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High frequency of double crossover recombination facilitates genome engineering in *Pseudomonas aeruginosa* PA14 and clone C strains

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Abstract

Pseudomonas aeruginosa is a key opportunistic human pathogen. An established procedure to replace a target gene is two-step allelic exchange, i.e. selection of single crossover at homologous sequences and subsequent counter selection to induce double crossover for excision of the suicide vector. In this study, we found that certain strains of *P. aeruginosa* display a high rate of instant double crossover upon introduction of a suicide vector containing an antibiotic resistance cassette flanked by adjacent sequences for gene replacement, making the counter selection step to achieve the second crossover superfluous. Assessment of a limited panel of target genes commonly showed negligible double crossover with a frequency <20 % in the genetic reference strain PAO1, whereas a high double crossover frequency of >70 % was observed for PA14 and clone C strains. Consequently, for certain *P. aeruginosa* strains replacement of an ORF by a antibiotic resistance cassette can be shortened by directly selecting for double crossover recombination.

Pseudomonas aeruginosa is ubiquitous in nature. As a facultative human pathogen *P. aeruginosa* causes a wide variety of infections such as wound, ear, lung, eye and urinary tract infections [1]. For example, *P. aeruginosa* is a prominent pathogen in chronic lung infections in cystic fibrosis patients [2]. Besides, *P. aeruginosa* is commonly distributed in various environmental niches including the aquatic habitat covering natural and man-made environments [3–6]. The importance of *P. aeruginosa* as a key human pathogen has earlier triggered extensive studies first with PAO1 [7] and, more recently, PA14 [8] as reference strains to understand *P. aeruginosa* physiology and virulence. We have recently extended genetic manipulation of *P. aeruginosa* to clone C strains [9, 10], which are a dominant group of *P. aeruginosa* strains in acute and chronic infections as well as environmental settings [9, 11]. In this study, we report the frequency of double crossover recombination at a target gene to differ dramatically between PAO1 compared to PA14 and the three clone C strains SG17M, SG31M and 8277, which facilitates allelic exchange by homologous recombination in the latter strains.

Allelic exchange, alteration of genetic elements based on homologous recombination, has been and is broadly used to manipulate bacterial genomes [12–14]. This approach, which has been applied to *P. aeruginosa* since the 1980s uses, e.g. suicide vectors to produce scarless mutations or replacement of an ORF by an antibiotic resistance cassette. Subsequent removal of the antibiotic resistance cassette flanked by recombination target sites can be achieved by temporal expression of heterologous site-specific recombinases such as Flp flippase and Cre topoisomerase [13, 15, 16]. A frequently used approach to manipulate genes in *P. aeruginosa* is two-step allelic exchange using, e.g a pEX18-based vector system (Fig. 1) [9, 13]. Thereby, the plasmid harbouring an antibiotic resistance marker and an origin of replication, ColE1, which enables replication in the cloning host *Escherichia coli*, but not in the target bacterium *P. aeruginosa* is used as a scaffold for the replacement cassette. The replacement cassette consists of a distinct antibiotic resistance marker flanked by sequences homologous to the regions up- and down-stream of the target gene cloned into the plasmid (Fig. 1). Besides, a selectable marker such as *sacB* encoding levansucrase, which

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Abbreviations: ORF, open reading frame.

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Supplementary material is available with the online version of this article.

