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Evidence of a conjugal erythromycin resistance element in the Lyme disease spirochete *Borrelia burgdorferi*

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Abstract

We report the identification of isolates of *Borrelia burgdorferi* strain B31 that exhibit an unusual macrolide–lincosamide (ML) or macrolide–lincosamide–streptogramin A (MLS_A) antibiotic resistance pattern. Low-passage isolates were resistant to high levels (>100 μg/mL) of erythromycin, spiramycin and the lincosamides but were sensitive to dalfopristin, an analogue of streptogramin B. Interestingly, the high-passage erythromycin-resistant strain B31 was resistant to quinupristin, an analogue of streptogramin A (25 μg/mL). Biochemical analysis revealed that resistance was not due to antibiotic inactivation or energy-dependent efflux but was instead due to modification of ribosomes in these isolates. Interestingly, we were able to demonstrate high-frequency transfer of the resistance phenotype via conjugation from *B. burgdorferi* to *Bacillus subtilis* (10^{-2}–10^{-4}) or *Enterococcus faecalis* (10^{-5}). An intergeneric conjugal system in *B. burgdorferi* suggests that horizontal gene transfer may play a role in its evolution and is a potential tool for developing new genetic systems to study the pathogenesis of Lyme disease.

Keywords

Borrelia burgdorferi; Erythromycin; Antimicrobial resistance; Conjugation

1. Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, is the number one vector-borne disease in the USA [1]. It is a multistage disorder that is difficult to diagnose at any stage of the disease as well as being difficult to treat during the later symptoms. However, during the early stages of the disease, treatment with various antibiotics such as amoxicillin or doxycycline is very efficacious [2]. In vitro, *B. burgdorferi* cells have high minimum inhibitory concentrations (MICs) for some aminoglycosides (e.g. gentamicin, MIC > 16 μg/mL), quinolones (e.g. nalidixic acid, MIC > 10 μg/mL) and first-generation cephalosporins (e.g. cefadroxil, MIC > 11 μg/mL) but are very susceptible to β-lactams, tetracyclines,

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fluoroquinolones, everninomycins and macrolides, suggesting that several of these antimicrobials would be effective for the treatment of Lyme disease [3–11]. However, therapeutic failures have been reported with most of the appropriate antimicrobials. In fact, poor treatment outcomes have caused the removal of some drugs (e.g. erythromycin) from the list of suitable antibiotics [12]. Whether these failures are due to poor pharmacokinetics in vivo or to the emergence of drug-resistant strains remains unclear. Clearly, both possibilities need to be investigated.

To date, there has been only one report of drug-resistant clinical isolates of *B. burgdorferi* [13]. This could be due to the difficulty in isolating *B. burgdorferi* from patients during later stages of the disease, making it hard to determine whether drug-resistant strains are a major cause of poor clinical outcomes. With other bacterial pathogens, the emergence of antibiotic-resistant strains due to mutational events or through the acquisition of foreign DNA has been a major problem in effective treatment. The ease with which resistance genes spread throughout bacterial populations via genetic exchange (e.g. conjugation, transduction or natural transformation) is a major contributing factor. Eggers et al. [14] have shown that *B. burgdorferi* phage ΦBB-1 can transfer genetic markers between different strains, suggesting that beneficial genetic traits can move within *B. burgdorferi* populations via transduction. However, owing to the specificity and limited host range of bacteriophages, it is unlikely that *B. burgdorferi* phage ΦBB-1 fosters the transfer of traits between different genera of bacteria.

We report the isolation of low-passage (LP) and high-passage (HP) strains of *B. burgdorferi* B31 that exhibit macrolide–lincosamide (ML) or macrolide–lincosamide–streptogramin A (MLS_A) resistance patterns. Characterisation of the resistance mechanism suggests that it is due to modification of the ribosomes. More importantly, we demonstrate that the erythromycin-resistant (Em^R) phenotype was transferred via conjugation from *B. burgdorferi* to *Bacillus subtilis* or *Enterococcus faecalis*.

2. Materials and methods

2.1. Bacterial strains and reagents

The *B. burgdorferi*, *Escherichia coli*, *E. faecalis*, *B. subtilis* and *Streptococcus pyogenes* strains used in this study are listed in Table 1. *Borrelia burgdorferi* were grown in BSK-II medium [23] at 34 °C under an atmosphere of 3–5% O_2, 5% CO_2 and 90% N_2 and cell numbers were determined by dark field microscopy. *Escherichia coli* and *B. subtilis* were grown in Lauria–Bertani (LB) and brain–heart infusion (BHI) medium, respectively. Quinupristin (streptogramin B) and dalfopristin (streptogramin A) were obtained from Aventis Pharma S.A. (Cedex, France). Rabbit serum was purchased from Atlanta Biologicals (Atlanta, GA). [*N*-methyl-^{14}C]-erythromycin (50 mCi/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma Aldrich (St Louis, MO).

2.2. Isolation and initial characterisation of Em^R B. burgdorferi strains

Em^R strains of *B. burgdorferi* were isolated by plating strains on BSK-II medium containing erythromycin (10, 25 or 50 μg/mL) as described by Samuels [24]. Plates were incubated in a BBL anaerobic GasPak jar without catalyst (Becton Dickinson, Sparks, MD) at 34 °C for 7–14 days. For induction, cultures were grown in BSK-II medium to a cell density of 2×10^7 cells/mL, erythromycin (0.04 μg/mL) was added and the incubation was continued for 24 h. Induced cultures were plated as described above. Resistant colonies were transferred to BSK-II medium containing erythromycin (50 μg/mL), grown to a cell density of 5×10^7 cells/mL and isolates were stored at −80 °C in 50% glycerol.

2.3. Resistance to lincosamides and streptogramins

Erythromycin-sensitive (Em^S) and Em^R *B. burgdorferi* isolates were grown in BSK-II medium to a density of ca. 5×10^7 cells/mL and plated on BSK-II medium containing erythromycin (1–100 μg/mL), lincomycin (0.5–15 μg/mL), clindamycin (0.5–10 μg/mL), virginiamycin M (1–15 μg/mL), quinupristin (1–15 μg/mL) or dalfopristin (1–15 μg/mL).

