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Genetic transformation of a Corynebacterial symbiont from the Chagas disease vector *Triatoma infestans*

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Abstract

Insect-borne diseases have experienced a troubling resurgence in recent years. Emergence of resistance to pesticides greatly hampers control efforts. Paratransgenesis, or the genetic transformation of bacterial symbionts of disease vectors, is an alternative to traditional approaches. Previously, we developed paratransgenic lines of *Rhodnius prolixus*, a vector of Chagas disease in Central America. Here, we report identification of a Corynebacterial species as a symbiont of *Triatoma infestans*, a leading vector of Chagas disease in South America. We have modified this bacterium to produce an immunologically active single chain antibody fragment, termed rDB3. This study establishes the basis for generating paratransgenic *T. infestans* as a strategy for control of Chagas disease.

Index Descriptors

Paratransgenesis; Chagas disease; *Triatoma infestans*; Corynebacteria

1. Introduction

Insect-borne diseases remain a leading cause of human illness throughout the world. Strategies for control of these diseases rely principally on chemical insecticides that eliminate or reduce numbers of vector insects. Chagas disease and its main vector, *Triatoma infestans*, are widely prevalent in the Gran Chaco, a semi-arid landscape extending over Argentina, Bolivia, Paraguay and southwestern Brazil. Several vector control programs, such as the Southern Cone Initiative for control of Chagas disease, are mostly based on the residual application of pyrethroid insecticides (Dias et al., 2002; Schofield and Dias, 1999). In the Argentine Chaco, however, symptomatic acute cases of Chagas disease are increasingly reported since 2001. Disorganized decentralization of vector control programs in the early 1980s followed by diminishing operational capacity since the 1990s, further compounded by the Argentine economic crisis in late 2001, contributed to the present scenario of persistent peridomestic infestation with recurrent domestic recolonization by *T. infestans* and renewed transmission to humans in the most affected regions (Gürtler et al., 2005).

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Most insecticides cause environmental toxicity and many classes of these chemicals harm humans. Insects have evolved resistance to many of these agents (World Health Organization, 1992) and insecticide failure is common. Resistance of triatomine populations to a variety of insecticides has been documented in other Chagas endemic regions of the world (Picollo et al., 2005). The poor effects of pyrethroid insecticides against peridomestic *T. infestans* and other triatomines are mainly caused by their short-lasting residual effects in outdoor sites exposed to sunlight, high temperatures, rain and dust (Gürtler et al., 2004). The suite of several insecticide intervention trials and the background experience of Chagas vector control services in the Gran Chaco clearly demonstrate that current tactics and procedures fail to eliminate populations of *T. infestans* in rural areas and need to be revised.

Control of vector-borne diseases may potentially be achieved by modifying insects, using foreign genes, to reduce their ability to transmit pathogens. This can involve direct germline transformation (Coates et al., 1998; Jasinskiene et al., 1998; Olson et al., 1996), or paratransgenic manipulation (Richards, 1993; Beard et al., 1993; Conte, 1997; Beard et al., 1998; Beard et al., 2000). In the reduviid bug vector of Chagas disease, *Rhodnius prolixus*, we have expressed the peptide, cecropin A, via an engineered symbiotic bacterium, *Rhodococcus rhodnii*, at concentrations that eliminate the parasite, *Trypanosoma cruzi* (Durvasula et al., 1997). We have demonstrated stable expression of an active antibody fragment in *R. prolixus* via genetically altered *R. rhodnii* (Durvasula et al., 1999a,b). Furthermore, we have shown under simulated field conditions that engineered bacteria can be delivered to populations of newly emerging nymphs of *R. prolixus*. These transformed bacteria predominate in the host insect throughout its life cycle (Durvasula et al., 1999a,b).

A wide variety of bacteria—including *Nocardia*, *Corynebacteria* and *Streptococci*—have been isolated from the midgut of *T. infestans* (Figueiro et al., 1995). These organisms play an important role in insect development and survival by providing essential nutrients such as pantothenic acid to nymphs (Dasch et al., 1984). Here we report the identification of a *Corynebacterial* species as a symbiont of *T. infestans* (Argentina) strain and the genetic transformation of this bacterium to produce an immunologically active single chain antibody fragment. Our test antibody fragment, termed DB3 V_H-Kappa (rDB3), is a murine antibody fragment that binds progesterone with affinity in the order of 1×10^9 l/mol (He et al., 1991). It is used as a model for expression of single chain antibodies that have activity against *T. cruzi*. This study establishes the basis for generating paratransgenic *T. infestans*—insects that carry genetically altered symbionts—as a strategy for control of Chagas disease transmission.