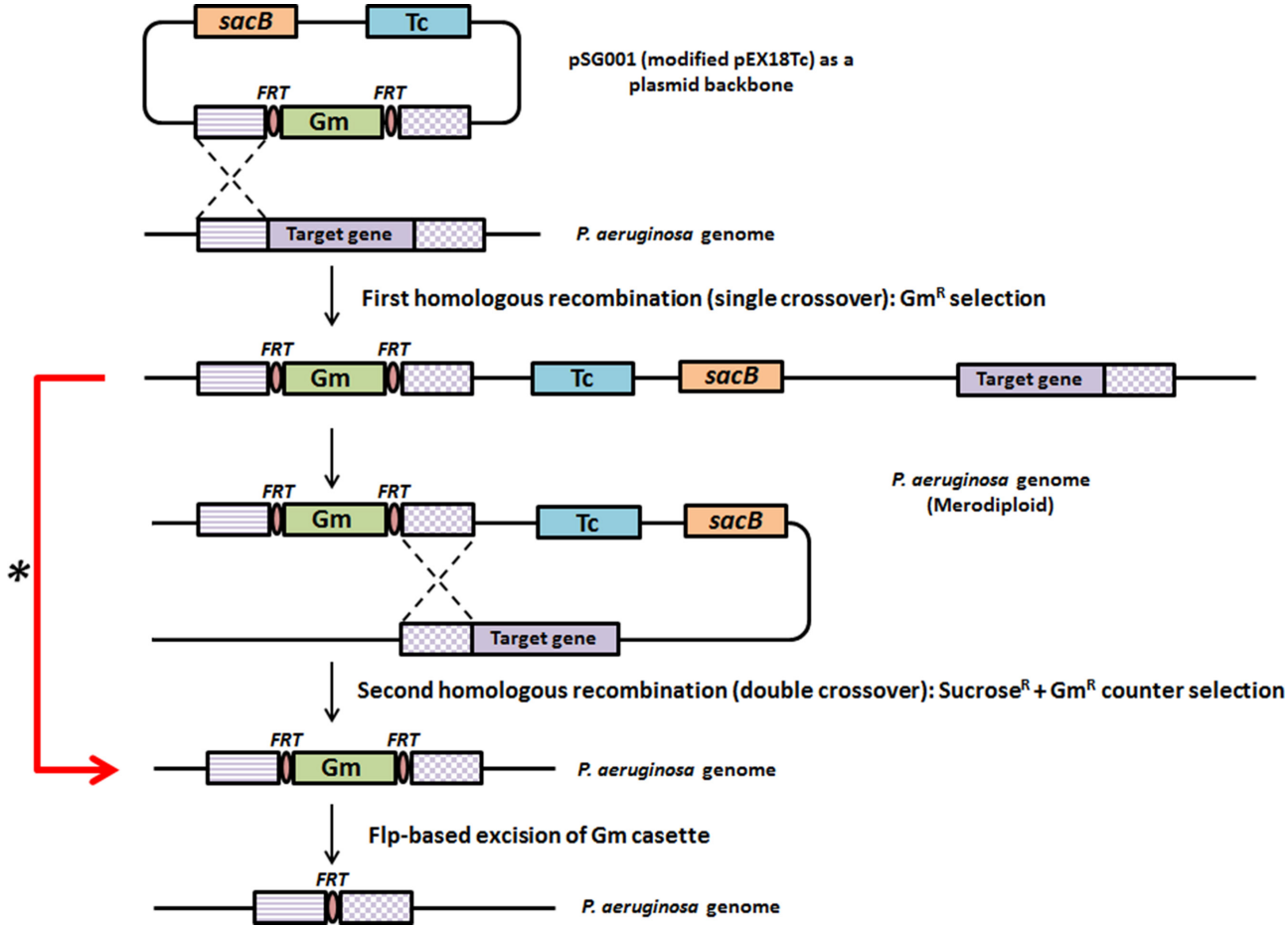

Fig. 1. Schematic view of homologous recombination events in *P. aeruginosa*. In strain PAO1, the first homologous recombination, a single crossover event, occurs between one of the regions homologous on the genome and the replacement cassette on the suicide plasmid, for example the pEX18Tc-derived vector pSG001 [9]. By selecting for Gm-resistant colonies, merodiploid colonies are selected. The merodiploid colonies with plasmid-encoded SacB levansucrase are subsequently subject to counter selection on sucrose-containing medium forcing the second homologous recombination event [15]. As a consequence, the plasmid backbone is excised from the chromosome accompanied by a loss of the tetracycline resistant marker (Tc) and *sacB*. Subsequently, the antibiotic resistance marker Gm that replaced the gene can be removed by expression of a heterologous recombinase, such as the tyrosine family site-specific recombinase Flp [13]. Flp recombinase acts at the engineered FRT sites to excise the antibiotic resistance marker. In conclusion, a double crossover event occurs with high frequency in *P. aeruginosa* PA14 and clone C strains without the requirement of a counter selection procedure (red arrow; *).