2.4. Erythromycin inactivation assay

Inactivation assays were performed as described by Clancy et al. [25]. BSK-II medium (80 μg/mL erythromycin) was inoculated with isolates B31(LP)-Em^R or B31(HP)-Em^R and incubated for 60 h at 34 °C. As a positive control, LB medium (80 μg/mL erythromycin) was inoculated with *E. coli* BM694 harbouring *ereA*, an erythromycin resistance esterase [16], and incubated at 37 °C for 72 h. Samples were taken at 12-h intervals, cells were removed by centrifugation (10 000 × *g*, 5 min) and the supernatant was filtered through a 0.22 μm filter (Fisher Scientific, Norcross, GA). Sterile filter disks were placed on plates spread with *E. faecalis* JH2-2 or *E. coli* JM109, 5 μL of the culture filtrate was applied to the disks and plates were incubated at 37 °C for 12 h. Zones of inhibition were measured in millimetres. Filtrates from medium with and without erythromycin were used as controls.

2.5. Erythromycin efflux assay

Energy-dependent efflux was assayed as described by Sutcliffe et al. [22] with the following modifications. Cultures of *S. pyogenes* strain O2C1064 (harbouring a Mef(A) efflux determinant), *B. burgdorferi* strain B31-Em^S and isolate B31-Em^R were grown to a cell density of 5×10^7 cells/mL. Cells were collected by filtration, the filters were washed three times with HEPES buffer (50 mM NaCl, 20 mM HEPES, pH 7.4) and cells were counted in a Beckman LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA).

2.6. Binding of [^{14}C]erythromycin to B. burgdorferi ribosomes

Ribosomes were isolated from Em^S or Em^R *B. burgdorferi*, *E. faecalis* and *B. subtilis* isolates using the method of Goldman et al. [26] and the protein concentration was estimated using the method of Whitaker and Granum [27]. Partially purified ribosomes (7.5 μg) were denatured and mixed with 2.5 nmol (0.25 μCi) [^{14}C]erythromycin (Amersham Biosciences). Ribosomes were collected on 0.2 μm nitrocellulose membranes (Bio-Rad, Hercules, CA), washed 10 times with 10 mM Tris–HCl, 5 mM $MgCl_2$ and 150 mM KCl pH 7.2, and radioactivity was determined using a LS 6000 Beckman scintillation counter (Beckman Instruments). The percent [^{14}C]erythromycin bound by resistant ribosomes was calculated using the following equation:

$$\frac{[^{14}\text{C}]\text{erythromycin bound to ery}^R\text{ ribosomes}}{[^{14}\text{C}]\text{erythromycin bound to ery}^S\text{ ribosomes}} \times 100 = \%\ \text{bound or sensitive}$$

2.7. Polymerase chain reaction (PCR) primers and DNA sequencing

All primers were purchased from Sigma/Genosys Biotechnologies (The Woodlands, TX). The sequences of PCR primers used for the detection of different classes of erythromycin resistance determinants were based upon those described by Sutcliffe et al. [28].

2.8. Genetic exchange from B. burgdorferi to B. subtilis or E. faecalis

Donor cells were prepared as follows: B31(LP)-Em^R, B31(HP)-Em^R and B31-5A3 cells were grown in BSK-II medium (50 μg/mL erythromycin) to a cell density of ca. 1×10^8 cells/mL. Cells were harvested by centrifugation (3000 × *g*, 10 min) and re-suspended in BSK-II medium to a cell density of ca. 5×10^7 cells/mL. Recipient cells were prepared as follows: *E. faecalis*

JH2-2 or *B. subtilis* PY79 were grown to an optical density at 600 nm (OD_{600}) of 0.8. Cultures were diluted 1:1 with fresh medium, harvested by centrifugation (9000 × *g*, 10 min) and re-suspended in BSK-II medium. Donor and recipient cells were mixed at a ratio of 1:1, 1:2 or 1:3, DNaseI was added and the cells were pelleted by centrifugation (6000 × *g*, 10 min) to promote cell-to-cell contact. The mixtures were incubated at 34 °C for 18–24 h. The cell pellet was suspended and the incubation continued for 2 h in the presence of 0.04 μg/mL erythromycin (*E. faecalis*) or 0.06 μg/mL lincomycin (*B. subtilis*). The *E. faecalis*/*B. burgdorferi* mating mixture was plated on BHI medium (25 μg/mL erythromycin), whilst the *B. subtilis*/*B. burgdorferi* mating mixture was plated on LB medium (25 μg/mL lincomycin, 1 μg/mL erythromycin). Plates were incubated at 37 °C for 1–3 days. Recipient cells were plated to determine the frequency of spontaneous antibiotic resistance. The frequency of transfer was expressed as the number of transconjugants per recipient cell.

To test for possible genetic transfer via transduction, B31(LP)-Em^R or B31(HP)-Em^R cells were grown as described. Then, 1 mL of the culture was removed, cells were removed by centrifugation (5000 × *g*, 15 min) and the supernatant was filtered using a 0.22 μm filter (Fisher Scientific). The filtrate was examined by dark field microscopy to ensure that all spirochetes had been removed. The filtrate was added to *E. faecalis* or *B. subtilis* (OD_{600} = 0.8), incubated at 37 °C overnight and the cells were plated as described above.

3. Results

3.1. Isolation of Em^R B. burgdorferi

While testing LP and HP *B. burgdorferi* B31 strains for susceptibility to various antibiotics, erythromycin-resistant B31(HP)-Em^R isolates were identified. These isolates were resistant to high levels of erythromycin (>100 μg/mL), whereas susceptibilities to tetracycline, chloramphenicol, kanamycin and gentamicin were very similar to those reported [2,4,7–11, 29]. Interestingly, no Em^R isolates were obtained for strain B31(LP) in initial experiments. Because the avirulent HP isolate was derived from the virulent LP strain, one would expect the parental strain to display a similar resistance pattern. However, B31(LP)-Em^R isolates were obtained when 3×10^7 cells/mL were incubated in BSK-II medium containing 0.04 μg/mL erythromycin prior to plating on BSK-II medium containing erythromycin, suggesting that resistance was inducible in this strain. Subsequent analysis of the LP Em^R isolates on higher concentrations of erythromycin indicated that they were also resistant to >100 μg/mL erythromycin. Two additional B31(LP) strains (B31-5A3 and B31-35210) were tested for erythromycin susceptibility; however, no spontaneous or inducible erythromycin resistance was detected in these strains.

