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Transformation of *Bartonella bacilliformis*

by Electroporation

by

Helen A. Grasseschi

B. S., The University of Montana--Missoula, 1992

**Presented in partial fulfillment of the requirements
for the degree of Master of Science in Microbiology**

The University of Montana

1994

Approved by

Chairman, Board of Examiners

Dean, Graduate School

April 28, 1994

Date

Transformation of *Bartonella bacilliformis* by ElectroporationDirector: Michael F. Minnick *mf*

Bartonella bacilliformis cannot be transformed by standard chemical methods. We report the first successful transformation of the bacterium by the method of electroporation. The optimal conditions for electroporation of *B. bacilliformis* include a field strength of 12.5 kV per cm and a time constant of 5 ms using 0.2 cm cuvettes. With these parameters and the cosmid pEST (possessing the RK2 origin of replication), a transformation efficiency of 7.8×10^5 CFU per μg of DNA was obtained. Transformants were readily cultured on medium containing kanamycin sulfate at concentrations up to 600 μg per ml. Bacterial survival was approximately 30% under optimal electroporation conditions, and the maximal number of transformants was obtained with 80 ng of pEST DNA. Verification of *B. bacilliformis* as the transformed organism was done by a comparison of transformant protein profiles to wild-type *B. bacilliformis* using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and detection of the presence of the exogenous plasmid in DNA from the transformed bacteria by DNA hybridization. Transformations using the plasmids pMK20, pML31, and pUCK18 (containing the replicons ColE1, F, and pMB1, respectively) were unsuccessful. Attempted mutagenesis of *B. bacilliformis* using the suicide vector pRKTV14 (ColE1 replicon, Tn5 insert containing a kanamycin resistance gene) was also unsuccessful.

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CHAPTER I

Introduction

1.1 Morphology and history.

Bartonella bacilliformis is an intracellular parasite of human cells, and is the etiologic agent of Oroya fever. *B. bacilliformis* is a gram negative bacterium in the eubacterial order *Rickettsiales* (27). The pathogen is the only known motile member of the order, and possesses a tuft of 1 to 10 unipolar flagellae. It is a small microorganism of approximately 0.25 to 0.5 μm in width by 1 to 3 μm in length (27), with a cell wall approximately 200 nm in thickness (30). *B. bacilliformis* is an obligate aerobe (27), and is one of the few rickettsial organisms which can be cultured on artificial media.

Bartonellosis has a very ancient history in the South American Andes, and is believed to be depicted on *huacas* (anthropoid ceramics) dating from about 100 A.D. to 1400 A.D. (44). Bartonellosis has been implicated in the epidemic which claimed the life of Huayana Capac, the last undisputed ruler of the Incan empire, as well as in the epidemic which later killed many of Pizarro's men (44). Due to the lack of accurate documentation and the existence of other tropical diseases which also fit the descriptions of high fever, delirium, and muscle and skeletal pains followed by an eruption of tumors (44), these

implications cannot be proven. It is apparent, however, that bartonellosis has existed in the Andes for many years. The first documented case of the disease occurred in 1871 when laborers were employed to build the Central Railroad of Peru from the city of Lima to the city of Oroya (19, 27). Hundreds of cases of bartonellosis occurred, and the mortality rate reached approximately 40% (27).

The first demonstration that the two distinct phases of bartonellosis were produced by the same organism occurred in 1885 by a Peruvian medical student, Daniel Carrion (27). Carrion entered a national scientific contest in which the best work on verruga peruana would win a prize (19). He decided to study the infectivity of verruga peruana by inoculating himself with scrapings from the lesions on an afflicted patient, and recording all signs and symptoms of the disease (19). He subsequently contracted fatal Oroya fever (27). A second demonstration in 1926 by Hideyo Noguchi showed that organisms isolated from patients with either the hematic phase or the tissue phase of bartonellosis could produce Oroya fever in Rhesus monkeys. *B. bacilliformis* was then re-isolated in pure culture to fulfill Koch's postulates. Unfortunately, some of these experiments have never been repeated. In fact, Oroya fever has never been demonstrated in animals except in an atypical form rarely seen in experimental monkeys (42). It was, however, demonstrated in 1913 by Rocha Lima that bartonellae are definitely present within the verrugas (1).

1.2 Transmission and infection.

The bacterium is transmitted through the bite of the female sandfly of the *Phlebotomus* genus (27). Transmission occurs at night when the female sandfly takes a blood meal, and is further restricted by the ecology of the vector (27). Bartonellosis occurs only in an area 20° north of the equator to 13° south latitude, and in a narrow region from 800 to 2600 meters above sea level (27). Humans are the only known reservoir (19).

In vitro, *B. bacilliformis* appears to enter erythrocytes through a process of forced endocytosis (27). *B. bacilliformis* binds to the surface of erythrocytes and induces deformation of the cell membrane, resulting in prominent pits and invaginations (3). A similar mechanism may be employed *in vivo*; bartonellae have been found in vacuole-like structures within erythrocytes (10). Due to the non-endocytotic nature of erythrocytes, the presence of bartonellae enclosed in vacuoles within the red blood cells (RBCs) implies a mechanism of entry initiated and mediated by the bacterium (34). *B. bacilliformis* can invade capillary endothelial cells, and has been shown to be capable of invading a variety of cell types such as human dermal fibroblasts, HEp-2 cells, HeLa cells, and human umbilical vein endothelial cells (25). This ability of bartonellae to invade both HEp-2 and endothelial cells implies that the apparent preference of *B. bacilliformis* for erythrocytes may be related more to the route of entry rather than to a cell-type specificity (25).

