp), and *E. coli* (Ec). Dots (•) in the sequence refer to nucleotides that are identical to the corresponding nucleotide in *B. subtilis*.

205 of the *B. subtilis* RecA protein. Various combinations of pairs of degenerate oligonucleotide primers specific for these regions were used to amplify DNA from a variety of gram-positive bacteria and mycoplasmas. Amplifications with the primers specific for DNA sequences encoding amino acids regions 90 to 96 (left primer) and 187 to 192 (right primer) yielded particularly impressive results; the desired PCR product was obtained from all species examined, and these results are the focus of this study. Combinations of primer pairs specific for the other conserved regions were not pursued beyond initial experiments (data not shown), indicating that they could amplify *recA* sequences from only a limited number of species.

DNAs were enzymatically amplified by using the Programmable Thermal Cycler (MJ Research, Watertown, Mass.) and Taq polymerase and buffer (Stratagene Cloning Systems, La Jolla, Calif.). Reaction mixtures (in 100-μl volumes) included 100 pmol of each degenerate oligonucleotide primer, as described previously (2). DNA primers were supplied by the Oligonucleotide Synthesis Core Facility,

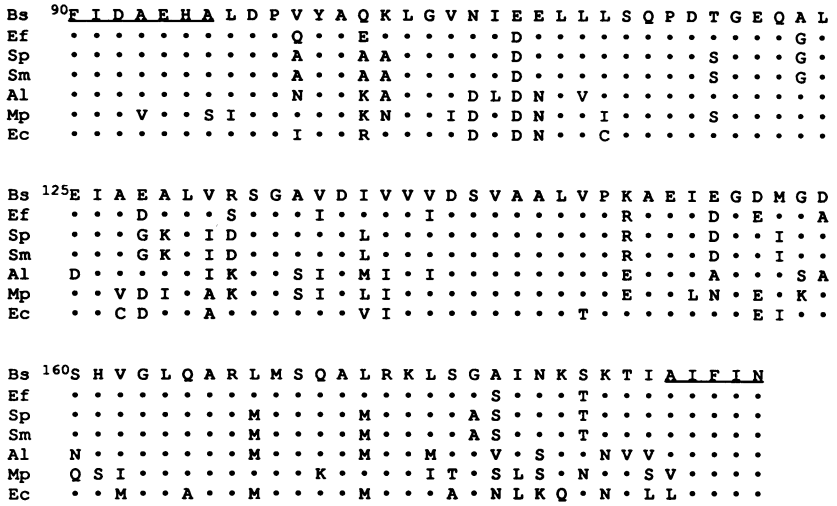

FIG. 3. Alignment of amino acid sequences of RecA from gram-positive bacteria and mycoplasmas. Amino acids sequences were deduced from the DNA sequences shown in Fig. 2. The first amino acid shown in the *B. subtilis* sequence is the 90th amino acid of this protein. Amino acids deduced from the primer-binding sites are underlined. Dots (•) in the sequence refer to amino acids that are identical to the corresponding amino acid in *B. subtilis*. See the legend to Fig. 2 for organism abbreviations.

TABLE 1. Percent identity and base composition of gram-positive bacterial *recA* nucleotides and encoded amino acids

Organism	Nucleotide and amino acid identity with[a]:							G+C[b]
	B. subtilis	*E. faecalis*	*S. pyogenes*	*S. mutans*	*A. laidlawii*	*M. pulmonis*	*E. coli*	
B. subtilis	100	71	69	68	67	61	67	49
E. faecalis	**86**	100	73	70	69	64	58	44
S. pyogenes	**81**	**83**	100	84	70	62	62	44
S. mutans	**81**	**83**	**100**	100	69	61	61	42
A. laidlawii	**72**	**73**	**75**	**70**	100	61	59	39
M. pulmonis	**64**	**66**	**62**	**62**	**64**	100	56	33
E. coli	**75**	**70**	**70**	**70**	**66**	**64**	100	58

[a] Nucleotide comparisons are shown above the diagonal (plain type), and amino acid comparisons are shown below the diagonal (boldface type).
[b] The base composition (in % G+C) of each DNA sequence is shown.