3.2. Resistance of B. burgdorferi to the lincosamide and streptogramin antibiotics

To test the resistance patterns of *B. burgdorferi* to the lincosamide and streptogramin antimicrobials, isolates B31(HP)-Em^R and B31(LP)-Em^R as well as strains B31-5A3-Em^S and B31-35210-Em^S were plated onto BSK-II medium containing 15 μg/mL lincomycin, 10 μg/mL clindamycin, 15 μg/mL quinupristin (streptogramin B) or 15 μg/mL dalfopristin (streptogramin A). As expected, LP strains B31-5A3 and B31-35210 were sensitive to all antibiotics tested (Table 2). However, isolate B31(LP)-Em^R was resistant to lincomycin and clindamycin but was sensitive to both streptogramins A and B. The B31(HP)-Em^R isolate was resistant to dalfopristin (streptogramin A) but sensitive to quinupristin (streptogramin B) (Table 2). These data suggest that these isolates (B31(HP)-Em^R and B31(LP)-Em^R) do not harbour a typical MLS_B resistance determinant. Because the binding site in the 50S ribosomal subunit for streptogramin A does not overlap with those for macrolide, lincosamide and streptogramin B antibiotics, isolate B31(HP)-Em^R might harbour an additional determinant conferring resistance to streptogramin A.

3.3. Screening B. burgdorferi Em^R isolates for erythromycin resistance determinants

PCR was used to screen *B. burgdorferi* B31(HP)-Em^R, B31(LP)-Em^R and B31-5A3-Em^S for known erythromycin and streptogramin resistance genes (e*rm, msr, mef, ere, mph* and *vga*). Although not all genes in these groups were tested, no homologues were found based on the primer sets designed by Sutcliffe et al. [28]. Additionally, analysis of the completed genome sequence did not reveal any potential erythromycin resistance determinants, as expected, since the DNA used for *B. burgdorferi* genome sequencing was isolated from an Em^S B31 strain.

3.4. Inactivation of erythromycin

Whilst rRNA methylase *erm* genes have been found, high levels of erythromycin resistance are more commonly due to macrolide-inactivating enzymes [30]. Therefore, *B. burgdorferi* isolates B31(HP)-Em^R and B31(LP)-Em^R were tested for inactivation of erythromycin. B31 (HP)-Em^R, B31(LP)-Em^R and *E. coli* BM694 (*ereA* or *ereB*) were grown in the presence of erythromycin and culture supernatants were collected at 12-h intervals and tested for activity against Em^S *E. faecalis*. No zones of inhibition were detected from *E. coli* (*ereB*) or *E. coli* (*ereA*) supernatants after 12 h and 36 h, respectively, indicating inactivation of erythromycin. However, the zones of inhibition remained constant at 3 mm for B31(HP)-Em^R and B31(LP)-Em^R (Fig. 1), indicating that erythromycin had not been inactivated. No zones were produced with *B. burgdorferi* cells or *E. coli* cells alone. These experiments indicated that the erythromycin resistance mechanism in *B. burgdorferi* was not due to inactivation of the antibiotic.

3.5. Efflux

Another possible mechanism for the observed erythromycin resistance in *B. burgdorferi* is active transport of erythromycin via an energy-dependent efflux pump. Uncouplers that poison the proton-motive force effectively block export of erythromycin, causing accumulation within cells. It has previously been demonstrated that the uncoupler carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) blocks the energy-dependent uptake of [^{54}Mn] in *B. burgdorferi* [31]. To determine whether resistance to macrolides was mediated by an efflux mechanism, B31(LP)-Em^R, B31-5A3-Em^S and *S. pyogenes* 02C1064 cultures were assayed for [^{14}C]erythromycin efflux with and without CCCP. Both Em^R and Em^S *B. burgdorferi* cells accumulated [^{14}C]erythromycin rapidly and the intracellular levels stabilised within minutes of exposure to labelled antibiotic (Fig. 2A,B, ⋄ solid lines). As expected, intracellular levels of [^{14}C]erythromycin were higher in the Em^S strain since sensitive ribosomes bind the antibiotic causing intracellular levels to be higher (Fig. 2B, ⋄ solid line). Interestingly, addition of CCCP prior to [^{14}C]erythromycin did not affect the intracellular levels of antibiotic in the Em^R isolate B31(LP) (Fig. 2A, □ dashed line). In contrast, *S. pyogenes* 02C1064, which harbours an energy-dependent efflux protein, showed rapid uptake of [^{14}C]erythromycin (Fig. 2C, ⋄ solid line) and CCCP dramatically affected the accumulation of [^{14}C]erythromycin (Fig. 2C, □ dashed line). These data suggest that erythromycin resistance in strain B31(LP)-Em^R was not dependent on an efflux mechanism.

3.6. Binding of [^{14}C]erythromycin to B. burgdorferi ribosomes

The pattern of macrolide and lincosamide resistance in Em^R *B. burgdorferi* isolates and data from the inactivation and [^{14}C]erythromycin uptake experiments suggested that the most likely mechanism of erythromycin resistance in *B. burgdorferi* was modification of the ribosomes. Since modified ribosomes do not bind erythromycin efficiently, *B. burgdorferi* ribosomes were analysed for [^{14}C]erythromycin binding [26]. Partially purified ribosomes from B31(LP)-Em^R, B31-5A3-Em^S, *E. faecalis* JH2-2 and *E. faecalis* JH2-2-[pAT28(*ermA*)] were denatured and mixed with 2.5 nmol (0.25 μCi) [^{14}C]erythromycin. Binding of [^{14}C]erythromycin to ribosomes purified from B31(LP)-Em^R was reduced compared with the Em^S strain, indicating

that ribosomes from the resistant isolate were modified (Fig. 3A,B). [^{14}C]Erythromycin binding to ribosomes isolated from *E. faecalis* JH2-2-[pAT28(*ermA*)] [20] was also reduced in comparison with ribosomes isolated from *E. faecalis* JH2-2 (Fig. 3E,G). These data suggest that ribosomes from the Em^R B31 isolate had been modified.

3.7. Transfer of erythromycin resistance from B. burgdorferi to E. faecalis and B. subtilis

Frequently, macrolide resistance determinants identified in Gram-positive bacteria [32] are able to transfer between genera via conjugation. To determine whether the *B. burgdorferi* Em^R phenotype could also be transferred from *B. burgdorferi* to Gram-positive bacteria, Em^R *B. burgdorferi* isolates were mated with Em^S strains of *E. faecalis* and *B. subtilis*. The frequency of transfer from B31(HP)-Em^R to *E. faecalis* JH2-2 was 4.5×10^{-5} (transconjugant/recipient), whilst transfer from B31(LP)-Em^R to the same recipient was 2.9×10^{-5} (Table 3). This was at least three orders of magnitude above the background for spontaneous resistance. Em^R *E. faecalis* transconjugants were resistant to high levels (>100 μg/mL) of erythromycin, whilst spontaneous mutants were sensitive to 25 μg/mL of erythromycin (data not shown). Transfer was not detected to *E. faecalis* UV202(*recA*$^-$) [18] from B31(LP)-Em^R or B31(HP)-Em^R, indicating that the transfer was dependent on recipient recombination functions (Table 3). Interestingly, *E. faecalis* transconjugants were able to transfer the erythromycin resistance to a second *E. faecalis* recipient, strain OG1-SSp, at a frequency ($1.0–1.6 \times 10^{-5}$) similar to that observed between *B. burgdorferi* and *E. faecalis* JH2-2. Finally, transfer was reduced dramatically if the *E. faecalis* recipient contained a resident plasmid (pAD1) [19] (Table 3). No transfer was observed if *B. burgdorferi* strain B31-5A3 was used as the donor.