Oroya fever (also called Carrion's disease) is endemic to the Andes regions of South America, namely Ecuador, Peru and Colombia (30). Greater than 60% of the asymptomatic population in these regions are seropositive for *B. bacilliformis* (29), and 5 to 10% of the population are active carriers of the disease (30). Bartonellosis manifests itself in two distinct phases, a primary hematic phase and a secondary tissue phase which produces localized cutaneous eruptions. The hematic phase of the disease presents with fever, malaise, diffuse bone and muscle pain, and perhaps the most severe hemolytic anemia known. *B. bacilliformis* invades nearly all erythrocytes in the blood (3), and reduces the erythrocyte population by nearly 80% (26). Within 4 to 8 weeks after recovery from the primary infection, the secondary phase presents in the form of hemangiomas, termed verruga peruana. This phase is likely due to the invasion of the capillary endothelial cells. *B. bacilliformis* enters the endothelial cells and replicates. The cells are then lysed by an as yet undiscovered mechanism, and the bartonellae are released and can then invade neighboring cells. The eruptions are seen on the face and extremities, and it is thought that they are caused by the hyperproliferation of the terminal vascular endothelial cells in response to an angiogenic factor released by the bacterium during infection (18). The secondary phase can also present with immunosuppression, hepatosplenomegaly, and lymphadenopathy (19). In addition, the verrugas are occasionally seen in individuals who have not displayed clinical symptoms of Oroya fever. Recovery

from bartonellosis is complete, but persistent carrier states can occur (23).

1.3 Phylogenetic relationships.

There are numerous clinically important rickettsial organisms. The order *Rickettsiales* encompasses a diverse group of human pathogens. In the family *Bartonellaceae* there are two genera and three species (5, 42), of which the species *B. bacilliformis* is the only human pathogen. The family *Rickettsiaceae*, however, contains several organisms which are infectious to humans. One example, *Rickettsia rickettsii*, is the etiologic agent of Rocky Mountain spotted fever and is naturally cycled through ticks and various small animals, with humans as an accidental host (27). *Rochalimaea quintana* is the etiologic agent of trench fever, and is transmitted in a human-louse-human cycle. It was seen in epidemic form in both World War's I and II. *Coxiella burnetti* and *Rickettsia prowazekii*, the etiologic agents of Q fever and epidemic typhus, respectively, are two other members of this family.

B. bacilliformis has been found to be most closely related to *R. quintana*, *Brucella abortus*, and *Rochalimaea henselae* based on sequence homology of both the 16S ribosomal RNA (38, 40) and the 5S ribosomal RNA (36) (Fig. 1.1). *B. abortus* causes fetal abortion in cattle as well as undulant fever in humans, and

Figure 1.1. Dendogram showing phylogenetic relationships of *Bartonella bacilliformis*. Family tree based on the sequence homology of the 16S ribosomal RNA or 16S ribosomal RNA genes of various microorganisms from D. A. Relman, et al. (40). BA-TF stands for bacillary angiomatosis-tumor factor, which has recently been identified as the microorganism *Rochalimaea henselae*.

R. henselae has recently been identified as the causative agent of bacillary angiomatosis (a common secondary infection in individuals infected with HIV-1) as well as cat-scratch disease. Research on the molecular biology of rickettsial pathogens is hampered by an inability to grow the majority of rickettsiae axenically, as well as an inability to perform routine genetic manipulations. Members of the order *Rickettsiales* have been notably difficult to transform.

1.4 Transformation

A good definition of natural transformation is "the process by which a bacterium absorbs pure DNA from it's surroundings and functionally integrates the exogenous DNA into it's own genetic material" (48). Natural transformation has been found to occur in many bacteria, including both Gram-positives and Gram-negatives. Transformation was initially thought to be accidental and of no particular significance, until the realization that some bacteria (e.g. *Haemophilus*, *Neisseria*) actually possess proteins that mediate the uptake of DNA as well as specifically recognizing DNA from related species (48). Organisms with this ability are called competent, and may have a distinct survival advantage over non-competent cells. The introduction of exogenous DNA to non-competent cells was accomplished through the infection of the bacteria by bacterial phages (i.e. transduction). However, in 1970, Mandel and Higa discovered that certain strains of

Escherichia coli could be made artificially competent to bacteriophage λ DNA in the absence of helper phage by the addition of Ca^{2+} (33). Subsequently, in 1972, Cohen, Chang, and Hsu reported the ability of *E. coli* to uptake plasmid DNA when treated with calcium chloride, and that the introduced plasmid persisted as an independently replicating unit (6). The ability to induce competence by the addition of ice-cold buffer containing Ca^{2+} followed by a brief heat treatment was found to be effective with many species of bacteria. This resulted in a simple method of manipulating the bacterial genome which could be used as an alternative to transduction. In transformation, plasmid vectors can be introduced into the host organism directly. In addition, transformation provides a means of introducing DNA to genetically engineer organisms and has been a major contributor to the development of recombinant DNA techniques (48).