2. Materials and methods

2.1. Identification of *Corynebacterium*

Adult *T. infestans* (Argentina) were kindly provided by Dr. Vaclav Hitsva. These insects were initially collected from the Gran Chaco region and had matured for generations under insectary conditions. Fecal droplets from 10 adult *T. infestans* were collected. Individual droplets were diluted in 100 µl of PBS and plated on BHI agar (Difco, Detroit, MI) for 24 h at 28 °C. Individual colonies of bacteria from a monoculture were identified by 16S rDNA sequence analysis. A 1.4 kb fragment was amplified using standard 16S primers, 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Lane, 1991) and the GeneAmp XL PCR Kit (PE Applied Biosystems, Foster City, CA) that contains the rTth DNA polymerase XL, a polymerase with proofreading activity. The resulting product was purified using the Wizard PCR Prep kit (Promega, Madison, WI). Both strands of the fragment were sequenced using the above primers and the internal primers 530F (GTGCCAGCMGCCGCGG) and 685R (TCTACGRATTTACCYCTAC) (Lane, 1991) and the ABI Prism BigDye Terminator Cycle sequencing kit. Unincorporated dyes were removed from reaction mix with Centri-sep columns (Princeton Separations, Inc., Adelphia, NJ). The

fluorescent dye-labelled fragments were electrophoresed on 5% Long Ranger polyacrylamide gel using an ABI 377 automated sequencer. Sequence alignment and contig assembly were done using Sequence Navigator (PE Applied Biosystems) and further analysis and sequence comparisons were done with the Wisconsin Genetics Computer Group (GCG) package.

2.2. Establishing *Corynebacterium* as the symbiont of *T. infestans*

Aposymbiotic first instar nymphs of *T. infestans* were generated as described (Durvasula and Taneja, 1999). Hundred and eighty nymphs were divided into four groups. Group 1 ($n = 60$) were kept in containers lined with filter paper stained by the feces of the adult *T. infestans*. Group 2 ($n = 40$) were maintained in a sterile container but fed two blood meals supplemented with the *Corynebacterium* at a concentration of 1×10^6 CFU/ml. Group 3 ($n = 40$) were kept in a sterile container and fed two blood meals supplemented with *R. rhodnii* at a concentration of 1×10^6 CFU/ml. Group 4 ($n = 40$) were kept in a sterile container until the sixth month. At that time, Group 4 was again divided. Group 4a ($n = 20$) received a blood meal supplemented with the *Corynebacterium* at a concentration of 1×10^6 CFU/ml; Group 4b ($n = 20$) received a sterile blood meal. All insects in the study were maintained on monthly meals of defibrinated sterile sheep's blood. The colonies were monitored for growth, mortality and egg production. At 12 months, a fecal droplet was collected from the living insects and cultured. Infection with the *Corynebacterium* was established as described above. Gut contents of insects that died during the course of the experiment were similarly cultured within 24 h of their death to assay for presence of the *Corynebacterium* or contaminating bacteria.

2.3. Construction of the shuttle plasmid, pRrMDWK6, and genetic transformation of *Corynebacterium*

Construction of the shuttle plasmid, pRrMDWK6, is described elsewhere (Durvasula et al., 1999a,b). Briefly, the replication origin fragment of *R. rhodnii* was cloned into the EcoRI restriction site of the DNA vector pBlue-script SK+ (Stratagene). The alpha antigen gene of *Mycobacterium kansasii* (Mk α), used to express a heterologous promoter-signal peptide, was ligated in frame to the rDB3 V_H-Kappa fragment. The Mk α /rDB3 fragment was cloned into the XbaI site of the shuttle vector. Cloning of a kanamycin resistance gene (Pharmacia) as a BamHI fragment yielded the final shuttle plasmid pRrMDWK6. Generation of electrocompetent *Corynebacterium* and transformation with pRrMDWK6 were done as previously described (Durvasula et al., 1999a,b). Transformed cells were selected on Luria Bertani (LB) agar that contained 50 μ g/ml of kanamycin.

2.4. Measurement of DB3 activity in transformed *Corynebacterium*

Individual colonies of the *Corynebacterium* that were genetically transformed with pRrMDWK6 were inoculated into 100 ml of LB broth containing kanamycin (50 μ g/ml) and grown for 16 h with gentle agitation at 28 °C. Cells were pelleted and lysed by incubation in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 100 μ g/ml phenylmethylsulfonyl fluoride and 1% Triton X-100 (Sigma) at 4°C for 2 h. The lysate was centrifuged at 12,000g for 5 min at 4°C. Serial dilutions of the supernatant were used for ELISA. All assays were performed in triplicate.

2.5. ELISA

Microtiter plates were coated with progesterone 3-(*O*-carboxymethyl)oxime:BSA conjugate (Sigma) using 2.0 μ g of conjugate per well. A volume of 150 μ l lysate of *Corynebacterium* were incubated in the progesterone-coated wells for 16 h at 4°C. After washing the wells five times with PBS, the bound V_H/K was detected using alkaline phosphatase-conjugated sheep anti-mouse IgG (Boehringer Mannheim) at 1:1000 dilution followed by *p*-nitrophenyl-

phosphate (Sigma). Absorbance at 405 nm was read using an MRX-HD automated kinetic plate reader (Dynex).

