Oligonucleotide-mediated genetic transformation of *Borrelia burgdorferi*

D. Scott Samuels^{1,2} and Claude F. Garon²

Author for correspondence: Scott Samuels. Tel: +1 406 243 6145. Fax: +1 406 243 4184.
e-mail: samuels@selway.umt.edu

¹ Division of Biological Sciences, University of Montana, Missoula, Montana 59812, USA

² Bacterial Pathogenesis Section, Rocky Mountain Laboratories Microscopy Branch, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840, USA

We have used short oligonucleotides to genetically transform the Lyme disease spirochaete *Borrelia burgdorferi*. The oligonucleotides are derived from the sequence of an Arg-133 to Ile mutant *gyrB* (chromosomal) gene that confers resistance to the antibiotic coumermycin A₁. Oligonucleotides were about 10 000-fold less efficient at transformation, on a molar basis, than longer PCR-generated substrates. All of the transformants tested contained the predicted site-directed silent mutation in their *gyrB* genes. Antisense oligonucleotides were more efficient at transformation than either sense or double-stranded oligonucleotides. This is the first demonstration of oligonucleotides used to introduce site-directed mutations directly into the genome of a bacterium.

Keywords: Lyme disease, *Borrelia burgdorferi*, oligonucleotide, genetic transformation, electroporation, *gyrB*

INTRODUCTION

Borrelia burgdorferi, a causative agent of Lyme disease (Baranton & Old, 1995; Barbour & Fish, 1993; Steere, 1994), is a spirochaete with a genome that consists of both linear and circular plasmids as well as a linear chromosome (Hinnebusch & Tilly, 1993; Saint Girons *et al.*, 1994). *B. burgdorferi* is highly susceptible to the coumarin antibiotic coumermycin A₁ (Samuels & Garon, 1993), which targets the B subunit of DNA gyrase (Maxwell, 1993). We have isolated and characterized spontaneous mutants of *B. burgdorferi* that are resistant to coumermycin A₁ (Samuels *et al.*, 1994b). Currently, these are the only available mutants of *B. burgdorferi* resistant to an antimicrobial agent. We previously reported on two classes of coumermycin A₁-resistant mutants that had single point mutations in their *gyrB* genes that converted Arg-133 to either Gly or Ile (Samuels *et al.*, 1994b). These mutant genes have been used as selectable markers for the first demonstration of genetic transfer in any species of *Borrelia* (Samuels *et al.*, 1994a). Successful transformation was confirmed using PCR to create site-directed mutant *gyrB* genes and introducing them into wild-type cells by electroporation. Recently, we have inserted a coumermycin A₁-resistant *gyrB* gene cassette into the 26 kb circular plasmid of *B. burgdorferi* (Rosa *et al.*, 1996).

Electroporation, the application of a high voltage electrical pulse to disrupt cell membranes, has been used to genetically transform a variety of micro-organisms (Nickoloff, 1995; Shigekawa & Dower, 1988; Trevors *et al.*, 1992). We have previously electroporated PCR-generated 840 bp DNA substrates into *B. burgdorferi* and selected for the homologous recombination of these DNA molecules into the chromosome (Samuels *et al.*, 1994a). The yeast *Saccharomyces cerevisiae* can be transformed with short oligonucleotides (Moerschell *et al.*, 1988). We now demonstrate that single-stranded oligonucleotides of 27 bases can be used to introduce site-directed mutations conferring coumarin resistance into the *gyrB* gene of *B. burgdorferi*.

METHODS

DNA. Oligonucleotides 385F/398T399T (5' GTTTATGTT-AATATTGATGGAAAAATT 3', M_r 8834) and 411R/398A399A (5' AATTTTCCATCAATATTAACATAAAC 3', M_r 8652) were used as transformation substrates. These oligonucleotides are inverse complements of each other and were used previously to create site-directed mutations (Samuels *et al.*, 1994a). They encode amino acids 129–137 of the *B. burgdorferi* strain B31 *gyrB* gene product and include two point mutations that change the Arg-133 AGA codon to an ATT Ile codon. The double-stranded oligonucleotide transformation substrate was generated by boiling 2.5 mg ml⁻¹ (~0.3 µmol ml⁻¹) each of 385F/398T399T and 411R/398A399A together for 1 min and cooling slowly to allow for annealing (100 °C–65 °C in ~10 min and 65 °C–25 °C in ~60 min).

Abbreviation: BSK II medium, Barbour–Stoenner–Kelly II medium.