The ability to manipulate the bacterial genome has been an important tool in understanding the various functions and mechanisms employed by the organisms. The introduction of functional DNA and the subsequent observation of the effect of that DNA on the normal activity of the bacteria, as well as the examination of the products of certain gene segments in cells which normally do not contain those segments, are methods of investigation which are revealing and relatively simple due to the tool of transformation. Since the initial experiments, many investigators have determined various ways of optimizing the

system of transformation. Transformation efficiencies (number of transformants per μg of DNA) can be increased through longer incubations with calcium chloride (11), the addition of several different cations to the reaction mixture (37), and the treatment of the bacteria with dimethyl sulfoxide (DMSO), hexaminecobalt, and dithiothreitol in the presence of monovalent and divalent cations (21). Manipulations of these protocols have extended the list of microorganisms which have been successfully and stably transformed. Unfortunately for rickettsiologists, these methods have proved incapable of rendering rickettsial organisms competent. To date, *Rochalimaea quintana* is the only member of the *Rickettsiales* order to be stably transformed (41).

1.5 Electroporation.

R. quintana was stably transformed with the cosmid vector pEST by the method of electroporation (41). Electroporation is a physical, rather than a chemical, method of transformation. It involves the introduction of an electric current to a solution containing both bacterial cells and exogenous DNA (15). This procedure was first developed for use in the introduction of exogenous DNA into both plant protoplasts (17) and mammalian cells (7), and has been shown to increase the frequency of eukaryotic cell fusion events (51). At this point, several investigators developed electroporation systems for use with bacterial cells. In general, bacterial electroporation involves

small-volume samples of high resistance, high voltages, and narrow electrode gaps. Intact bacteria of many species have been successfully transformed by electroporation, but the actual mechanism of exogenous DNA uptake has not been elucidated.

In prokaryotic electroporation, an electric pulse of high voltage is applied to a DNA-plus-bacteria cell suspension for a period of time using a suitable capacitor, e.g. the Bio-Rad Gene Pulser. The electric field is defined as the voltage applied across the distance between electrodes:

$$E = V/d$$

Both the electric field and the voltage applied quickly rise to a peak (E_0 and V_0 , respectively) and then decline over time,

$$E_{(t)} = E_0[e^{-(t/\tau)}]$$

$$V_{(t)} = V_0[e^{-(t/\tau)}]$$

where τ is the RC time constant (resistance [ohms] times capacitance [farads]) and is recorded in seconds or milliseconds (ms). Using a Bio-Rad Pulse Controller unit, the sample is placed in series with a 20 ohm (Ω) resistor and in parallel with a selection of 100 to 1000 Ω resistors (15). The total resistance of the system is then

$$1/R_{\text{total}} = 1/R_{\text{series}} + 1/R_{\text{sample}} + 1/R_{\text{parallel.1}} + 1/R_{\text{p.2} \dots} + 1/R_{\text{p.x}}$$

where x equals the total number of resistors set in parallel to the sample. If the resistance of the sample is high, then the resistance of the system is determined by the resistors in parallel to the sample. As a result, the duration of the pulse (RC time constant) can be determined manually, and held constant.

The 20 Ω resistor is used to limit the current and protect the instrument should arcing of the sample occur (15).

The mechanism of electroporation appears to rely on a reversible permeabilization of the cellular membrane. Tsong (47) suggested several properties of cellular membranes which could lend themselves to permeabilization by electrical currents. First, the charges on the lipid molecules would cause them to reorient in an intensely applied electric field, resulting in hydrophilic pores and an inability to function as a barrier against ions. Also, the finite permeability of the membrane to small ions would conduct current and generate local Joule heating, which would result in thermal phase transitions of the bilayer. Both of these events would result in lipid bilayer rearrangement; the expansion or creation of hydrophobic pores and the formation of structurally stable hydrophilic pores. Another possibility is that electroporation in a cell membrane can also occur in protein channels, which are also known to depend on transmembrane electric potential (22, 46).

There is a compensatory relationship between the amplitude of the electric field and the duration of the pulse (15, 47). As the electric field is increased, the time constant must be decreased in order to maintain optimal transformation efficiencies. The opposite of this is also true; as the time constant is increased the amplitude of the electric field must be decreased. This relationship is due to the reversible nature of the conformational changes induced in the cell membrane. If the

electric field is high, the amount of rearrangement and molecular inversion will be high. In order to avoid irreversible damage to the cell membrane, the duration of the pulse must be short enough to allow the cell membrane to 'reseal' itself in a functional manner. If the electric field applied is of low magnitude, the cell will be able to tolerate the applied current for a longer period of time without undergoing irreversible damage.

The actual uptake of DNA into the host cell appears to be accomplished by either passive diffusion of the DNA into the cell or bulk flow of the medium containing the DNA into the cell (15). The observation that a pre-electroporation incubation does not significantly change efficiencies of transformation implies that there is no need for the DNA to bind to the cells prior to transformation. Whatever the actual mechanism, electroporation appears to depend upon a physical property of bacterial cells (i.e. the presence of a cellular membrane) (7, 37) and this may account for the high rate of success seen with many different organisms as well as cell types.

1.6 Specific aims and goals.

Given the close phylogenetic relationship between *Rochalimaea* species and *B. bacilliformis* (Fig. 1) (36, 38), as well as the ability to grow bartonellae axenically, we wanted to

determine if *B. bacilliformis* could be stably transformed using the method of electroporation. Several attempts to transform *B. bacilliformis* using classical chemical methods had previously failed in our hands.