University of Alabama, Birmingham. The left primer was 5′-CGTAAGCTTYATHGAYGCNGARCAYGC-3′, and the right primer was 5′-CTCAAGCTTGRTTDATRAADATN GC-3′ (sequences not homologous to *recA* and incorporated into the primers for cloning purposes are underlined). The temperature profile of the first three PCR cycles was 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C with a slow ramp time (2 min) for adjustment of the temperature from 37 to 72°C. After these initial cycles, reaction mixtures were subjected to another 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C.

PCR products were initially analyzed on 2% agarose gels, and several of the PCR products were cloned into *E. coli* vectors (usually plasmid pUC18 [19] was used) for further analysis and DNA sequencing. For cloning convenience, a *Hin*dIII recognition site had been incorporated into the PCR primers. Therefore, PCR products were digested overnight with an excess of *Hin*dIII and ligated into alkaline phosphatase-treated, *Hin*dIII-digested vector DNA. The *E. coli* host for most of the cloning experiments was strain JM103 (17). DNA sequencing was performed by the dideoxynucleotide-chain termination method, with a double-stranded DNA template and the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). In all cases, both strands of DNA were sequenced.

By using the primers described above, we amplified DNAs from *M. pulmonis*, *E. faecalis*, *S. pneumoniae*, *S. aureus*, *S. lactis*, *S. mutans*, and *A. laidlawii*. All of these PCR reactions resulted in a 323-bp product, the correct size for the expected *recA* fragment (Fig. 1). Similar results were obtained when DNAs from *S. pyogenes* and *M. mycoides* subsp. *mycoides* were amplified (data not shown), but the yield of the desired PCR product from *M. mycoides* subsp. *mycoides* was relatively low.

The 323-bp PCR products from *S. mutans*, *S. pyogenes*, *M. pulmonis*, and *E. faecalis* OGIX were cloned, and the DNA sequence of each fragment was determined. An alignment of these DNA sequences with the *recA* sequences from *E. coli*, *B. subtilis*, and *A. laidlawii* is shown in Fig. 2. Although these sequences are clearly related, the high level of homology between them is most evident when the encoded amino acid sequences are similarly aligned (Fig. 3). From these data, we conclude that the 323-bp PCR products are indeed *recA* fragments.

The percent identity between the *recA* nucleotides and between the encoded amino acids for each species examined is given in Table 1. The *S. mutans* and *S. pyogenes* sequences were most closely related: 84% of their nucleotides and 100% of their amino acids were identical. The *E. coli* and *M. pulmonis* nucleic acid sequences were the most dissimilar, partially owing to differences in base composition (Table 1). *M. pulmonis* had the most divergent amino acid sequence, which is something of a surprise because this organism is phylogenetically much more closely related to the gram-positive bacteria than is *E. coli*. The *M. pulmonis* sequence was even significantly divergent from the sequence from *A. laidlawii*, the other mycoplasma used in this study.

One way to compare the RecA sequences aligned in Fig. 3 is to examine amino acids that tend to be conserved across genera. Of the 102 aligned amino acids, 49 are invariant. Another 15 amino acids are conserved in six of the seven organisms. In these 15 cases, the organism with the variant amino acid was *M. pulmonis* seven times, *A. laidlawii* four times, *E. coli* three times, and *B. subtilis* once. These data are similar to comparisons of base conservation between rRNA genes from numerous microorganisms. Mycoplasmal rRNA genes tend to have more variation in what are usually conserved bases than do other bacteria, giving rise to the suggestion that mycoplasmas are evolving more rapidly than other procaryotes (27).

The isolation of an internal fragment of *recA* should provide a means of constructing *recA* mutants of species for which genetic transformation methods have been developed. The internal *recA* fragment, e.g., a PCR product, when combined with an appropriate selectable marker on a plasmid that does not replicate in gram-positive bacteria (or mycoplasmas), could be used to insertionally inactivate the *recA* gene by plasmid integration mutagenesis (20). The resulting *recA* mutant should be stable because the integration event (in the absence of RecA) would be irreversible. By using this approach, *recA* mutants of *A. laidlawii* have recently been constructed (9).

Nucleotide sequence accession numbers. The sequences of the *recA* PCR products from the following organisms have been submitted to GenBank and assigned the indicated accession numbers: *E. faecalis*, M81466; *M. pulmonis*, M81467; *S. mutans*, M81468; and *S. pyogenes*, M81469.

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