Electroporation. Wild-type *B. burgdorferi* cells were made competent for electrotransformation as described previously (Samuels, 1995; Samuels *et al.*, 1994a). Briefly, cells were grown to $\sim 5 \times 10^7$ cells ml^{-1} , washed twice in Dulbecco's phosphate-buffered saline and three times in 272 mM sucrose and 15% (v/v) glycerol (EPS), and resuspended in EPS at $\sim 10^{10}$ cells ml^{-1} . Cells were mixed with 5–10 μg (570–1140 pmol) oligonucleotide or 0.5–1.5 μg (1–3 pmol) of an 840 bp PCR product, transferred to a 0.2 cm electroporation cuvette (BTX), and subjected to a single pulse of 2.5 kV, 25 μF and 200 Ω from a Gene Pulser with Pulse Controller (Bio-Rad).

Electron microscopy. Fractions of competent cells were removed from the cuvette both before and immediately after electroporation and applied to the surface of a parlodion-coated grid, negatively stained for 30 s with a 2% (w/v) ammonium molybdate solution, air-dried and examined in a Philips CM10 transmission electron microscope at 80 kV accelerating voltage.

Selection and analysis of transformants. Cells were recovered in 10 ml of Barbour–Stoenner–Kelly II (BSK II) medium without antibiotics for 19–25 h at 32 °C and plated in solid medium containing 0.2 μg coumermycin A_1 ml^{-1} as described previously (Rosa & Hogan, 1992; Samuels, 1995; Samuels *et al.*, 1994b). Individual clones were expanded and DNA was isolated from each clone. A portion of the *gyrB* gene was then amplified from each clone by PCR and sequenced using a dsDNA Cycle Sequencing System (Life Technologies) as described previously (Samuels *et al.*, 1994b).

RESULTS

The structural effects of a single electric field pulse on *B. burgdorferi* are shown in Fig. 1(b). The darkly stained regions, suggesting marked cell surface disruptions, are seen along the entire length of the spirochaete. Although the number of these darkly stained regions per cell appears to vary broadly, most of the spirochaetes in a field appear to have them. These surface perturbations, however, are not visible before the electroporation pulse is delivered (Fig. 1a). In addition, these structures are no longer visible after the cell is allowed to recover from the pulse in BSK II medium for 30 min (data not shown).

We electroporated *B. burgdorferi* strain B31 with oligonucleotides encoding a mutation in the *gyrB* gene that confers coumermycin A_1 resistance in three separate experiments (Table 1). The transformation efficiency of the oligonucleotides was considerably lower than that obtained using the 840 bp PCR-generated substrates, which typically yield about 20 000 coumermycin A_1 -resistant colonies per 10 μg of input DNA (2000 transformants per electroporation reaction) (Samuels *et al.*, 1994a). In experiment 2, competent cells were frozen and stored at -70 °C before electroporation. This treatment lowers the transformation efficiency with PCR-amplified substrates by less than twofold. In all of the experiments, the 411R/398A399A oligonucleotide, which is the inverse complement of the coding strand (antisense), resulted in more transformants than the 385F/398T399T oligonucleotide (Table 1). Although the effect was small, mixing the two oligonucleotides immediately prior to electroporation resulted in a lower transformation efficiency than using either 411R/

Fig. 1. Electron micrograph of *B. burgdorferi* before and after electroporation. Cells were washed extensively and either not subjected (a) or subjected (b) to an electrical pulse, followed by negative staining and observation by transmission electron microscopy. Periplasmic flagella were exposed or liberated during preparation of competent cells, which probably disrupted the outer membrane. In addition, membranous vesicles (blebs) are occasionally seen both free in the background and associated with spirochaetes (both electroporated and not electroporated).

398A399A by itself or a double-stranded oligonucleotide (Table 1), which suggests that the 385F/398T399T oligonucleotide is slightly inhibitory.

The spontaneous rate of mutation was two or fewer coumermycin A_1 -resistant colonies per electroporation reaction, as assessed using wild-type DNA sequences and irrelevant oligonucleotides as electroporation substrates (data not shown). To test if the coumermycin A_1 -resistant colonies represented genuine transformants, DNA was isolated and sequenced from five colonies electroporated with 411R/398A399A in experiment 1. Spontaneous mutants would be expected to have a single G to T transversion changing the Arg-133 AGA codon in *gyrB* to an ATA Ile codon, while transformants would have a double mutation changing the Arg-133 AGA codon to an ATT Ile codon (Samuels *et al.*, 1994a). All five coumermycin A_1 -resistant clones contained the double (site-directed) mutant ATT Ile codon, demonstrating that they resulted from successful transformation.