enables counterselection on sucrose containing medium in Gram-negative and Gram-positive bacteria is encoded on the suicide vector. SacB hydrolyses sucrose and synthesizes levan polysaccharides, but the molecular mechanism of toxicity is not well established [17]. The plasmid containing the replacement cassette is subsequently transferred from *E. coli* to *P. aeruginosa* by triparental conjugation. A site-specific low-frequency genomic integration occurs by homologous recombination, conventionally by a single crossover event (Fig. 1). As a consequence of single crossover, the plasmid is integrated into the chromosome via one of the two regions for homologous recombination. Subsequently, the plasmid backbone can be excised by a second homologous recombination event leading to double crossover on sucrose containing counter selection medium. The ultimate outcome is the replacement of the target gene by an antibiotic resistance marker. Subsequently, the antibiotic marker can be removed by Flp-catalysed excision if flanked by FLP-sites (Fig. 1). A Flp-mediated recombination will leave the 34 bp long FLP recombinase target (FRT) scar sequence.

Upon gene replacement, we previously observed double crossover events in *P. aeruginosa* SG17M [9]. To investigate whether such an event occurs only in this particular strain or with a particular gene, we used the reference strains PAO1 and PA14 and clone C strains SG17M, SG31M and 8277 to delete a panel of target genes, namely *clpB*, *clpG, ibpA* and *exsA*. We used the modified pEX18Tc vector pSG001 containing a gentamicin (Gm)-resistant cassette flanked by Flp sites in the multiple cloning site (Fig. 1) [9]. Approximately 1 kbp of