In the transformation of *R. quintana* by electroporation (41), several vectors, were used. We concerned ourselves only with those which contained a gene for kanamycin resistance. These vectors were pEST (41, Fig. 1.2), pUCK18, pMK20, and pML31 (41, 28, and 32 respectively, described in Table 1.1).

In this study we optimized the conditions for stable transformation of *B. bacilliformis* by electroporation using the cosmid vector pEST (41). We then attempted transformation of *B. bacilliformis* with several other vectors possessing different origins of replication to determine which replicons are functional in *B. bacilliformis*. Expression of the exogenous DNA by the bacterium was demonstrated by stable growth on selective media containing kanamycin sulfate, and transformation was verified by both DNA hybridization and SDS-PAGE analysis of cell lysates from the transformed organisms.

Figure 1.2. Diagram of the cosmid pHK17; the parent of the cosmid vector pEST (41). The tetracycline resistance gene was excised from the cosmid pHK17, and the resulting cosmid was called pEST (41). Abbreviations: tet^r tetracycline resistance; kan^r kanamycin resistance; *cos* represents a λ *cos* site present on the vector. The RK2 *ori* is the RK2 origin of replication.

pHK17

CHAPTER II

Materials and Methods

Bacterial growth conditions. *B. bacilliformis*, strains KC583 and KC584 were purchased from the American Type Culture Collection, Rockville, Md. The bacteria were grown in high humidity at 30°C on HIBB [heart infusion agar, (Difco Laboratories) enriched with 5% defibrinated sheep erythrocytes (v/v) and 5% filter-sterilized sheep serum (v/v) (Colorado Serum Co.)]. Bartonellae were typically harvested at 4 days post-inoculation. *Escherichia coli* strains containing the plasmids of interest (Table 1.1) were routinely grown overnight in Luria-Bertani medium (12) at 37°C with constant shaking. When required, kanamycin sulfate (Sigma Chemical Co.) was added to the growth medium at concentrations ranging from 15 to 600 µg per ml.

DNA isolation. Plasmids used in this study are listed in Table 1.1, and were extracted and purified from *E. coli* using standard alkaline extraction (4), or by a Quiagen Midi Prep kit

(Quiagen, Inc.). For the alkaline extraction, the cells were lysed with lysozyme, 0.01 M EDTA, and 0.25 M Tris (pH 8.0). The proteins were denatured by the addition of 400 μ l 10% (w/v) sodium dodecyl sulfate (SDS), and the total DNA was precipitated with 300 μ l 3 M sodium acetate (pH 5.0). The plasmids were then shaken out of the protein/DNA clot. After centrifugation, the supernatant was aspirated and the plasmid DNA was precipitated with isopropanol, resuspended in 0.052 M Tris (pH 8.0) plus 0.102 M sodium acetate, and precipitated again with 95% ethanol. Isolated plasmids were further purified by a chloroform:isoamyl alcohol (24:1; v/v) extraction followed by a 95% ethanol precipitation. Plasmid concentrations were obtained visually by comparing to DNA standards of known concentration on ethidium bromide-stained agarose gels and/or by optical density readings at 260 nm. The cosmid pEST was used to optimize the transformation of *B. bacilliformis* by virtue of its successful use in *R. quintana* (41) and is diagrammed in Fig. 1.2.

The chromosomal DNA of both transformed and untransformed *B. bacilliformis* was extracted by standard protocol (2). Briefly, bacteria were harvested at 4 days post-inoculation into 100 μ l TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 7.4), containing 5 μ l of 10% SDS (w/v) and 40 μ g of proteinase K (Sigma). The mixture was incubated for 1 h at 50°C. The DNA was subsequently extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v), gently shaken, and centrifuged for 1 min at 16,000 x g in a microcentrifuge. The aqueous phase was

TABLE 1. List of cosmids and plasmids with kanamycin-resistance genes used to transform *B. bacilliformis* by the method of electroporation

<u>Vector</u>	<u>Origin</u>	<u>Transformation^a</u>	<u>Reference^b</u>
pUCK18	pMB1	-	41
pMK20	ColE1	-	28
pML31	F	-	32
pEST	RK2	+	41
<u>pRKTV14</u>	<u>ColE1</u>	<u>-</u>	<u>52</u>

^aSuccessful transformation was verified by DNA hybridization. Successful transformation was determined by a minimum of six experiments.

^bOriginal description of vector.

aspirated and extracted with 1 volume of chloroform-isoamyl alcohol (24:1; v/v) and was vortexed and centrifuged again. The aqueous phase was collected, and 15 μ l of 2 M ammonium acetate (pH 7.0) was added and mixed. The DNA was precipitated by adding 1 ml of 95% ethanol and incubating for 20 min at 22°C. The precipitate was then centrifuged for 15 min at 16,000 x g. The resulting DNA pellet was dried and resuspended in 50 μ l sterile distilled water and frozen at -20°C until needed.