A heterologous strain, HB19, was also transformed and the results were qualitatively similar to that of strain B31, with the antisense oligonucleotide yielding the most transformants and the sense oligonucleotide demonstrating inhibitory activity (data not shown). However, compared to B31, the transformation efficiency of HB19 was about fivefold less using oligonucleotides and 10- to 100-fold lower using a PCR product (amplified

Table 1. Transformation efficiency of oligonucleotides

Oligonucleotide	Coumermycin A ₁ -resistant colonies per 10 µg DNA*		
	Experiment 1	Experiment 2†	Experiment 3
385F/398T399T	< 20	18	27
411R/398A399A	350	52	150
Double-stranded	ND	24	70
Mixed	ND	6	10

* ND, not done.

† Competent cells were frozen prior to electroporation.

from coumermycin A₁-resistant B31). Five coumermycin A₁-resistant clones from the PCR product electroporation and five from the 411R/398A399A oligonucleotide electroporation were isolated and their *gyrB* genes were partially sequenced. All 10 contained the marker mutation as described above, indicating that strain HB19 can be transformed with either oligonucleotides or PCR-generated substrates.

DISCUSSION

The effects of electroporation on the ultrastructure of *B. burgdorferi* are profound as judged by negative staining and electron microscopy. We believe that these micrographs represent the first visualization of electroporated bacteria. Darkly staining regions appear on the surface of the spirochaetes subjected to electroporation, but not on control cells. We suspect that these regions represent pores, induced by the electrical pulse, through which DNA can enter the cell (Nickoloff, 1995; Shigekawa & Dower, 1988; Trevors *et al.*, 1992).

The method presented here for the introduction of site-directed mutations *in vivo* is both simple and convenient. Although the transformation efficiency is low, all of the isolated clones tested contained the mutations encoded by the oligonucleotide. We suspect that the transformation efficiency will increase with oligonucleotide length, as this would allow more target sequence for homologous recombination. Oligonucleotides as small as 20 bases can transform yeast, although transformation is optimal with 50- to 60-base oligonucleotides (Moerschell *et al.*, 1988). There were ~1000 times more oligonucleotide molecules than PCR-generated molecules per electroporation reaction in our experiments. Therefore the 840 bp PCR product results in ~10000-fold more transformants, on a molar basis, than the 27-base oligonucleotide.

The antisense oligonucleotide (411R/398A399A) produced 3- to >30-fold more transformants per molecule than the sense oligonucleotide (385F/398T399T). We do not know what mechanism may be responsible for this phenomenon, but the opposite preference occurs in yeast, with the sense oligonucleotide resulting in 20-fold more transformants than the anti-

sense oligonucleotide (Yamamoto *et al.*, 1992). This implies that if interference with transcription by binding of the sense oligonucleotide to the mRNA occurs in *B. burgdorferi*, it is not a general phenomenon. The mixing experiment suggests that the sense oligonucleotide inhibited transformation in *B. burgdorferi*, while the antisense oligonucleotide did not inhibit transformation in yeast (Yamamoto *et al.*, 1992). Therefore, different mechanisms may be responsible for the different strand preferences in the two organisms. Another possibility for the difference between the transformation efficiencies of the two oligonucleotides in *B. burgdorferi* is that purines are mismatched (G/A and A/A) with the antisense oligonucleotide, while pyrimidines are mismatched (T/C and T/T) with the sense oligonucleotide during recombination. The antisense oligonucleotide, which encodes the reverse complement of an Ile codon (3' TAA 5'), hybridizes with the wild-type chromosomal sense strand, which carries an Arg codon (5' AGA 3'), and the sense oligonucleotide (5' ATT 3') hybridizes with the wild-type chromosomal antisense strand (3' TCT 5'). The homologous recombination system in *Borrelia* may prefer purine mismatches, which are bulkier. A third explanation is that the ends of the two oligonucleotides, which are different (C and A for the antisense and G and T for the sense oligonucleotide), or overall structure, contribute to either their capability to enter cells, stability in cells, or ability to recombine into the chromosome.

Strain HB19 has a lower transformation efficiency than strain B31, although there is a greater difference in transformation efficiencies between B31 (the source of the transformation substrate) and HB19 using the larger PCR-generated substrate compared to using the oligonucleotides. Besides the predicted mutations conferring coumermycin A₁ resistance, there are two nucleotide differences between the 840 bp region of the PCR-generated substrate from B31 and the homologous 840 bp region of the HB19 *gyrB* gene (data not shown). These two point mutations, which are outside of the sequence encoded by the oligonucleotides, may decrease transformation efficiency of the PCR product because mismatch repair could be initiated at more than one site after formation of the heteroduplex and a lethal double strand break might form if the excision tracts overlap.

We plan to use oligonucleotide electroporation to assess the ability of other site-directed mutations in *gyrB* to confer coumarin resistance in *B. burgdorferi*, but the technique may be generally useful for introducing mutations into other genes or into other bacteria. If a convenient positive selection for a phenotype is available, then this method of site-directed mutagenesis is simple, straightforward and inexpensive.

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