The frequency of transfer from *B. burgdorferi* isolates B31(HP)-Em^R and B31(LP)-Em^R to *B. subtilis* was two to three orders of magnitude higher than that observed with *E. faecalis* and five to six orders above background levels of spontaneous resistance (Table 3). As observed in the previous matings, *B. subtilis* transconjugants were resistant to >100 μg/mL erythromycin. Transfer was reduced by two orders when Em^R *B. burgdorferi* were mated with *B. subtilis* strain BD224(*recA*$^-$) [21] (Table 3), indicating that, unlike *E. faecalis*, *B. subtilis* did not require RecA function for efficient transfer. Again unlike *E. faecalis*, Em^R *B. subtilis* transconjugants were not able to transfer the resistance phenotype to other *B. subtilis* strains (Table 3). Although we were able to detect high-frequency conjugal transfer of the Em^R phenotype to *E. faecalis* and *B. subtilis*, we have not been able to demonstrate transfer of the erythromycin resistance between *B. burgdorferi* Em^R and Em^S strains owing initially to a lack of reliable selectable markers in the recipient strains. More recently, identification of a transduction system in *B. burgdorferi* has complicated the analysis of potential conjugal transfer between *Borrelia* species.

Further analysis of the transconjugants yielded interesting information. First, Em^R *B. subtilis* and *E. faecalis* transconjugants did not contain any new extrachromosomal elements, suggesting that *B. burgdorferi* plasmids were not replicating autonomously in recipient strains. Second, pulsed-field gel electrophoresis analysis of chromosomal DNA from Em^R transconjugants showed no large changes to any DNA fragments, indicating that only the transfer of small amounts of DNA was required. Finally, ribosomes purified from Em^R *B. subtilis* and *E. faecalis* transconjugants were protected from [^{14}C]erythromycin binding (Fig. 3D,F). Taken together, these data indicate the mechanism of erythromycin resistance in the transconjugants was the same as that characterised in the *B. burgdorferi* Em^R isolates.

4. Discussion

Use of erythromycin has had mixed results in the treatment of Lyme disease. Previously, this failure has been attributed to poor penetration of tissues by erythromycin and low attainable serum levels in the human host [29,33] since in vitro data on the sensitivity of virulent *B.*

burgdorferi to the macrolide antibiotics [29,33] indicate that these strains are sensitive to low levels (<0.06 μg/mL) of erythromycin and lincomycin (<0.25 μg/mL). Terekhova et al. [13] identified clinical isolates of *B. burgdorferi* that exhibited high-level resistance to erythromycin (100–500 μg/mL) and the resistance was inducible in some strains. However, these strains were not tested for susceptibility to MLS antibiotics nor were the mechanisms of resistance tested experimentally. The Em^R strains of *B. burgdorferi* identified in our laboratory were resistant to high levels of erythromycin (>100 μg/mL). Although the resistance patterns in bacteria are commonly macrolide (M), lincosamide (L), macrolide–streptogramin (MS) or MLS_B type [30], Hamilton-Miller and Shah [34] reported similar ML and MLS_A resistance patterns in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, but the mechanism(s) has not been identified.

Because we have been unsuccessful at cloning the erythromycin resistance determinant or identifying the gene by PCR, we tried to define the mechanism in the Em^R *B. burgdorferi* strains biochemically. However, testing of the Em^R *B. burgdorferi* for the presence of an antibiotic-inactivating enzyme was negative. Similarly, we found no indication that resistance was associated with an efflux system similar to that previously described in *S. aureus* (e.g. multiprotein system encoded by *msrA*) [35], *Neisseria gonorrhoeae* (*mtrRCDE* system) [36], *Pseudomonas aeruginosa* (*mexAB-oprK* system) [37], *E. coli* [38] or *Streptococcus pneumoniae* [22]. Interestingly, the *B. burgdorferi* genome contains an open reading frame encoding a putative multidrug efflux transporter on linear plasmid 28-4 (lp28-4) with 55% identity to a putative tetracycline resistance protein, TetA(P), from *Helicobacter pylori* [39]. Since all Em^S and Em^R *B. burgdorferi* isolates in this study are sensitive to tetracycline (<0.25–2 μg/mL), it seemed unlikely that this gene encoded a tetracycline efflux protein. However, it was possible that this or other proteins were involved in active efflux of erythromycin in Em^R B31 isolates. This possibility was investigated using [^{14}C]erythromycin uptake assays in the presence and absence of CCCP. For *B. burgdorferi*, addition of CCCP in [^{14}C]erythromycin uptake assays did not affect the incorporation of labelled erythromycin in resistant or sensitive cells, suggesting that the mechanism of erythromycin resistance in these cells was not due to an energy-dependent drug efflux pump.

The most common mechanism for resistance to MLS antibiotics is modification of the ribosome, particularly by methylation of 23S rRNA, with 30 different *erm* genes described (http://faculty.washington.edu/marilynr/). A less common mechanism of ribosome modification involves amino acid changes in key ribosomal proteins that encompass the antibiotic binding site(s) [40–44]. To test whether ribosome modification was responsible for the Em^R phenotype in *B. burgdorferi* Em^R strains, we partially purified ribosomes from different isolates and assayed for [^{14}C]erythromycin binding. Equimolar amounts of ribosomes isolated from Em^R *B. burgdorferi* strains bound 85% less [^{14}C]erythromycin than Em^S ribosomes, strongly suggesting that the ribosomes had been modified. The gene(s) responsible for this modification and erythromycin resistance in *B. burgdorferi* have not been identified.