Southern blotting. Chromosomal or plasmid DNA was digested to completion with *Sma*I (GIBCO-BRL), and the resulting digest was run on an ethidium bromide-stained-0.8% agarose (w/v) gel at 15 V overnight. Blotting of the gel to nitrocellulose (0.45 μ m pore size) (Schleicher and Schuell, Inc.) was accomplished by the methods of Southern (45). The gel was washed in 0.25 N HCl for depurination for 15 min. It was then incubated in the Southern base solution (0.5 N NaOH, 1.5 M NaCl) for 15 min, rinsed briefly in distilled water, and washed in the Southern base for an additional 30 min. The gel was neutralized to a pH of 8.0 to 8.5 in Southern neutralization buffer (0.5 M Tris-HCl, 3.0 M NaCl, pH 7.0), and rinsed briefly with 2X SSC (90 mM-sodium chloride plus 9.0 mM-sodium citrate) to remove the neutralizing buffer. The gel was placed on Whatman filter paper on a wick blotter.

Nitrocellulose paper (NCP) was cut to the size of the gel, wetted and equilibrated with 20X SSC, and placed onto the gel surface, followed by Whatman paper and paper towels. The nitrocellulose filter paper (NCP) was weighted to aid in the wicking process,

and after 12 to 16 h was dried, baked at 80°C for 60 min, and placed in a pre-hybe solution (6X SSC plus 10X Denhardt's reagent [43]) for 1 h. This was followed by a 4 h equilibration in the hybridization solution (5X SSC, 5X Denhardt's, 20 mM Tris-HCl [pH 8.0], 1.0 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], and 20 µg/ml salmon sperm DNA). The NCP was then hybridized overnight at 45°C with *Sma*I-digested pEST cosmid (41) which had been radiolabeled with [α -³²P]dCTP by random primer extension (16). For radiolabeling, the pEST cosmid probe was digested to completion with *Sma*I, and heat denatured for 10 min. The digested, denatured DNA was immediately placed on ice to prevent reannealing. OLB buffer (0.10 mM dATP, 0.10 mM dGTP, 0.10 mM dTTP, random hexamers [27 OD units/ml], and buffer [0.24 M Tris-HCl, 1.0 M Hepes, pH 6.6]), bovine serum albumin (BSA, 20 µg), 50 µCi [α -³²P]dCTP (NEN), and 2 units of *E. coli* DNA polymerase I klenow fragment (GIBCO-BRL) were all added, and the reaction mix was incubated at room temperature for 4 h. After incubation, the probe was heat denatured and added to the hybridization mixture. After hybridization, the blot was washed four times at high stringency for 30 min at 65°C with 1X SSC with 0.1% SDS (w/v) and 5 mM-EDTA added. The blot was then exposed onto XAR-5 X-OMAT (Kodak Co.) film to visualize the hybridized DNA bands.

SDS-polyacrylamide gel electrophoresis. Transformed or wild type strains (KC583 and KC584) of *B. bacilliformis* were harvested from HIBB, pelleted by centrifugation at 2940 x g for 5 min, and resuspended in 75 µl ice-cold, sterile distilled water. An equal

volume of Laemmli sample buffer (31) was added, and the mixture was boiled for 5 min to denature the proteins. Denatured proteins were then electrophoresed on SDS-PAGE (12.5% acrylamide; w/v) gel by the general methods of Laemmli (31). The gel was subsequently fixed for 30 min in 50% methanol (v/v) and silver stained by the methods of Wray et al. (49).

Electroporation of *B. bacilliformis*. Bartonellae were aseptically harvested from HIBB plates into 1 ml of heart infusion broth (Difco) at 22°C. The suspension was centrifuged at 2940 x g for 5 min at 4°C. The pellet was then washed with 1 ml of ice-cold 10% glycerol-water (v/v), and centrifuged again for 5 min at 2940 x g. The bacterial cell concentration was obtained using a Petroff-Hauser counter and adjusted to 1×10^{10} cells/ml with 10% glycerol. Forty μ l of the bacterial suspension (4×10^8 cells) was then mixed with 20 ng of plasmid DNA in a microcentrifuge tube, transferred to a 0.2 cm gap electroporation cuvette (Bio-Rad Laboratories), and chilled on ice for 5 min. Electroporation was then performed using a Bio-Rad Gene Pulser (model #1652076) with an exponential decay waveform set at field strengths of 12.5, 10, 8, and 6 kv/cm, and at pulse times of 5 and 10 ms, (i.e. resistance values of 200 and 400 Ω , respectively). The capacitance was held constant at 25 μ F for all experiments.

Immediately following electroporation, bacteria were rinsed with 1 ml of sterile recovery broth [heart infusion broth containing 0.5% (w/v) bovine serum albumin, and 5% (v/v) human

erythrocyte lysate (3) prepared by mixing 1 volume of outdated human red blood cells (American Red Cross) with 3 volumes of cold distilled water, gently shaking to lyse, and centrifuging at 3000 x g for 15 min at 4°C to remove debris] and incubated at 30°C for 14 h, i.e. approximately two bartonellae generation times (3). Following incubation, the electroporated bacteria were plated onto HIBB plates containing kanamycin sulfate at 15 µg per ml. Suspected transformants were subcultured at 6 days post-inoculation onto HIBB-kanamycin plates for use in SDS-PAGE and/or DNA isolation. Colony forming units (CFU) were counted to determine transformation efficiencies and the percent of bacterial cell survival, as well as to determine the effect of plasmid DNA concentration on the transformation efficiency.

Statistical analysis. Numerical values in this study are expressed as the mean $\pm$ standard error of the mean (SEM), with the number of trials equal to 3 (n=3). Statistical significance of the data was determined through the use of Students t test, where a P value <0.05 was considered significant.