For reactions that involved excess progesterone-CMO as a competitive inhibitor, 100 μ l of the cell lysate were mixed with 50 μ l of a 260 mM solution of progesterone 3-(*O*-carboxymethyl) oxime (Sigma).

3. Results

3.1. Identification of *Corynebacterium*

FASTA and NetBlast searches of the GenBank databases were used to find sequences similar to the 1.4 kb sequence fragment (Accession No. AF322369). No sequences with 100% identity were found. The highest sequence identities (99%) observed were with *Corynebacterium* sp. CNJ954 PL04 (Accession No. DQ448694), with a single gap in the sequence and with *Corynebacterium* sp. BBH8 (Accession No. AM183334), where three separate gaps in the sequence was observed. This sequence also showed 98.8% sequence identity with *Corynebacterium variabilis* (Accession No. AJ222815) although a 10 bp gap had to be inserted around base 410 in *C. variabilis* for the alignment. Sequence similarity of 98.1, 96.6 and 96.1 were observed with *Corynebacterium terpenotabidum* (AB004730), *Corynebacterium falsenii* (Y13024) and *Corynebacterium jeikeium* (X84250), respectively.

3.2. Tests for symbiosis

Of the 60 first instar nymphs in Group 1 (bugs exposed to feces of adult *T. infestans*), 50 survived to month 12 (see Table 1). Faecal droplets collected from half of the insects at 12 months were positive by culture for the *Corynebacterium*. No other bacteria were isolated. By month 11, nine sexually mature females had produced 100 eggs. Thirty eight of the 40 insects in Group 2 (bugs fed the *Corynebacterium* via initial blood meals) survived to month 12. At month 11, 9 sexually mature females had produced 80 eggs.

None of the 40 nymphs in Group 3 (bugs fed *R. rhodnii* via initial blood meals) survived to month 12. About 90% of these insects reached the third or fourth instar stage by month 6 but all were dead by month 9. No eggs were produced. Cultures of the gut contents of these insects within 24 h of their death yielded no bacteria.

At month 6, all of the 40 nymphs in Group 4 (bugs maintained in sterile environment for 6 months) were alive and had reached the third to fourth instar stages. The 20 nymphs removed and fed the *Corynebacterium* (Group 4a) all survived to month 12; of these, 10 insects were sampled and found to be culture positive for the *Corynebacterium*. By month 12, 4 sexually mature females had produced 40 eggs. In contrast, insects that were not fed the *Corynebacterium* (Group 4b) died by month 12. No eggs were produced and all of the 10 insects sampled were negative by culture for the *Corynebacterium*.

There was no significant difference in survival to month 12 between the control group ($n = 60$) and the groups fed *Corynebacterium* at month 0 or month 6 ($p = .085$). The difference in survival to month 12 between the aposymbiotic group and the control group was highly significant ($p \ll .0001$). Also significant was the difference in survival to month 12 between the group that was fed *R. rhodnii* and the control group ($p \ll .0001$).

3.3. Measurement of DB3 activity in transformed *Corynebacterium*

Fig. 1 provides evidence that rDB3 is being produced by the transformed *Corynebacterium*. The cell lysate binds strongly to the progesterone-coated plate, giving rise to an OD_{405nm} of $.59 \pm .073$. Serial dilutions of the lysate yield decreased binding to the progesterone-coated

plate. When a competitive inhibitor, progesterone-3-CMO, is added to the lysate, binding to the progesterone-coated plate is drastically reduced. The lysate of the untransformed *Corynebacterium* does not bind to the progesterone-coated plate.

4. Discussion

This study establishes a *Corynebacterium* species as a symbiont of the *T. infestans* (Argentina) strain. This organism was isolated from an insectary colony of *T. infestans* as a monoculture, and is required for the maturation of the triatomine bug. It is therefore highly likely that this *Corynebacterium* sp. represents the intrinsic flora of *T. infestans*. The DNA sequence encoding the 16S ribosomal subunit of this bacterium shares 99% homology to corresponding 16S rRNA gene sequences of *Corynebacterium* sp. CNJ954 PL04 (Accession No. DQ448694), and *Corynebacterium* sp. BBH8 (Accession No. AM183334). It is interesting to note that both organisms were cultured from marine sediments. The 16S rRNA gene sequence of this *Corynebacterium* is available (GenBank Accession No. AF322369).

