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Invited Review

Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease

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Abstract

The triatomine vectors of Chagas disease are obligate haematophagous insects, feeding on vertebrate blood throughout their entire developmental cycle. As a result of obtaining their nutrition from a single food source, their diet is devoid of certain vitamins and nutrients. Consequently, these insects harbour populations of bacterial symbionts within their intestinal tract, which provide the required nutrients that are lacking from their diet. We have isolated and characterised symbiont cultures from various triatomine species and developed a method for genetically transforming them. We can then reintroduce them into their original host species, thereby producing stable paratransgenic insects in which we are able to express heterologous gene products. Using this methodology, we have generated paratransgenic *Rhodnius prolixus* that are refractory for infection with *Trypanosoma cruzi*. Two examples of potentially refractory genes are currently being expressed in paratransgenic insects. These include the insect immune peptide cecropin A and active single chain antibody fragments. We have also developed an approach that would allow introduction of genetically modified bacterial symbionts into natural populations of Chagas disease vectors. This approach utilises the coprophagic behaviour of these insects, which is the way in which the symbionts are transmitted among bug populations in nature. The production and ultimate release of transgenic or paratransgenic insects for public health applications is potentially very promising but also worthy of much careful consideration with respect to environmental, political, and human safety concerns.

Keywords: Chagas disease; *Rhodnius prolixus*; Paratransgenic; Symbiosis

1. Introduction

Chagas disease is caused by the parasitic protozoan *Trypanosoma cruzi* and currently affects over 16 million people throughout South and Central America, where it results in significant morbidity and mortality (WHO, 1991). While transmission has been shown to occur through other routes such as blood transfusion, over 80% of all cases are vector-borne (Dias and Schofield, 1999), being transmitted by insects in the family Reduviidae and subfamily Triatominae, known commonly as kissing bugs, chinches, vinchucas, or barbeiros.

2. Blood-feeding and symbiosis in triatomines

Because these insects feed exclusively on blood throughout their entire developmental cycle, their diet is deficient in certain vitamins and nutrients. To overcome this obstacle, they harbour intestinal symbiotic flora that produce or otherwise provide the nutrients lacking in their diet. *Rhodnius prolixus* is an important domestic vector of Chagas disease throughout northern South America and Central America. This species maintains a symbiotic association with the actinomycete bacteria *Rhodococcus rhodnii* (Dasch et al., 1984). These bacteria grow within the gut of the insect and reach numbers approaching 10^9 organisms following ingestion of a bloodmeal. They are essential for growth and development of the insects and are transmitted efficiently from adult bugs to their progeny through coprophagy (i.e. the ingestion of faeces), a behaviour that is common among triatomines (see Fig. 1). Insects that do

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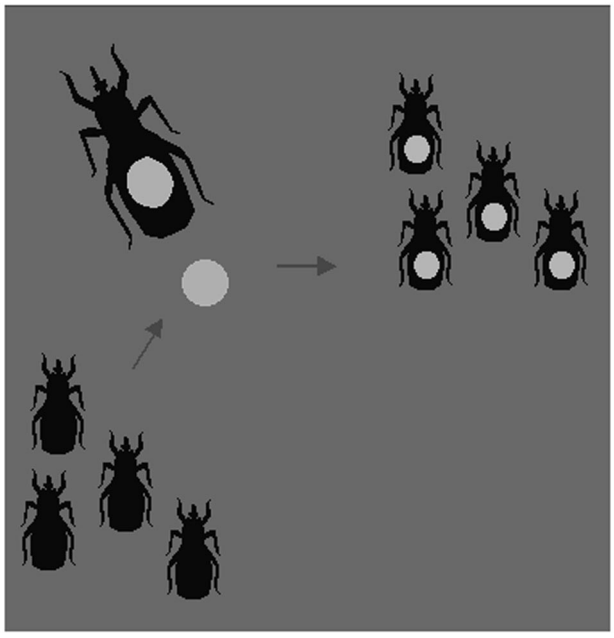

Fig. 1. Symbionts are transmitted by coprophagy from adults to nymphs. Insects that do not acquire the symbionts fail to reach reproductive maturity (modified from Beard et al., 2000).

not become colonised with the bacteria have higher mortality rates between moults and fail ultimately to reach adulthood (Dasch et al., 1984).