CHAPTER III

Results

3.1 Optimization of electroporation conditions.

Initial transformants were obtained by applying a field strength of 12.5 kV per cm to the bacterial cell-pEST DNA suspension for 10 ms. Colonies began to appear at approximately 6 days following electroporation and were typically smaller than colonies formed by untransformed *B. bacilliformis* at the same time after inoculation. There was an apparent growth lag time of approximately 2 days as compared to untransformed bartonellae. Growth was observed on HIBB plates containing kanamycin sulfate at concentrations ranging from 15 μ g per ml (usual selection pressure) to 600 μ g per ml. The transformants could be subcultured repeatedly on the selective medium, suggesting that stable transformation had occurred. Upon subculturing, the growth of the transformants resembles that of the wild-type bartonellae (i.e. colony size, rate of growth).

To determine which voltages produced optimal transformation efficiencies, *B. bacilliformis* was transformed with the cosmid vector pEST at field strengths of 12.5, 10, 8, and 6 kV per cm and at time constants of 5 (Fig. 3.1) and 10 (Fig. 3.2) ms. At both time constants, the optimal transformation efficiency occurred when an electric field of 12.5 kV per cm was applied to

Figure 3.1. The effect of field strength on the transformation efficiency of *B. bacilliformis*. *B. bacilliformis* was electroporated with 25 ng of pEST DNA at a resistance of 200 Ω and a capacitance of 25 μF , which translates to a time constant of 5 ms. Each data point represents the average of three experiments $\pm$ SEM. Transformation efficiency is recorded as CFU $\times 10^5$ per μg of pEST DNA.

Figure 3.2. The effect of field strength on the transformation efficiency of *B. bacilliformis*. *B. bacilliformis* was electroporated with 25 ng of pEST DNA at a resistance of 400 Ω and a capacitance of 25 μ F, which translates to a time constant of 10 ms. Each data point represents the average of three experiments $\pm$ SEM. Transformation efficiency is recorded as CFU $\times 10^5$ per μ g of pEST DNA.

transformation mixture. Although not statistically significant, the transformation efficiencies obtained at 5 ms (Fig. 3.1) were slightly higher than the efficiencies obtained at 10 ms (Fig. 3.2). As a result, all subsequent experiments were performed using a time constant of 5 ms.

The transformation efficiencies showed a direct correlation with the field strength applied during electroporation (Fig. 3.1). Optimal transformation efficiencies of 7.8×10^5 were obtained at a voltage of 2.5 kV, which corresponds to a field strength of 12.5 kV per cm. These results are similar to those obtained for the closely-related bacterium, *R. quintana* (41). However, due to the limits of the Bio-Rad Gene Pulser and the 0.2 cm electroporation cuvettes, field strengths beyond 12.5 kV per cm could not be analyzed. It is possible that greater field strengths may yield greater transformation efficiencies since an apparent peak value was not obtained (Fig. 3.1 and Fig 3.2).

3.2 The effect of DNA concentration on the number of transformants recovered and the effect of variable field strength on the percent of bacterial cell survival.

The effect of DNA concentration on the transformation efficiency under optimal conditions was also analyzed using Quiagen-purified pEST cosmid (Fig. 3.3). Serial dilutions of the pEST DNA were carried out, and *B. bacilliformis* was electroporated under optimal conditions (i.e. 12.5 kV per cm, 5

Figure 3.3. The effect of pEST cosmid DNA concentration on the transformation of *B. bacilliformis*. Electroporation conditions were optimal, i.e. 12.5 kV per cm, 5 ms, and 25 μ F. Each data point represents the mean of three experiments $\pm$ SEM.

ms) with DNA concentrations ranging from 2 to 300 ng. The greatest number of transformants (determined by counting colony forming units [CFU]) was observed when the bacteria were electroporated with 80 ng of pEST DNA (Fig. 3.3).

Bacterial cell survival during electroporation was also determined (Fig. 3.4). *B. bacilliformis* was electroporated in the absence of exogenous DNA at 12.5, 10, 8, and 6 kV per cm. The percentage of cells surviving electroporation (i.e. CFU on the growth media) as a function of field strength was then calculated using un-treated bartonellae as a negative control. The percent survival for *B. bacilliformis* at optimal conditions was approximately 30% (Fig. 3.4). This value compares favorably to the percent survival at optimal electroporation conditions for *R. quintana*, where survival values of approximately 20% were obtained (41).

3.3 Verification of transformation.

Southern blot analysis was performed on the extracted chromosomal DNA of transformed bacteria to verify the presence of the pEST cosmid. DNA hybridization was performed as described above. The radiolabeled pEST hybridized to comigrating bands of 16,000 and 6,000 base pairs (bp) in both the *Sma*I-digested pEST cosmid and in the *Sma*I-digested DNA obtained from transformed *B. bacilliformis* (Fig. 3.5B, lanes 4 and 3, respectively). The hybridization data demonstrates that the fragments contained

Figure 3.4. The effect of field strength on the post-pulse survival of electroporated *B. bacilliformis*. Bacteria were electroporated at 5 ms and 25 μ F. Each data point represents the mean percent survival of three experiments +/- SEM.

within the transformed *B. bacilliformis* total DNA are fragments of the pEST cosmid and that no hybridization occurs on the chromosomal DNA of untransformed bartonellae (Fig. 3.5B, lane 2). The slight hybridization signal in the λ HindIII size standard (Fig. 3.5B, lane 1) is presumably due to homologous regions common to both the pEST cosmid and the λ marker (i.e. λ cos sites). Comparison of the DNA-fragment banding patterns for both the native and transformed strains of *B. bacilliformis* (Fig. 3.5A) demonstrates comigration of all bands except for the 16,000 and 6,000bp fragments seen in the chromosomal digest of the transformed strain. This suggests that the transformed bacteria is *B. bacilliformis*.

