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Insertion-duplication mutagenesis of *Salmonella enterica* and related species using a novel thermosensitive vector

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Abstract

We constructed a novel temperature-sensitive vector as a tool for gene disruption by insertion-duplication mutagenesis (IDM) in *Salmonella enterica* and related species. A *phoN* insertion mutant was proven highly stable during growth in LB medium and during infection of macrophage cells in the absence of selection pressure. By progressive shortening of a *phoN* fragment, the minimal length for effective insertional mutagenesis driven by homologous recombination was determined to be 50 bp, allowing to disrupt even short genes that could not yet be subjected to site-specific IDM. We also showed that plasmid excision from the chromosome restores the wild-type genotype with a reliability of 98%. Intracellular recovery of the excised vector provides the option to switch between two genotypes and thus to rapidly attribute the observed mutant phenotype to the targeted gene. In addition, a fragment library was used to measure the integration rate at various chromosomal sites that varies greatly by at least 2.5 magnitudes, independently from the length of the cloned fragment.

Keywords: *Salmonella enterica* sv. Typhimurium; Insertion-duplication mutagenesis; Homologous recombination; Vector excision; Temperature-sensitivity

1. Introduction

A common method of gene knockout for functional studies is insertion-duplication mutagenesis (IDM), which has the strong advantage to be usable for direct targeting of selected genes. This technique requires a conditionally replicating vector that cannot proliferate under nonpermissive growth conditions. In general, IDM bases on homologous (Campbell-type) recombination between the bacterial chromosome and a plasmid carrying a chromosomal fragment, resulting in

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plasmid integration into the chromosome and fragment duplication. If the replication ability of the used plasmid is temperature-sensitive, strains that harbour the plasmid on the chromosome can be selected by growth at the nonpermissive temperature, since only these cells remain resistant against the applied antibiotic.

Conditionally replicating vectors are versatile tools for genetic manipulations like the delivery of transposable elements (Michiels and Cornelis, 1986), the expression of essential gene products (Dai and Lutkenhaus, 1991), the construction of plasmids for biological containment (Hashimoto-Gotoh et al., 1981), or the construction of conditional mutants, which is important for studies of genes essential for cell survival (Jana et al., 2000). The most prominent application of thermosensitive plasmids is gene disruption and allelic exchange with the chromosome of Gram-positive and Gram-negative bacteria. The temperature-sensitive shuttle vectors pTSV32(Ts) and pLSV1 have been used to construct insertional knockouts or deletion mutants of the two pathogens *Listeria monocytogenes* (Wünscher et al., 1991) and *Streptococcus pneumoniae* (Lee et al., 1998), respectively. Thermosensitive derivatives of the rolling circle plasmid pGK12 are examples for common conditionally replicating plasmids that were often applied for gene inactivation and replacement in *Lactobacillus* spp. and *Bacillus subtilis* (Biswas et al., 1993; Law et al., 1995). A pGK12 derivative also allowed the systematic, genome-wide construction of regulatory mutants to confirm the essentiality of *B. subtilis* genes (Kobayashi et al., 2003). Other examples for temperature-sensitive vectors are pME487, which is specific for *Pseudomonas aeruginosa* (Reimmann et al., 1988), pTV10K used in Group B streptococci (Framson et al., 1997), and pCL derivatives replicating in *Staphylococcus aureus* (Hahn et al., 1999; Sau et al., 1997). In all these cases, insertional knockout mutants can be obtained directly by incubating transformed strains at nonpermissive temperature, or by growth at permissive conditions followed by a shift to higher temperatures. Thermosensitive mutants of pSC101-derivatives like pKO3, pMAK705, HSG422 or pHS1 are proven tools for genetic manipulations of *Escherichia coli* and *Salmonella* spp. (Hamilton et al., 1989; Hashimoto-Gotoh et al., 1981; Link et al., 1997; Merlin et al., 2002; Phillips, 1999). Before the development of the more efficient one-step inactivation method basing on the phage λ Red recombinase (Datsenko and Wanner, 2000), those vectors were the preferred mean to generate gene deletions. For this purpose, the plasmids, which incorporate cloned DNA flanking the target gene, are transformed into an appropriate host, and integrants are selected at 42–44 °C (Hahn et al., 1999) (Hamilton et al., 1989). Subsequent growth of these insertion mutants at permissive temperature leads to a second recombination event that results in plasmid excision.

Here, we describe the features of a novel temperature-sensitive plasmid and its use for genetic analyses of *E. coli* and *S. enterica*. This plasmid, pIDM1, carries several recognition sites for restriction enzymes, encodes a tetracycline efflux mechanism, and enables blue/white selection for efficient cloning. A low shutoff temperature for replication was determined, and it was shown that integrants are highly stable at nonpermissive temperature in the absence of tetracycline. Therefore, pIDM1-insertion mutants can be used in cell culture experiments or for in vivo studies. One practical conclusion from our data on homologous recombination is that 50 bp is a sufficient fragment length for targeting the disruption of individual genes of *S. enterica* sv. Typhimurium. The site-specific variability of integration rates in the *Salmonella* genome is discussed.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and general procedures

The bacterial strains used in this study are DH5α (Sambrook and Russell, 2001), helper strain EC101 harbouring a wild-type *repA* allele (Law et al., 1995), *S. enterica* sv. Typhimurium ATCC14028 (Institute Pasteur, Paris), 14028*phoP*::Tn10d tet^R and 14028Δ 2582(18)::kan^R (J. Klumpp, unpublished). Luria–Bertani (LB) broth consisted of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. For solid media, 1.5% bactoagar was added. Minimal