3. Cultivation and genetic transformation of triatomine symbionts

The actinomycete symbionts of *R. prolixus* and other Triatominae can be readily isolated and grown in a variety culture media. Furthermore, genetic transformation protocols have been developed, primarily using electroporation (Beard and Aksoy, 1996), that allow efficient production of transformed bacteria. In the *R. prolixus–R. rhodnii* system, transformation was initially achieved (see Fig. 2) using shuttle plasmids that contained dual replication origins for the rhodococci and for *Escherichia coli* and independent selectable antibiotic marker genes (Beard et al., 1992). These studies showed that it was possible to introduce genetically modified (GM) symbionts into aposymbiotic bugs (insects that have been raised under sterile conditions) and get stable expression of the marker in insects that have been reconstituted with these bacteria (see Fig. 3). Furthermore, it was shown that insects containing GM symbionts completed normal development. In more recent studies, shuttle plasmids have been replaced with DNA integration elements that are inserted in the bacterial genome and appear to be even more stable than the low-copy-number shuttle plasmids that were used initially (Dotson et al., unpublished data).

4. Expression of heterologous genes in Triatominae

Using shuttle plasmids or DNA integration elements, representatives of three broad groups of gene products have been successfully expressed in reconstituted paratransgenic insects. These include selectable marker genes, trypanocidal peptides, and active single chain antibody fragments.

4.1. Marker genes

In initial transformation studies with shuttle plasmids, the thiostrepton resistance marker gene was used to select for and identify GM symbionts (Beard et al., 1992). The benefits of using this marker are that thiostrepton is fairly efficient at killing non-transformed symbionts and it is an antibiotic not likely to be used for therapy in humans or animals. Later constructs, however, utilised the kanamycin resistance gene. This marker is more effective in *Rhodococcus*, resulting in cleaner selection and the removal of non-transformed symbiont colonies that would grow occasionally on thiostrepton plates. This marker, however, has the additional benefit of being effective for selection in *E. coli*, which is naturally resistant to thiostrepton, thereby allowing the use of a single antibiotic resistance marker gene that is utilisable in both *E. coli* and in *R. rhodnii*. Successful transformation vectors have also been developed that utilise the hygromycin resistance gene as a selectable marker (Dotson et al., unpublished data).

In addition to antibiotic resistance markers, colorimetric biochemical markers have also been utilised for identifying transformed symbionts. The two markers that have been most studied are beta-galactosidase and the green fluores-

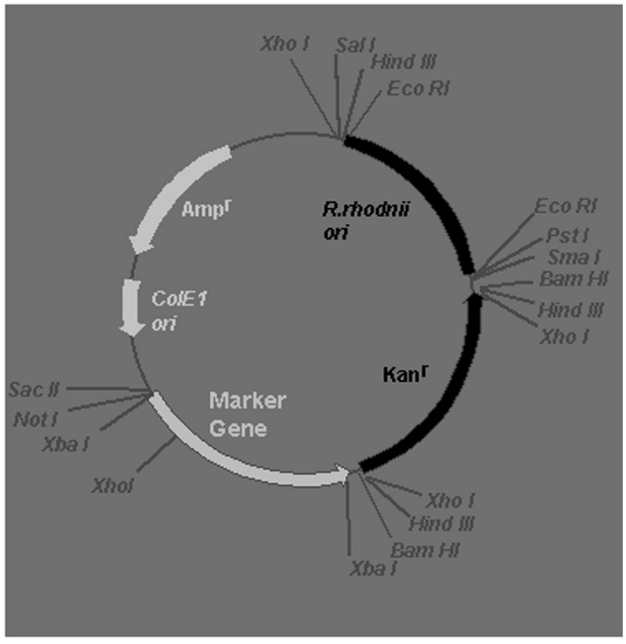

Fig. 2. Shuttle plasmid for genetic transformation and expression of marker genes in *R. prolixus* (from Beard et al., 1998).