The most interesting finding in the study was the ability to transfer the Em^R phenotype via conjugation to two Gram-positive bacteria, *E. faecalis* and *B. subtilis*. Many of the MLS genes are associated with conjugative elements and can move into a variety of hosts [30]. Most of the characteristics of the transfer of *B. burgdorferi* MLS_A resistance phenotype resemble those of constins or integrating conjugative elements (ICEs). For example, the *B. burgdorferi* MLS_A resistance transferred by conjugation at high frequency to *B. subtilis* (10^{-2}–10^{-3}) and *E. faecalis* (10^{-5}–10^{-6}). Additionally, the absence of plasmid DNA in the *E. faecalis* or *B. subtilis* transconjugants suggested that the resistance determinant was probably integrating into the recipient chromosome [30]. Conversely, integration of *B. burgdorferi* MLS_A resistance, unlike most constins that contain genes encoding recombinases, appeared to be partially or completely dependent on recipient *recA* function after transfer. Transfer decreased by a factor

of 10^2–10^4 when a *recA*$^-$ *E. faecalis* recipient was used in matings, whilst transfer to *recA*$^-$ *B. subtilis* was reduced by 10–10^2. Although unusual, a 20–50-fold lower frequency of transfer of a *Vibrio cholerae* conjugative element, SXT, into *recA*$^-$recipients has been described [44]. The fact that transfer to *recA*$^-$ *B. subtilis* was still occurring at a high frequency (10^{-4}) suggests that one of the numerous recombinases in *B. subtilis*, such as RecR, YefB, RecG, etc. (TIGR database) might be facilitating integration in that strain. The exact role of RecA in the recipients is unknown.

Both Em^R *B. burgdorferi* isolates B31(HP) and B31(LP) contain numerous plasmids that could be involved in transfer of the *B. burgdorferi* MLS_A resistance determinant. Two observations hint that they may play a role. First, transfer of the Em^R phenotype to *E. faecalis* decreased dramatically when the recipient strain contained the conjugative plasmid pAD1. Whilst inhibition of transfer of conjugal plasmids due to incompatibility with resident plasmids in recipient strains has been described [45], it is rarely observed with conjugal integrating elements [46,47]. Thus, it seems plausible that transfer from *B. burgdorferi* to *E. faecalis* may involve donor plasmids that are incompatible with some resident *E. faecalis* plasmids. Second, a homologue to *traB* from *E. faecalis* encoding a pheromone shutdown protein has been identified in *B. burgdorferi* [39]. TraB is involved in transfer of pheromone-responding plasmids in members of the genus *Enterococcus* [48]. Unfortunately, no other genes encoding potential transfer factors have been identified on *B. burgdorferi* plasmids. Despite our limited knowledge of the *B. burgdorferi* MLS_A resistance phenotype and the mechanism of transfer to Gram-positive bacteria, its discovery does suggest that interspecies gene transfer may play a role in the evolution of *B. burgdorferi*. Additionally, it also provides potential tools for developing new genetic systems to study the pathogenesis of Lyme disease. Studies are ongoing to identify the gene(s) conferring resistance to erythromycin in *B. burgdorferi* as well as those required for efficient transfer.

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References

1. Centers for Disease Control and Prevention (CDC). Lyme disease—United States, 2003–2005. MMWR Morb Mortal Wkly Rep 2007;56:573–6. [PubMed: 17568368]
2. Berger BW. Current aspects of Lyme disease and other *Borrelia burgdorferi* infections. Dermatol Clin 1997;15:247–55. [PubMed: 9098634]
3. Agger WA, Callister SM, Jobe DA. In vitro susceptibilities of *Borrelia burgdorferi* to five oral cephalosporins and ceftriaxone. Antimicrob Agents Chemother 1992;36:1788–90. [PubMed: 1416868]
4. Berger BW. Treatment of erythema chronicum migrans of Lyme disease. Ann N Y Acad Sci 1988;539:346–51. [PubMed: 3190102]
5. Dattwyler RJ, Halperin JJ, Pass H, Luft BJ. Ceftriaxone as effective therapy in refractory Lyme disease. J Infect Dis 1987;155:1322–5. [PubMed: 3572042]
6. Dattwyler RJ, Halperin JJ, Volkman Luft BJ. Treatment of late Lyme borreliosis—randomised comparison of ceftriaxone and penicillin. Lancet 1988;1:1191–4. [PubMed: 2897008]
7. Johnson RC. Isolation techniques for spirochetes and their sensitivity to antibiotics in vitro and in vivo. Rev Infect Dis 1989;11(Suppl 6):S1505–10. [PubMed: 2682963]

8. Johnson RC, Kodner C, Russell M. In vitro and in vivo susceptibility of the Lyme disease spirochete, *Borrelia burgdorferi*, to four antimicrobial agents. Antimicrob Agents Chemother 1987;31:164–7. [PubMed: 3566246]
9. Johnson SE, Klein GC, Schmid GP, Feeley JC. Susceptibility of the Lyme disease spirochete to seven antimicrobial agents. Yale J Biol Med 1984;57:549–53. [PubMed: 6516457]
10. Preac-Mursic V, Wilske B, Schierz G. European *Borrelia burgdorferi* isolated from humans and ticks: culture conditions and antibiotic susceptibility. Zentralbl Bakteriol Mikrobiol Hyg [A] 1986;263:112–8.
11. Wretlind B, Johnson RC, Hansen K, Preac-Mursic V. Antibiotic susceptibility of *Borrelia burgdorferi* in vitro and in animal models. Scand J Infect Dis Suppl 1991;77:143–4. [PubMed: 1947803]
12. Dattwyler RJ, Grunwaldt E, Luft BJ. Clarithromycin in treatment of early Lyme disease: a pilot study. Antimicrob Agents Chemother 1996;40:468–9. [PubMed: 8834900]
13. Terekhova D, Sartakova ML, Wormser GP, Schwartz I, Cabello FC. Erythromycin resistance in *Borrelia burgdorferi*. Antimicrob Agents Chemother 2002;46:3637–40. [PubMed: 12384380]
14. Eggers CH, Kimmel BJ, Bono JL, Elias AF, Rosa P, Samuels DS. Transduction by ΦBB-1, a bacteriophage of *Borrelia burgdorferi*. J Bacteriol 2001;183:4771–8. [PubMed: 11466280]
15. Zhang JR, Hardham JM, Barbour AG, Norris SJ. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell 1997;89:275–85. [PubMed: 9108482]
16. Arthur M, Autissier D, Courvalin P. Analysis of the nucleotide sequence of the *ereB* gene encoding the erythromycin esterase type II. Nucleic Acids Res 1986;14:4987–99. [PubMed: 3523438]
17. Jacob AE, Hobbs SJ. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. zymogenes. J Bacteriol 1974;117:360–72. [PubMed: 4204433]
18. Yagi Y, Clewell DB. Recombination-deficient mutant of *Streptococcus faecalis*. J Bacteriol 1980;143:966–70. [PubMed: 6782083]
19. Tomich PK, An FY, Damle SP, Clewell DB. Plasmid-related transmissibility and multiple drug resistance in *Streptococcus faecalis* subsp. zymogenes strain DS16. Antimicrob Agents Chemother 1979;15:828–30. [PubMed: 112916]
20. Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in gram-positive bacteria. Nucleic Acids Res 1990;18:4296. [PubMed: 2143017]
21. Dubnau D, Davidoff-Abelson R, Scher B, Cirigliano C. Fate of transforming deoxyribonucleic acid after uptake by competent *Bacillus subtilis*: phenotypic characterization of radiation-sensitive recombination-deficient mutants. J Bacteriol 1973;114:273–86. [PubMed: 4349031]
22. Sutcliffe J, Tait-Kamradt A, Wondrack L. *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob Agents Chemother 1996;40:1817–24. [PubMed: 8843287]
23. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 1984;57:521–5. [PubMed: 6393604]
24. Samuels DS, Mach KE, Garon CF. Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant *gyrB*. J Bacteriol 1994;176:6045–9. [PubMed: 7928965]
25. Clancy J, Dib-Hajj F, Petitpas JW, Yuan W. Cloning and characterization of a novel macrolide efflux gene, *mreA*, from *Streptococcus agalactiae*. Antimicrob Agents Chemother 1997;41:2719–23. [PubMed: 9420045]
26. Goldman RC, Zakula D, Flamm R, Beyer J, Capobianco J. Tight binding of clarithromycin, its 14-(R)-hydroxy metabolite, and erythromycin to *Helicobacter pylori* ribosomes. Antimicrob Agents Chemother 1994;38:1496–500. [PubMed: 7979278]
27. Whitaker JR, Granum PE. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal Biochem 1980;109:156–9. [PubMed: 7469012]
28. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 1996;40:2562–6. [PubMed: 8913465]

