Optimization of gene expression in the radioresistant bacterium *Deinococcus radiodurans*

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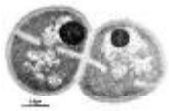

Introduction

Because of their tolerance to very high levels of ionizing radiation, members of the genus *Deinococcus* have received considerable attention over the past years. The type species of the genus, *Deinococcus radiodurans*, has been studied extensively in several labs. Although researchers are only beginning to understand the mechanisms by which this Gram-positive bacterium is able to repair massive DNA damage after radiation dosages as high as 1.5 Mrad, it has become evident that its recombination machinery has several unique characteristics (1-4).

The aim of the present studies is to engineer *D. radiodurans* into a detoxifier for bioremediation of complex waste mixtures, containing heavy metals, haloorganics and radionuclides, making use of its ability to be biologically active in environments where they will be exposed to high levels of radiation. For that purpose, we aim to clone and express several broad spectrum oxygenases and heavy metal resistance determinants, and test survival and activities of these strains in artificial mixtures of contaminants, designed to simulate DOE mixed waste streams.

Results and Discussion

To be able to construct strains for bioremediation, our initial studies have focused on the development of an insertional expression system for *D. radiodurans* R1. Several studies have shown that the expression signals used by *D. radiodurans* differ considerably from those found in other bacteria. Although *D. radiodurans* contains a typical eubacterial RNA polymerase core enzyme (TBLASTN searches on the genome sequence), *Escherichia coli* promoters are not recognized in *D. radiodurans* and *vice versa* (5). To expand our basic understanding of the requirements for transcription, and to optimize gene-expression, we are following two strategies. First, a promoter-probe vector is being developed for the selection of promoter sequences from the *D. radiodurans* R1 genome. This system will then be used for the construction of a shotgun-library of promoter fragments and subsequent screening of *D. radiodurans* transformants for expression of β-galactosidase or fluorescence. The second strategy involves primer extension studies of a number of genes which are expected to be transcribed at a substantial level. This will enable us to map transcription start sites and identify possible -35 and -10 sequences.

A. Promoter-probe vectors

❸ single-copy integrations in non-essential genes via replacement or double cross-over (DCO) recombination using suicide vectors (*i.e.* unable to replicate in this host). Genes were annotated based on TBLASTN similarity searches (*http://www.ncbi.nlm.nih.gov/BLAST/tigrbl.html*) on the R1 sequence established by TIGR (*http://www.tigr.org/tdb/mdb/mdb.html*)

❸ Two systems:
(i) pUC-derived vectors carrying the target gene, disrupted by insertion of a selectable marker (*Fig. 1*)

(ii) pMUTIN2-derived system, based on "back-to-back" cloning, upstream of a reporter gene, of gene-flanking regions and linearization through a unique *Eco*R1 site (*Fig. 3*)

(i) **pUC-derived replacement vectors.** Two integration systems were developed, based on insertion into *amyE* (α-amylase) and *amyX* (pullulanase), respectively. A kanamycin-resistance marker (Km^R) was used to test these systems and both appear to be good candidates for an insertion vector (up to 10^5 transformants/ug DNA; see *Table I*). In both cases, DCO-recombinants were obtained showing both Km-resistance and loss of function, *i.e.* failure to grow on the appropriate substrate (starch or pullulan; *Table II*).

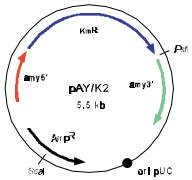

Figure 1. *An integrational vector for double cross-over recombination in* D. radiodurans *R1. pAY/K1 was constructed by PCR cloning of the amyE gene and subsequent insertion of the Tn907 Km^R cassette of pUC4K. Filling in, using T4 DNA polymerase, of a PstI site between amy5' and Km^R, and a second site in the pUC19 polylinker, resulted in a unique PstI site for cloning of promoter-probe genes (pAY/K2).*

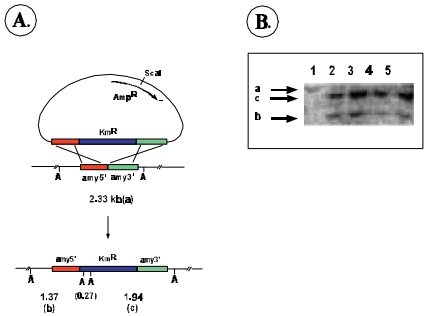

Figure 2. *Replacement recombination in* D. radiodurans *R1. **(A)** Schematic representation of double cross-over recombination in the* amyE *locus using plasmid pAY/K1. **(B)** Southern hybridization of* AvaI *(A)-restricted chromosomal DNA of wild-type R1 (lane 1) and several Km^R* amyE*⁻ integrants (lanes 2-5). The arrows indicate the position of the fragments corresponding to those outlined in panel A.*