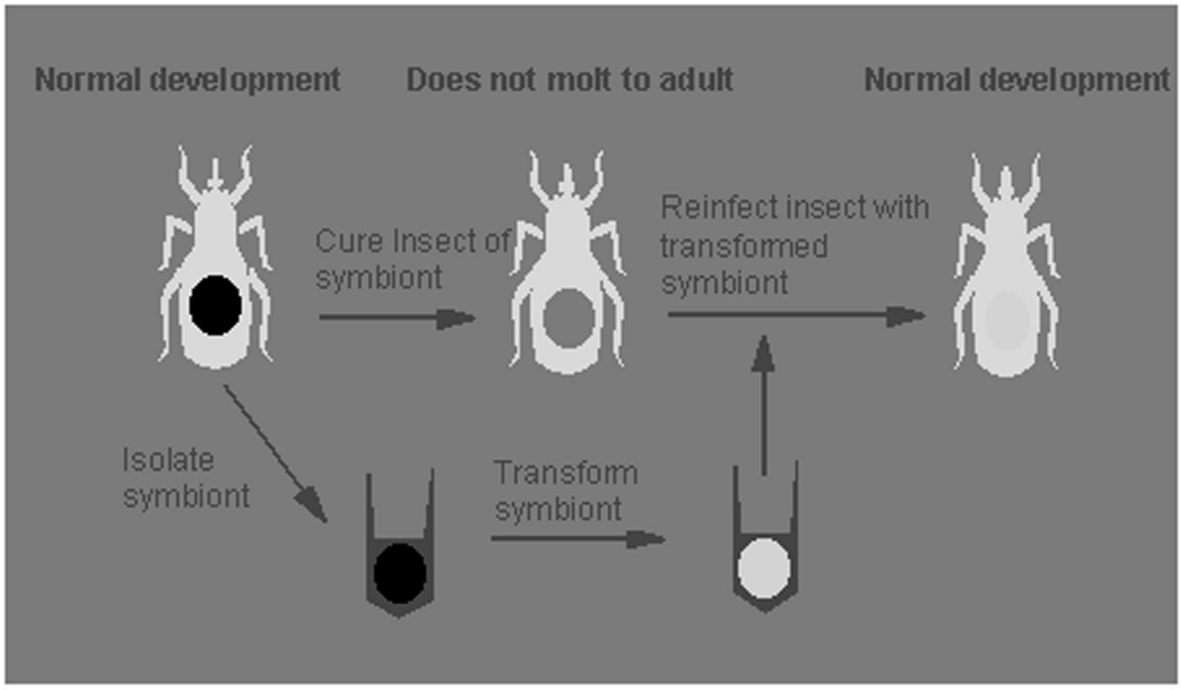

Fig. 3. Reconstitution experiments with genetically modified bacterial symbionts (from Beard et al., 1998).

cence protein (GFP). Both of these genes are currently being used in DNA integration elements, either by themselves or in conjunction with kanamycin or hygromycin resistance genes (Dotson et al., unpublished data). These types of markers, used alone, are less effective for general laboratory use because while they positively identify successful transformants, they do not eliminate non-transformed bacteria.

In constructs being developed for potential field pilot studies, colorimetric markers are essential due to the fact that it would not be responsible to release bacteria that contained antibiotic resistance genes. Another consideration for these types of applications is the fact that many bacteria naturally produce beta-galactosidase; consequently, it becomes preferable to use a marker such as GFP, which is unlikely to occur in soil bacteria.

4.2. Trypanocidal genes

To date, only a single actual trypanocidal gene, the insect immune peptide cecropin A, has been expressed in paratransgenic triatomines (Durvasula et al., 1997). In these studies, a gene fragment encoding the mature cecropin A peptide, driven from the *Streptomyces*-derived thiostrepton promotor, was inserted into a shuttle plasmid. The Chagas disease vector *R. prolixus* was then reconstituted with transformed symbionts, and the resulting paratransgenic bugs were either completely refractory (65/100) or 90–99% refractory (35/100) (see Figs. 4 and 5). Some degree of construct toxicity and instability was noted, however, suggesting that the peptide had slightly deleterious effects on its bacterial host. Studies currently in progress are aimed at expressing the inactive propeptide form of the molecule, which would be expected to be non-toxic to the rhodococci (Dotson et al., unpublished data). Digestive enzymes that are released in the insect gut following feeding would subsequently activate the propeptide.

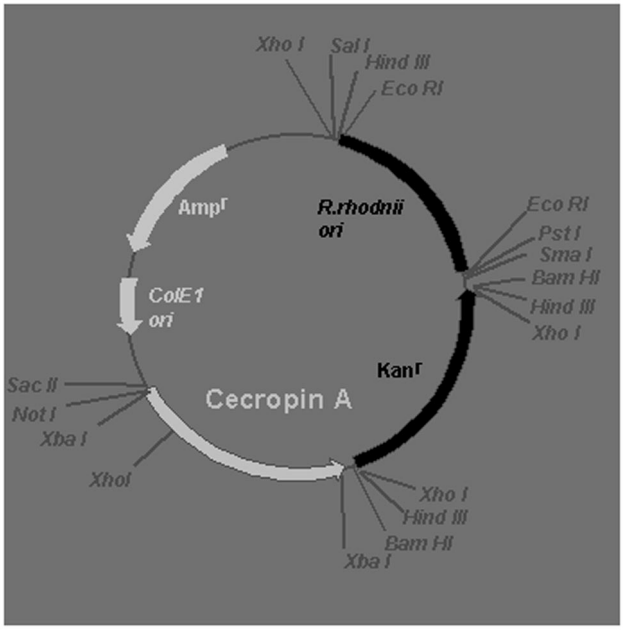

Fig. 4. Shuttle plasmid for genetic transformation and expression of cecropin A in *R. prolixus*.