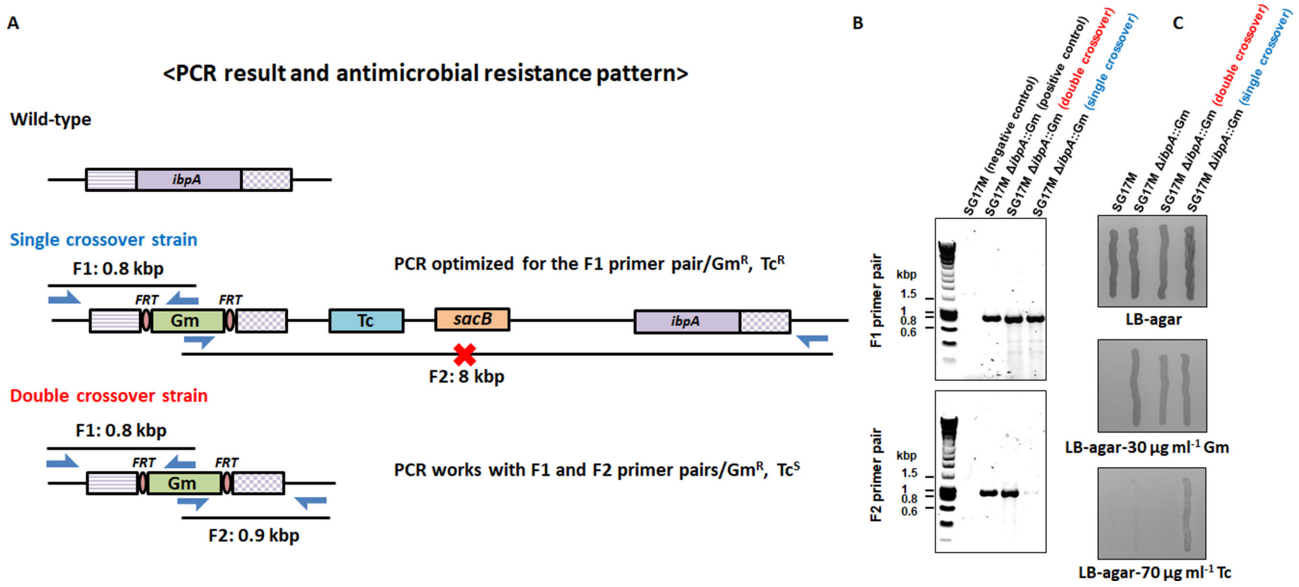

Fig. 2. Confirmation of single and double crossover by PCR and antibiotic resistance test. (a) The recombinant strain is verified by assessment of antibiotic resistance and subsequent testing of the recombination event by PCR. Two primer pairs to amplify up- and down-stream homologous recombination regions, named as F1 and F2, respectively, were used. Depending on the side of homologous recombination, either the F1 or F2 primer set amplifies a short fragment in the single crossover strain with the chosen extension time. However, two fragments are amplified in the double crossover strain. As an example, single and double crossover events to replace the *ibpA* gene in strain SG17M are shown (b). Consistent with PCR verification, assessment of antibiotic resistance is shown accordingly (c).

the flanking regions up- and down-stream of the target gene were cloned into the vector as sequences to be targeted by homologous recombination (Fig. 1) [10]. The vector with the gene replacement cassette was subsequently transferred from *E. coli* to recipient *P. aeruginosa* strains by triparental mating and recombinants were selected on Vogel-Bonner minimal medium (VBMM) containing Gm, which allows growth of *P. aeruginosa* upon genomic integration of the vector and replacement cassette by single or double crossover, respectively, but not *E. coli* [18]. Subsequently, we distinguished between single and instant double crossover recombination by screening colonies for resistance towards tetracycline (Tc) (Fig. 1). The recombination event was further selectively assessed by PCR using primers that allow discrimination between single and double crossover (Fig. 2). To this end, we used two primer combinations with one primer to bind in the Gm cassette and the other primer up- or down-stream of the region of homologous recombination, respectively. As the major results, double crossover was generally observed with a frequency of >70 % in PA14 and clone C strains (Table 1). In the case of PAO1, double crossover occurred with a frequency not higher than 20 % using the same gene replacement cassettes. Of note, *ibpA* could not be deleted in PA14. Thus, the conventional gene replacement protocol expects a single crossover event to occur upon selection for suicide plasmid integration into the chromosome, however,

Table 1. Frequency of double crossover events

Target gene/strain	SG17M*	PA14*	PAO1*	SG31M*	8277*
clpB	97/97, 100 %	62/62, 100 %	1/100, 1 %	45/54, 83 %	48/52, 92 %
clpG	18/24, 75 %	13/18, 72 %	0/24, 0 %	not tested	not tested
ibpA	57/60, 95 %	no colonies	0/62, 0 %	67/68, 99 %	35/35, 100 %
exsA	67/72, 93 %	63/76, 83 %	14/69, 20 %	48/72, 67 %	76/78, 97 %

Number of strains with a double crossover event/total number of examined strains with percentage calculated (* positive/tested, % positive). The strains, which are not double crossover are single crossover strains. Gm was used as the selection marker for the integration event. Colonies were subsequently streaked on Gm and Tc containing LB medium. Selected colonies with single or double crossover were tested by PCR. Primers and protocols used to isolate and confirm single or double crossover strains are described (Table S1, available in the online version of this article [9, 10]). The targeted homologous recombination regions were amplified from genomic DNA of SG17M and cloned into the pSG001 plasmid [9], except for the target regions flanking the *clpG* gene, which were cloned into pMQ150 [10]. *Ibp*A could not be deleted in PA14. The target regions in PA14 and PAO1 were more than 97.91 % identical (Supplemental data).

when selecting for the antibiotic resistance cassette for gene replacement double crossover events were observed with high frequency in particular strains.

The recombination fragments were derived up- and downstream of target genes in SG17M. PAO1 and PA14 have >97.91 % sequence identity for *clpB, clpG, ibpA* and *exsA* (Supplemental Material, available with the online version of this article). The degree of nucleotide sequence identity, though, was not directly correlated with double crossover frequency.