29. Preac-Mursic V, Wilske B, Schierz G, Süss G, Gross B. Comparative antimicrobial activity of the new macrolides against *Borrelia burgdorferi*. Eur J Clin Microbiol Infect Dis 1989;8:651–3. [PubMed: 2550233]
30. Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. Nomenclature for macrolide and macrolide–lincosamide–streptogramin B resistance determinants. Antimicrob Agents Chemother 1999;43:2823–30. [PubMed: 10582867]
31. Posey JE, Gherardini FC. Lack of a role for iron in the Lyme disease pathogen. Science 2000;288:1651–3. [PubMed: 10834845]
32. Bacterial conjugation. 1. New York, NY: Plenum Press; 1993.
33. Bryskier, AJ.; Butzler, JP.; Neu, HC.; Tulkens, PM. Macrolides: chemistry, pharmacology, and clinical uses. Boston, MA: Arnette Blackwell; 1993.
34. Hamilton-Miller JM, Shah S. Patterns of phenotypic resistance to the macrolide–lincosamide–ketolide–streptogramin group of antibiotics in staphylococci. J Antimicrob Chemother 2000;46:941–9. [PubMed: 11102413]
35. Wondrack L, Massa M, Yang BV, Sutcliffe J. Clinical strain of *Staphylococcus aureus* inactivates and causes efflux of macrolides. Antimicrob Agents Chemother 1996;40:992–8. [PubMed: 8849266]
36. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the mtrRCDE efflux system. Microbiology 1995;141:611–22. [PubMed: 7711899]
37. Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J Bacteriol 1993;175:7363–72. [PubMed: 8226684]
38. Kobayashi N, Nishino K, Yamaguchi A. Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. J Bacteriol 2001;183:5639–44. [PubMed: 11544226]
39. Fraser CM, Casjens S, Huang WM, et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 1997;390:580–6. [PubMed: 9403685]
40. Nagai K, Appelbaum PC, Davies TA, et al. Susceptibility to telithromycin in 1,011 *Streptococcus pyogenes* isolates from 10 central and Eastern European countries. Antimicrob Agents Chemother 2002;46:546–9. [PubMed: 11796375]
41. Pihlajamaki M, Kataja J, Seppala H, et al. Ribosomal mutations in *Streptococcus pneumoniae* clinical isolates. Antimicrob Agents Chemother 2002;46:654–8. [PubMed: 11850244]
42. Tait-Kamradt A, Davies T, Appelbaum PC, et al. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. Antimicrob Agents Chemother 2000;44:3395–401. [PubMed: 11083646]
43. Tait-Kamradt A, Davies T, Cronan M, Jacobs MR, Appelbaum PC, Sutcliffe J. Mutations in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. Antimicrob Agents Chemother 2000;44:2118–25. [PubMed: 10898684]
44. Waldor MK, Tschape H, Mekalanos JJ. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J Bacteriol 1996;178:4157–65. [PubMed: 8763944]
45. Le Bouguenec C, Horaud T, Bieth G, Colimon R, Dauguet C. Translocation of antibiotic resistance markers of a plasmid-free *Streptococcus pyogenes* (group A) strain into different streptococcal hemolysin plasmids. Mol Gen Genet 1984;194:377–87. [PubMed: 6330497]
46. Burrus V, Pavlovic G, Decaris B, Guedon G. Conjugative transposons: the tip of the iceberg. Mol Microbiol 2002;46:601–10. [PubMed: 12410819]
47. Whittle G, Shoemaker NB, Salyers AA. The role of *Bacteroides* conjugative transposons in the dissemination of antibiotic resistance genes. Cell Mol Life Sci 2002;59:2044–54. [PubMed: 12568330]
48. Fujimoto S, Tomita H, Wakamatsu E, Tanimoto K, Ike Y. Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J Bacteriol 1995;177:5574–81. [PubMed: 7559345]

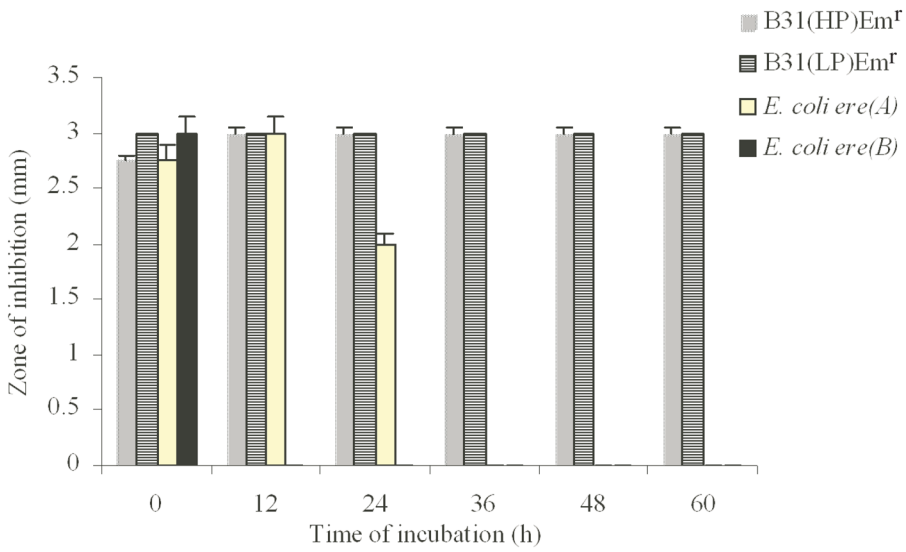

Fig. 1.