In the laboratory colonies of *T. infestans* used in this study, this bacterium is required for sexual maturation of nymphs. Nymphs maintained in the aposymbiotic state failed to mature beyond the fourth instar stage. Administration of the *Corynebacterium* to aposymbiotic nymphs at the first instar stage, either via a blood meal or via natural feces, allowed sexual maturation within 8 months, a rate comparable to the development of first instar nymphs in our insectary which are not aposymbiotic. Furthermore, administration of this bacterium to third and fourth instar aposymbiotic nymphs which were arrested in their development allowed completion of sexual maturation. *R. rhodnii*, a related nocardiform actinomycete which is a symbiont of *R. prolixus* (Baines, 1956), did not serve as a symbiont in this strain of *T. infestans*. Aposymbiotic first instar nymphs which were fed *R. rhodnii* via a blood meal suffered a growth arrest at the third and fourth instar stages and subsequently died, suggesting a specific relationship between the *Corynebacterium* and the Argentina strain of *T. infestans*.

The *Corynebacterium* transformed with the shuttle plasmid pRrMDWK6 was resistant to kanamycin and expressed an immunologically active single chain antibody, rDB3. Both the origin of replication, derived from *R. rhodnii*, and the MK α promoter for the rDB3 gene, derived from *M. kansasii*, were functional in the transformed *Corynebacterium*. Though we could not detect progesterone binding activity in cell-free supernatants of cultures of transformed *Corynebacterium*, assays of cell lysates were positive. Furthermore, binding activity decreased with serial dilutions of the cell lysate and was virtually eliminated by the addition of the competitive inhibitor, progesterone-3-CMO. No progesterone binding was detected in cell lysates from the untransformed *Corynebacterium*. These findings suggest that the progesterone binding activity observed in the transformed symbionts is due to expression of the functional single chain antibody, rDB3. We are currently modifying the signal peptidase recognition region of the MK α fragment to allow cleavage of the signal peptide and secretion of rDB3 from the transformed *Corynebacterium*.

This study establishes the foundation for generation of paratransgenic *T. infestans*. *In vivo* transgene expression by the engineered *Corynebacterium* in laboratory colonies of *T. infestans* is currently being assessed. We are also working on the transformation of symbiotic bacteria of other strains of *T. infestans* as well as the delivery of transformed symbionts to populations of *T. infestans* in simulated field settings. Comprehensive microbiology studies in the Argentine Chaco are underway to better understand the bacterial flora of *T. infestans*. Furthermore, single chain antibodies targeting key surface antigens of *T. cruzi* are in development; the rDB3 fragment is used in this study as a model for single chain antibody expression. These studies aim to develop a safe and effective strategy for delivery of molecules

with anti-trypanosomal activity to field populations of *T. infestans*. Such a strategy could serve as a powerful tool to complement current insecticide-based control programs.

The use of trade names does not constitute endorsement by the US Public Health Service or The Centers for Disease Control and Prevention.

Abbreviations

PCR, polymerase chain reaction; rDNA, ribosomal deoxyribonucleic acid; PBS, phosphate buffered saline; BHI, brain–heart infusion.

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Fig. 1.

Binding of serial dilutions of the cell lysate from the *Corynebacterium* transformed with pRrMDWK6 to progesterone-coated wells. OD_{405 nm} denotes readings obtained from an ELISA plate-reader. Inhibitor used was a 260 μ M solution of progesterone-3-CMO. Binding of lysates to progesterone 3-CMO on the plates was markedly reduced in the presence of free progesterone as evidenced by greatly reduced OD_{405 nm} values. Cell lysate of untransformed *Corynebacterium* did not bind progesterone, giving rise to an OD_{405 nm} of 0.0 (not shown).

Table 1

Percent survival of experimental *T. infestans* at months 6 and 12 and percent of insects infected with the *Corynebacterium* at month 12

	Number surviving to 6 months (%)	Number surviving to 12 months (%)	Percentage of sampled insects infected with <i>Corynebacterium</i> (n)
GROUP 1 (n = 60)	58 (96.6)	50 (83.3)	100% (20)
GROUP 2 (n = 40)	38 (95.0)	38 (95.0)	100% (19)
GROUP 3 (n = 40)	36 (90.0)	0	N/A
GROUP 4a (n = 20)	20 (100)	0	0 (10)
GROUP 4b (n = 20)	20 (100)	20 (100)	100% (10)

Group 1 were kept in containers lined with filter paper stained by the feces of the adult *T. infestans*.

Group 2 were maintained in a sterile container but fed two blood meals supplemented with the *Corynebacterium* at a concentration of 1×10^6 CFU/ml.

Group 3 were kept in a sterile container and fed two blood meals supplemented with *R. rhodnii* at a concentration of 1×10^6 CFU/ml.

Group 4 were kept in a sterile container until the sixth month. Group 4a received a blood meal supplemented with the *Corynebacterium* at a concentration of 1×10^6 CFU/ml; Group 4b received a sterile blood meal.