Further verification of the transformed bacteria as *B. bacilliformis* was determined by SDS-PAGE protein profiles of the cell lysate (Fig. 3.6). SDS-PAGE protein profiles of cell lysates comparing transformed *B. bacilliformis* to untransformed *B. bacilliformis* were electrophoresed and subsequently silver stained. The resulting protein profile seen in this gel (Fig. 3.6) was identical for both strains of native *B. bacilliformis* (KC583 and KC584) as well as the transformed bartonellae. These data again demonstrate that the transformed organism was *B. bacilliformis*.

3.4 Electroporation with additional vectors and mutagenesis.

Transformation by electroporation of *B. bacilliformis* was

Figure 3.5. Chromosomal digest and DNA hybridization analysis comparing total DNA of untransformed *B. bacilliformis* and *B. bacilliformis* transformed with pEST. (A) Ethidium bromide-stained agarose gel (0.8%, w/v). Lane 1, λ Hind III size standard (indicated to the left in kb); lane 2, *Sma*I-digested total DNA from native *B. bacilliformis* (KC584); lane 3, *Sma*I-digested total DNA from transformed *B. bacilliformis* (KC584); lane 4, *Sma*I-digested pEST cosmid (39) DNA. (B) Corresponding DNA hybridization of gel pictured in (A) blotted and subsequently probed with 32 P-labeled pEST DNA. Co-migrating hybridization signals (lanes 3 and 4) are arrowed.

attempted using the vectors pUCK18, pML31, and pMK20 possessing the origins of replication pMB1, F, and ColE1, respectively (Table 1.1). Electroporations were performed at optimal conditions for transformation with pEST. At approximately 8 days post-electroporation possible transformants were observed. However, upon prolonged incubation (i.e. 14 days) and attempts at subculturing, the transformation was seen to be unstable. No stable transformants were recovered through the course of 6 trials, and Southern blot analysis showed no *B. bacilliformis* in the samples collected at 8 days post-electroporation. These results were consistent with those for *R. quintana* using the same replicons (41).

Mutagenesis of *B. bacilliformis* was attempted using the suicide vector pRKTV14 (52). This vector possesses the ColE1 replicon as well as a Tn5 insert which contains a gene for kanamycin resistance. Upon introduction into the host cell, the vector undergoes several rounds of replication before failing. This would allow the transposase gene of Tn5 to be transcribed, and the transposon could then randomly insert itself into the host chromosome. Electroporation of *B. bacilliformis* was performed at optimal conditions with the suicide vector pRKTV14. The results were as described for transformation by vectors other than the pEST cosmid. Possible transformants were observed at 8 days post-inoculation, but were not stable under prolonged incubation (14 days) or attempted subculturing.

Figure 3.6. SDS-PAGE (12.5% acrylamide, w/v) protein profile comparing the banding patterns of cell lysates of native *B. bacilliformis* and *B. bacilliformis* transformed with the pEST cosmid. Lane 1, protein molecular mass standards; lane 2, pEST-transformed *b. bacilliformis* (KC584); lane 3, *B. bacilliformis* (KC583); lane 4, *B. bacilliformis* (KC584). M_r values for the protein standards are given to the left in kDa.

CHAPTER IV

Discussion

This study describes the first successful transformation of a member of the *Bartonellaceae* family, and only the second member of the *Rickettsiales* order. Using the pEST vector (41) and parameters which had been used with success for the closely-related bacterium *R. quintana* (41), we developed and optimized a system for the stable transformation of *B. bacilliformis*, obtaining transformation efficiencies up to 7.8×10^5 CFU/ μ g DNA. A further study involving either increased voltages or decreased cuvette gaps would allow the investigator to determine if electric fields greater than 12.5 kV per cm (the maximal amplitude used in this study) would result in increased transformation efficiencies. Dower et al. electroporated *E. coli* using electroporation cuvettes with a gap distance of 0.15 cm (15). At 2.5 kV, this translates to a field strength of 16.7 kV per cm. Transformation efficiencies for *E. coli*, when subjected to a field strength of this magnitude, were decreased as compared

to the efficiencies obtained at 12.5 kV per cm. Both *B. bacilliformis* and *R. quintana* displayed optimal transformation efficiencies at 12.5 kV per cm, with no apparent peak value. In light of the data obtained for *E. coli* and the ease with which *E. coli* is transformed as compared to the rickettsial organisms, it is possible that if *B. bacilliformis* were exposed to higher field strengths the transformation efficiencies would decline. The efficiencies obtained in this study, however, are adequate for further research requirements, and subculturing provides vigorous growth of the transformants. The transformants are viable after freezing at -70°C in 12.5% (v/v) glycerol, and regain vigorous growth after a 2 to 3-day lag period. The transformed bacteria were able to grow on HIBB plates containing kanamycin sulfate at concentrations ranging from 15 μg per ml up to 600 μg per ml. Both newly transformed bartonellae and specimens frozen at -70°C displayed stable growth when subcultured on media containing the antibiotic at 600 μg per ml.