Erythromycin inactivation. Filtered culture supernatant from *Borrelia burgdorferi* isolate B31 (HP)-Em^R, isolate B31(LP)-Em^R, *Escherichia coli* harbouring *ereA* or *E. coli* harbouring *ereB* was tested for its ability to inactivate erythromycin as describe in Section 2.4. Samples were collected at 12-h intervals (0, 12, 24, 36, 48 and 60 h) and zones of inhibition (mm) were measured. Standard error is indicated by error bars. HP, high-passage; LP, low-passage; Em^R, erythromycin-resistant.

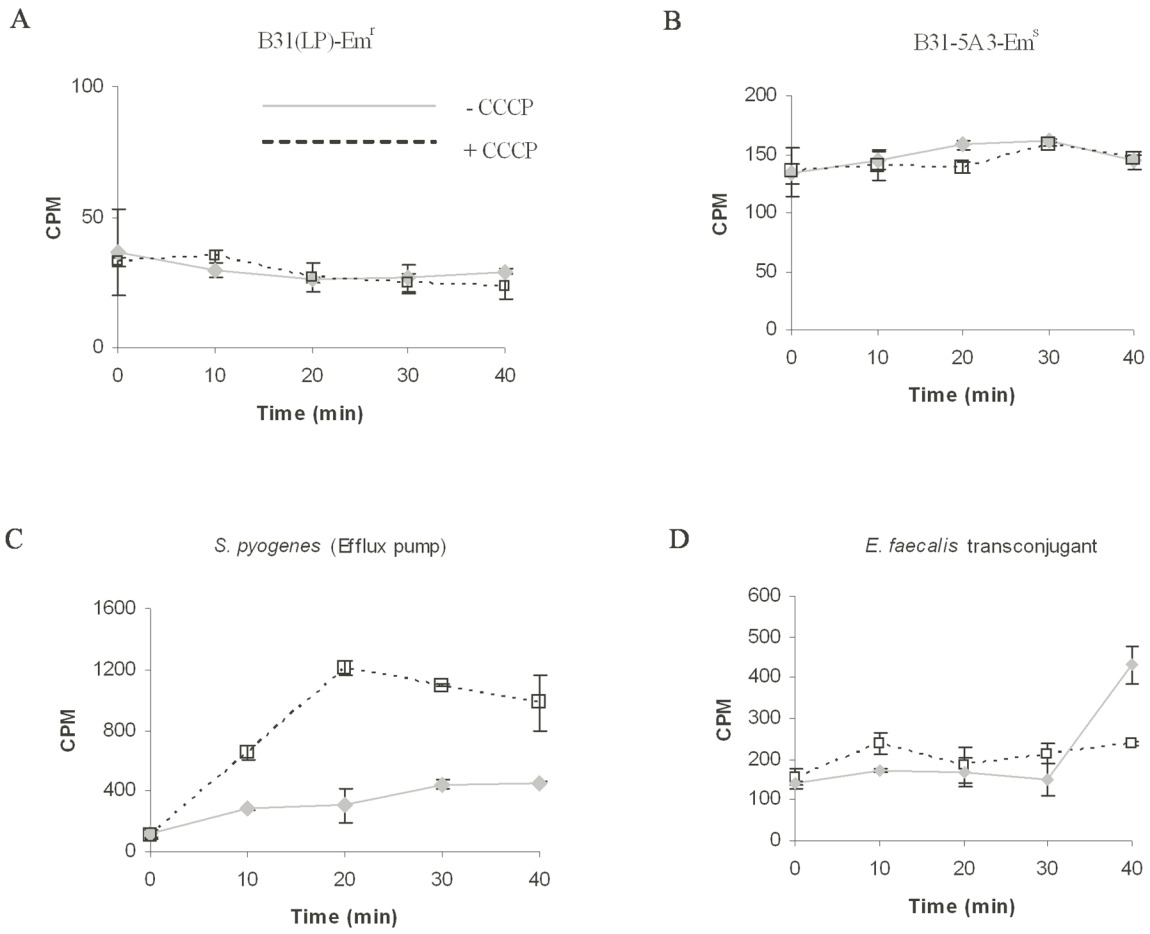

Fig. 2.

[^{14}C]Erythromycin uptake assays in the presence or absence of carbonyl cyanide *p*-chlorophenylhydrazone (CCCP). [^{14}C]Erythromycin was added to cells, aliquots were collected at 10-min intervals (0, 10, 20, 30 and 40 min) and the samples were processed as described in Section 2.5. For some samples, CCCP (100 μm) was added 10 min before the addition of [^{14}C]erythromycin. Incorporation of [^{14}C]erythromycin into (A) *Borrelia burgdorferi* isolate B31(LP)-Em^{R}, (B) *B. burgdorferi* strain B31-5A3-Em^{S}, (C) Em^{R} *Streptococcus pyogenes* (efflux pump) or (D) *Enterococcus faecalis* strain JH2-2 Em^{R} transconjugant was measured with or without the addition of CCCP. Standard error for each sample set is indicated by error bars. LP, low-passage; Em^{R}, erythromycin-resistant; Em^{S}, erythromycin-susceptible; CPM, counts per min.