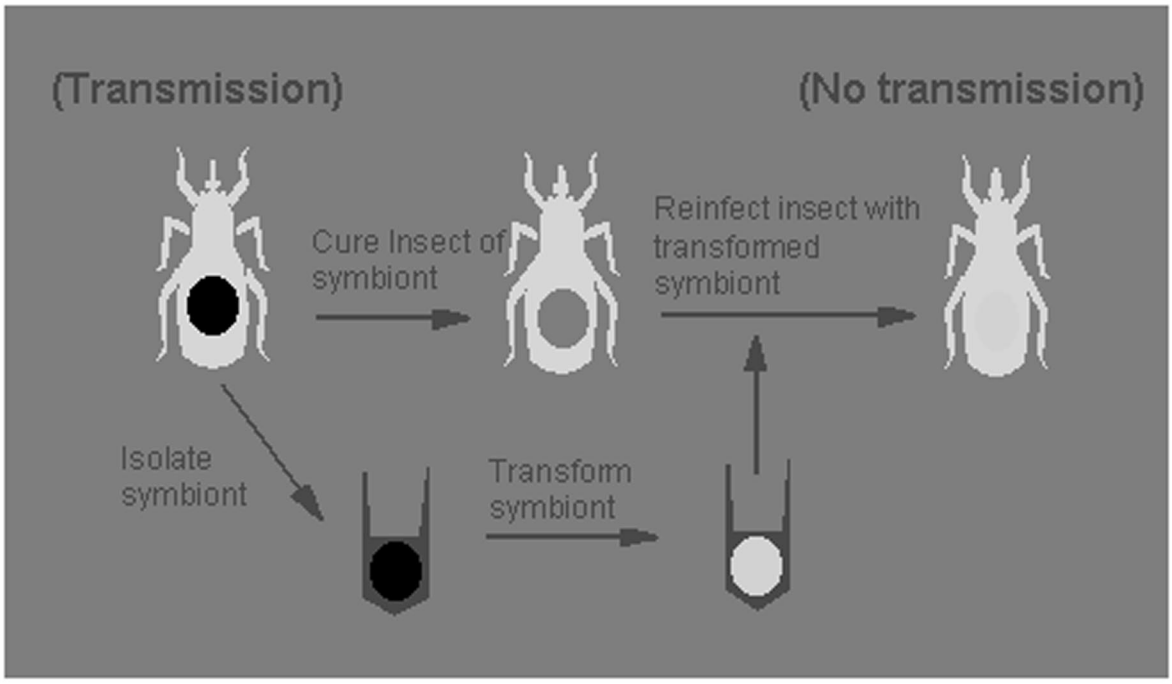

Fig. 5. Generation of *T. cruzi*-refractory *R. prolixus* via genetically modified symbionts.

4.3. Active antibody fragments

It is now possible to develop by phage display, single chain antibody genes that can be expressed in heterologous systems (Huse et al., 1986; Winter and Milstein, 1991). Using this approach, we have expressed and secreted a single chain functional mouse antibody fragment in the intestinal tract of paratransgenic *R. prolixus* that demonstrates high-affinity binding for the target (Durvasula et al., 1999a). Expression and secretion of the single chain antibody fragment was achieved using the *Mycobacterium kansasii* alpha antigen promoter and signal peptide (Fig. 6).

The purpose of these studies was to show that an antibody fragment could be expressed and that it is not degraded within the insect digestive tract but remains functional against a specific target, which in this case was the steroid hormone progesterone. Work now in progress is aimed at selecting transmission-blocking antibodies that successfully interfere with the trypanosome–vector relationship or otherwise disrupt trypanosome development in the insect. Once selected, purified hybridoma mRNA will be used in a phage display protocol to develop a transmission-blocking antibody cassette that can be expressed in paratransgenic Chagas disease vectors.