The effect of exogenous DNA concentration on the transformation efficiency of *B. bacilliformis* was consistent with the literature for other organisms. The transformant recovery of *B. bacilliformis* increased linearly with increasing DNA concentrations up to 80 ng (Fig. 3.3). For *E. coli*, transformant recovery increased linearly with DNA concentrations over a range from 0.4 pg to 0.3 μg of added DNA, with no peak value reached (15). The same was true for *Acidiphilium* strains up to a DNA concentration of 10 $\mu\text{g}/\text{ml}$ (20).

Our data displayed a peak number of bartonellae transformants at 80 ng, with a subsequent decrease at higher concentrations of exogenous DNA (Fig. 3.3). A similar pattern of the number of transformants recovered was also seen in the yeast *Saccharomyces cerevisiae*, with the number of transformants recovered increasing up to a concentration of 1000 ng DNA. At higher concentrations of added DNA, the number of transformed yeast cells decreased without obtaining a steady level (13). This type of curve appears to be fairly common among electroporated bacteria, with absolute values varying with the bacteria and vector used. It is apparent that exogenous DNA concentrations higher than 80 ng are in some manner detrimental to the survival of the bartonellae (as well as the yeast). Unfortunately, this type of experiment was not performed with *R. quintana* and we have no other data of this type for relatives of *B. bacilliformis*. A study of the effect of DNA concentration on the percent of bacterial cell survival could be beneficial in understanding this decline in the number of stably transformed organisms.

The survival percentages obtained (Fig. 3.4) were also consistent with the data obtained for other microorganisms. Throughout the literature, optimal transformation efficiencies range from 20 to 50% cell survival (15, 41, 14, 13).

This optimized system allows the introduction of exogenous DNA into the previously untransformable *B. bacilliformis* at rates of survival and with transformation efficiencies similar to several other bacterial species including *R. quintana* and

Pseudomonas aeruginosa (41, 14). A comparison of chromosomal digests of both transformed and untransformed bartonellae (Fig. 3.5), as well as comparison of SDS-PAGE cell lysate protein profiles (Fig. 3.6), suggest that *B. bacilliformis* is surviving the electroporation protocol and subsequently replicating. This study shows that *B. bacilliformis* is capable of utilizing the RK2 origin of replication, as well as having the ability to express the kanamycin resistance gene of pEST (Fig. 1.2).

The lack of success in the transformation of *B. bacilliformis* with the vectors pUCK18, pML31, and pMK20 (pMB1, F, and ColE1 origins, respectively) was not surprising. None of these vectors had been used with success in the transformation of the closely-related bacterium, *R. quintana* (41). In addition, we restricted our vectors to those which contained only a kanamycin resistance gene, due to the inability to use kanamycin as a chemotherapeutic agent in clinical cases of Oroya fever. Because of this restriction, we were unable to attempt transformation of *B. bacilliformis* with the vector pSUP204Ts (chloramphenicol resistance, replicons pMB1 and RSF1010 [39]), which had been the only other vector successful in the transformation of *R. quintana* (41). It is apparent that of the four replicons used, only the RK2 origin of replication is stably functional in *B. bacilliformis*.

Upon electroporation of the bartonellae with the additional vectors, there was apparent *B. bacilliformis* growth evident at approximately 8 days of incubation. We feel that this was due to

unstable replication of the exogenous DNA and transient kanamycin resistance. The colonies observed were smaller than those seen with pEST-transformed bartonellae, and additional growth time was required to visualize the colonies. In addition, the number of colonies observed was very low as compared to that of the pEST-transformed bacteria. These observations can possibly be explained by the generation time of *B. bacilliformis* (~14 h); unstable transformation would occur in a reduced number of bacteria, and it would subsequently take longer to visualize the colonies (~8 days instead of 6). In addition, the 'transformants' would eventually lose their resistance to the kanamycin (i.e. no plasmid replication) resulting in smaller, as well as fewer, colonies formed.

The lack of transformation success using these vectors is beneficial in determining what requirements *B. bacilliformis* might have to accept and utilize exogenous DNA. We now know that several replicons do not function in bartonellae. We also know that *B. bacilliformis* is capable of utilizing a kanamycin resistance gene as demonstrated by both the successful transformation with pEST and the unstable transformation exhibited by bartonellae electroporated with the other three vectors.

Mutagenesis of *B. bacilliformis* with the Tn5-containing suicide vector pRKTv14 (Table 1.1, [52]) was also unsuccessful. This lack of success is most likely due to the ColE1 replicon present on the vector. This replicon was present on the plasmid

pMK20, and does not appear to be functional in bartonellae. As with the three unsuccessful vectors, bartonellae exhibited unstable transformation when electroporated with pRKTV14, suggesting that an unstable transformation event occurred.

To successfully mutagenize *B. bacilliformis*, a suicide vector should be constructed containing both an RK2 origin of replication as well as Tn5, which carries a kanamycin resistance marker. A search of the literature indicates that there are several vectors which could be manipulated to fulfill these requirements. Once constructed, a vector compatible with *B. bacilliformis* could be introduced into bartonellae with relative ease using the system of electroporation which we have specifically optimized for this bacterium.

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