Table I. D. radiodurans *R1 transformation frequencies using plasmids pAY/K1 and pPU/K3, respectively. Frequencies are expressed as the number of transformants per ug of DNA.*

Transformation	pAY/K1 (*amyE*)	pPU/K3 (*amyX*)
Uncut plasmid	5.6×10^4	1.1×10^7 [a]
Linear (x *Sca*I)	6.7×10^4	3.8×10^4

[a], preliminary Southern hybridization results indicate that the majority of these are the result of single cross-overs (data not shown)

Table II. *Growth of randomly selected* D. radiodurans *R1 transformants on chemically defined media supplemented with either glucose, starch or pullulan (each at 0.5%).*

Strain	TGY [a]	Glucose	Starch	Pullulan
R1	+	+	+	+
AY1.1	+	+	-	+
AY1.2	+	+	-	+
AY6.2	+	+	-	+
PU2.1	+	+	+	-
PU3.1	+	+	+	-
PU7.1	+	+	+	+/- [b]

[a], TGY broth contained: tryptone (0.5%), yeast extract (0.3%), and glucose (0.5%)

[b], this mutant possibly still contained a wild-type copy of *amyX* on at least one of the chromosome copies

(ii) **pMUTIN2-derived DCO vector for integration in the *thyA* and *dfrA* (*folA*) genes.** Mutations in the *thyA* and *dfrA* genes, encoding thymidilate synthase and dihydrofolate reductase, respectively, render bacterial cells resistant to trimethoprim (*e.g.* 6). Constructing insertions in these genes may obviate the need of an efficiently expressed antibiotic resistance marker for the initial selection of transformants.

❸ Two vectors, carrying 3' and 5' flanking sequences of these genes, which are located in an operon, were constructed (Fig. 3).

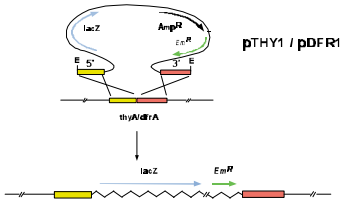

Figure 3. *pMUTIN2-based DCO vector for insertion in the* thyA *or* dfrA *genes. Cloning the flanking regions of the genes in reverse order, creating a single* EcoRI *site (E) in between, enables integration of the entire plasmid using linearized vector DNA for transformation*

❸ Tmp^R colonies harboring these vectors were not obtained; moreover, the frequency of spontaneous Tmp-resistance was relatively high

❸ Similar vector constructed carrying the *amyE*::Km^R cassette of pAY/K1; in this case, the 3' end of *amyE* was inserted upstream of the 5' end fused to KmR. This vector will now be tested for integration

B. Primer extension analyses

A more direct approach towards identifying promoters in *D. radiodurans* is based on mapping transcriptional start-sites of genes, which are expected to be either (i) expressed at a substantial level, or (ii) expressed by controllable promoters. For this purpose we cloned the upstream regions (Å 250 bp) of a number of genes that were annotated by comparison with their homologues in *E. coli* and *Bacillus subtilis*:

- ✓ *amyE*: α-amylase; promoter may be subject to catabolite repression
- ✓ *amyX*: pulullanase-encoding gene
- ✓ *groES*: heat-shock controlled promoter
- ✓ *lexA*: damage-inducible promoter

❸ Primer extension studies and Northern analyses will be initiated in the near future.

Conclusions and Future Directions

To date, a number of integration vectors have been constructed. Derivatives of these vectors, carrying reporter genes (*e.g. lacZ* and *gfp*) for the selection of promoter fragments, are currently being developed. In addition, several chromosomal fragments were cloned that will be tested for promoter activity by primer extension and Northern analysis.

Future work

❸ Clone genomic bank in pAY/K2 carrying either *lacZ*, *gfp* or *xylE*

❸ Transform R1 with bank and screen integrants for expression

❸ Sequence fragments and establish promoter sequence

❸ Fuse highly expressed and/or inducible promoters to various genes required for remediation (*i.e. mmo*, encoding methane monooxygenase, and *tod*, encoding toluene dioxygenase)

References

1) Daly, M.J., *et al*. (1996) *J Bacteriol* **178**: 4461-4471
2) Daly, M.J., and Minton, K.W. (1995) *Science* **270**: 1318
3) Minton, K.W., and Daly M.J. (1995) *Bioessays* **17**: 457-464
4) Battista , J.R. (1997) *Annu Rev Microbiol.* **51**: 203-224
5) Smith, M.D., *et al*. (1991) *Gene* **98**:45-52
6) Stacey, K.A., and Simson, E. (1965) *J Bacteriol* **90**: 554-555

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