To date, genetic engineering of *P. aeruginosa* is readily performed. Nevertheless, a manipulation strategy including a two-step allelic exchange is more time consuming than a one-step allelic exchange. Here, we describe the double crossover event to spontaneously and efficiently occur in PA14 and clone C strains at such high frequency that a subsequent counter selection step can be omitted. This 'short-cut' procedure facilitates allelic replacement of a target gene by an antibiotic resistance cassette. In addition, 5–7 days of handling can be saved compared to the two-step procedure, which includes counter selection to induce the second crossover. Therefore, it takes less than 6 days to generate a mutant strain. This method is also 2–3 days faster than allelic exchange using a suicide plasmid without selective marker to create a scareless mutant [19]. However, efficient double crossover does not apply for all *P. aeruginosa* strains as double crossover events have only infrequently been observed for PAO1, if at all. While PA14 and clone C isolates belong to major clonal groups, PAO1 constitutes a minor clonal group of *P. aeruginosa* [11]. In this context, it is important to note that genes involved in DNA replication, recombination, modification and repair are highly variable in PA14 and clone C strains [20]. This observation suggests that the ability to facilitate recombination might contribute to the survival and transmission of abundant clonal strains.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Kerr KG, Snelling AM. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *J Hosp Infect* 2009;73:338–344.
2. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002;15:194–222.
3. Grobe S, Wingender J, Trüper HG. Characterization of mucoid *Pseudomonas aeruginosa* strains isolated from technical water systems. *J Appl Bacteriol* 1995;79:94–102.
4. Pirnay JP, Matthijs S, Colak H, Chablain P, Bilocq F *et al*. Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. *Environ Microbiol* 2005;7:969–980.
5. Römling U, Kader A, Sriramulu DD, Simm R, Kronvall G. Worldwide distribution of *Pseudomonas aeruginosa* clone C strains in the aquatic environment and cystic fibrosis patients. *Environ Microbiol* 2005;7:1029–1038.
6. Römling U, Wingender J, Müller H, Tümmler B. A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl Environ Microbiol* 1994;60:1734–1738.
7. Holloway BW, Römling U, Tümmler B. Genomic mapping of *Pseudomonas aeruginosa* PAO. *Microbiology* 1994;140:2907–2929.
8. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG *et al*. Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 1995;268:1899–1902.
9. Lee C, Wigren E, Trček J, Peters V, Kim J *et al*. A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ Microbiol* 2015;17:4511–4526.
10. Lee C, Franke KB, Kamal SM, Kim H, Lünsdorf H *et al*. Stand-alone ClpG disaggregase confers superior heat tolerance to bacteria. *Proc Natl Acad Sci USA* 2018.
11. De Soyza A, Hall AJ, Mahenthiralingam E, Drevinek P, Kaca W *et al*. Developing an international *Pseudomonas aeruginosa* reference panel. *Microbiologyopen* 2013;2:1010–1023.
12. Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 1997;179:6228–6237.
13. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 1998;212:77–86.
14. Ruvkun GB, Ausubel FM. A general method for site-directed mutagenesis in prokaryotes. *Nature* 1981;289:85–88.
15. Schweizer HP. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable *Bacillus subtilis sacB* marker. *Mol Microbiol* 1992;6:1195–1204.
16. Quénée L, Lamotte D, Polack B. Combined *sacB*-based negative selection and *cre-lox* antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. *Biotechniques* 2005;38:63–67.
17. Steinmetz M, Le Coq D, Djemia HB, Gay P. Genetic analysis of *sacB*, the structural gene of a secreted enzyme, levansucrase of *Bacillus subtilis* Marburg. *Mol Gen Genet* 1983;191:138–144.
18. Choi KH, Schweizer HP. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* 2005;5:30.
19. Hmelo LR, Borlee BR, Almblad H, Love ME, Randall TE *et al*. Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nat Protoc* 2015;10:1820–1841.
20. Fischer S, Klockgether J, Morán Losada P, Chouvarine P, Cramer N *et al*. Intraclonal genome diversity of the major *Pseudomonas aeruginosa* clones C and PA14. *Environ Microbiol Rep* 2016;8:227–234.