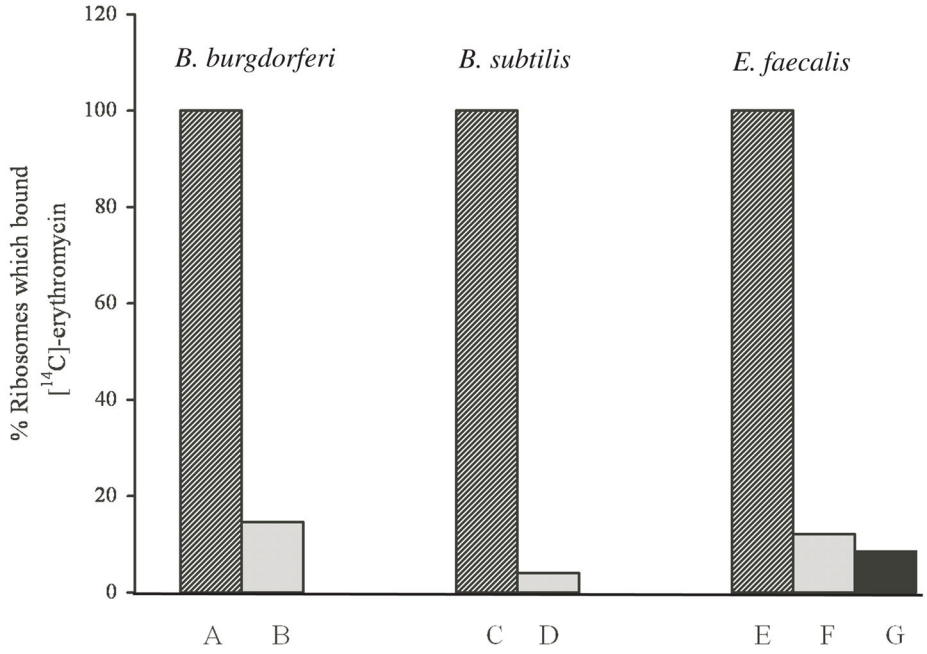

Fig. 3.

Binding of [^{14}C]erythromycin to ribosomes. [^{14}C]Erythromycin was mixed with partially purified ribosomes isolated from (A) *Borrelia burgdorferi* strain B31-5A3-Em^{S}, (B) *B. burgdorferi* strain B31(LP)-Em^{R}, (C) *Bacillus subtilis* strain PY79, (D) *B. subtilis* strain PY79 Em^{R} transconjugant, (E) *Enterococcus faecalis* JH2-2, (F) *E. faecalis* strain JH2-2 Em^{R} transconjugant or (G) *E. faecalis* JH2-2-[pAT28(*ermA*)] and assayed as described in Section 2.6. Standard error for each sample set is indicated by error bars. Em^{S}, erythromycin-susceptible; Em^{R}, erythromycin-resistant; LP, low-passage.

Table 1

Bacterial strains used in the study

Strain	Description	Source
Borrelia burgdorferi		
B31(HP)	High-passage, avirulent, passages >100, Em^R	This study
B31(LP)	Low-passage, virulent, passages 5, Em^R	This study
B31-5A3	Low-passage, highly virulent	Zhang et al. [15]
B31-35210	*B. burgdorferi* type strain	ATCC
Escherichia coli		
BM694	pAT63 *ereA*, Ap^R	Arthur et al. [16]
BM694	pAT72 *ereB*, Ap^R	Arthur et al. [16]
Enterococcus faecalis		
JH2-2	Fus^R, Rif^R	Jacob and Hobbs [17]
UV202	Fus^R, Rif^R, $recA^-$	Yagi and Clewell [18]
JH2-2(pAD1)	Fus^R, Rif^R, Hem–Bac	Tomich et al [19]
JH2-2[pAT28 (*ermA*)]	Fus^R, Rif^R, Spc^R, Em^R	Trieu-Cuot et al. [20]
OG1-SSp	Str^R, Spc^R, Tet^R	M. Roberts
Bacillus subtilis		
PY79	Prototrophic, rec^+, Tr^R	P. Youngman
PY1197(pHV1431)	Prototrophic, rec^+, Cm^R	P. Youngman
BD224	*recA4 thr-5 trpC2*	Dubnau et al. [21]
PY79N	Nal^R	This study
Streptococcus pyogenes		
O2C1064	Em^R	Sutcliffe et al. [22]

Em, erythromycin; R, resistant; Ap, ampicillin; Fus, fusidic acid; Rif, rifampicin; Hem–Bac, haemolysin, bacteriocin; Spc, spectinomycin; Str, streptomycin; Tet, tetracycline; Tr, trimethoprim; Cm, chloramphenicol; Nal, nalidixic acid.

Table 2

Macrolide–lincosamide–streptogramin (MLS) resistance patterns of *Borrelia burgdorferi* strain B31

Antibiotic	*B. burgdorferi* strain B31 from different sources (μg/mL)			
	(HP)-Em^R	(LP)-Em^R	5A3(LP)	35210
Macrolide				
Erythromycin	100	100	<1[a]	<1
Lincosamides				
Lincomycin	15	15	<1	<1
Clindamycin	10	10	<1	<1
Streptogramins				
Virginiamycin M[b]	15	1	1	<1
Quinupristin (B)	1	1	1	N.D.
Dalfopristin (A)	25	1	1	N.D.

HP, high-passage; LP, low-passage; Em^R, erythromycin-resistant; N.D., not determined.

[a] Cells did not grow at an antibiotic concentration of 1 μg/mL.

[b] >90% streptogramin A.

Table 3

Transfer frequency of *Borrelia burgdorferi* erythromycin resistance

Donor	Recipient	Transfer frequency[a]	Spontaneous frequency
B31(HP)-Em^R	*Bacillus subtilis* PY79	1.2×10^{-3}	10^{-8}
	B. subtilis BD224	1.0×10^{-4}	$<10^{-9}$
	Enterococcus faecalis JH2-2	4.5×10^{-5}	5.4×10^{-8}
	E. faecalis UV202	6.0×10^{-9}	2.5×10^{-8}
	E. faecalis JH2-2(pAD1)	7.0×10^{-7}	2.3×10^{-8}
Transconjugant from B31 (HP)-Em^R × PY79	*B. subtilis* PY79N	$<10^{-9}$	$<10^{-9}$
	B. subtilis PY1197	$<10^{-9}$	$<10^{-9}$
Transconjugant from B31 (HP)-Em^R × JH2-2	*E. faecalis* OG1-SSp	1.0×10^{-5}	8.7×10^{-8}
B31(LP)-Em^R	*B. subtilis* PY79	1.1×10^{-2}	10^{-8}
	B. subtilis BD224	2.5×10^{-4}	$<10^{-9}$
	E. faecalis JH2-2	2.9×10^{-5}	5.4×10^{-8}
	E. faecalis UV202	1.7×10^{-7}	2.5×10^{-8}
	E. faecalis JH2-2(pAD1)	3.3×10^{-7}	2.3×10^{-8}
Transconjugant from B31 (LP)-Em^R × PY79	*B. subtilis* PY79N	$<10^{-9}$	$<10^{-9}$
	B. subtilis PY1197	$<10^{-9}$	$<10^{-9}$
Transconjugant from B31 (LP)Em^R × JH2-2	OG1-SSp	1.6×10^{-5}	8.7×10^{-8}

HP, high-passage; LP, low-passage; Em^R, erythromycin-resistant.

[a] Per recipient.