5. Methods for introduction of GM symbionts into insects

In the earliest studies (Beard et al., 1992), GM symbionts were introduced into *R. prolixus* by membrane feeding. Bacteria that were grown in culture were added to sterile defibrinated rabbit blood that was then provided to the insects through a glass feeding apparatus, using an artificial membrane. We have shown more recently that it is possible to introduce GM symbionts using a method that involves the

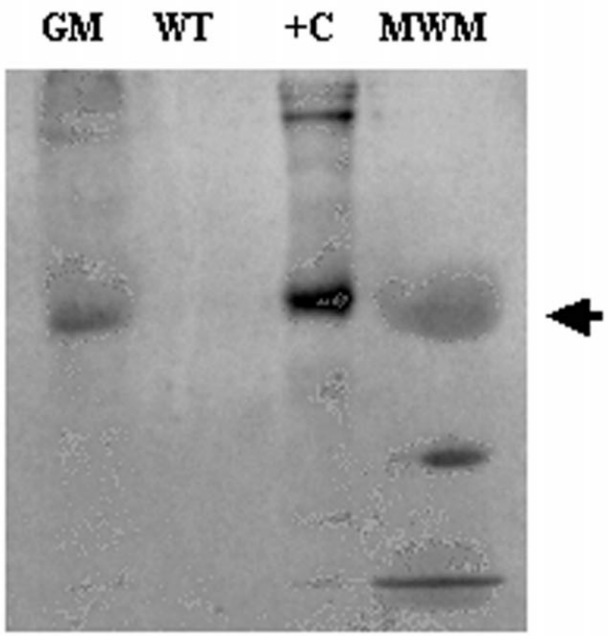

Fig. 6. Expression of a recombinant single chain antibody in *R. prolixus* via transformed *R. rhodnii*. Western blot using culture supernatant from genetically modified symbionts. WT, wild-type symbionts; +C, positive control from recombinant *E. coli*; MWM, 42 kDa molecular weight markers (modified from Durvasula et al., 1999a,b).

coprophagic mechanism used by these insects for acquisition of symbionts in nature (Durvasula et al., 1999b). In brief, large numbers of the bacteria are grown in culture. They are then purified and added to an inert polysaccharide carrier to which is added ammonia and a small amount of India ink. This formulation (called CRUZIGARD) approximates natural insect faeces and is actively ingested by triatomine nymphs that are coprophagic. This method has proven to be an effective way to introduce GM symbionts into *R. prolixus* in the laboratory. In one set of studies, CRUZIGARD was added to frames that were constructed to simulate the adobe or thatch materials that are used for house construction in rural South or Central America. Wild-collected adult *R. prolixus* were then introduced to these frames, which were then placed in plexiglass enclosures. The bugs were fed weekly and the F1 progeny tested for the presence of GM symbionts. At the end of the 8 week study, approximately 70% of the progeny contained GM symbionts, showing that CRUZIGARD competed favourably with the wild-type flora in native bug faeces.

6. Potential applications

One of the most useful potential applications of generating paratransgenic triatomines is to study various characteristics of trypanosome–vector interaction. By examining the effects of antibodies directed against specific surface proteins on the parasite or vector in paratransgenic insects, it may be possible to identify new targets for vaccines or new therapeutic agents.

Additionally, paratransgenic insects could potentially be useful someday as a part of an integrated pest management program (see Fig. 7). One can envisage a theoretical strategy in which CRUZIGARD is introduced into new or recently insecticide-treated homes. As insects re-invade these homes from the peridomicile or from untreated homes, they feed and lay eggs. By introducing GM symbionts in a stable bait formulation, applied in cracks and crevices in the treated homes, it may be possible to introduce GM symbionts into coprophagic nymphs which would then amplify and distribute these bacteria through natural mechanisms. This approach would not be used to replace insecticide-based control procedures but to supplement them by further reducing the capacity of domestic bug populations to transmit *T. cruzi* (Beard et al., 1998).

In addition to the studies done in *R. prolixus*, bacterial symbionts have been isolated and characterised from several important triatomine species, including *Triatoma infestans*, *Triatoma dimidiata*, and *Triatoma sordida* (Durvasula et al., unpublished data; Eichler et al., unpublished data; Pennington et al., unpublished data). For each of these species, the symbiont has been transformed using either DNA integration elements or shuttle plasmids. These results suggest a fairly broad potential use of GM symbionts for the two applications listed above.

- Genetically modified bacterial formulation are applied to new homes or to insecticide-treated homes.
- Insects infest or reinfest homes.
- Triatomine nymphs ingest modified bacteria.
- Genetically modified symbionts are amplified and dispersed by newly infected insects.

Fig. 7. A theoretical strategy for controlling Chagas disease transmission using genetically modified symbionts.

7. Concerns relating to research and potential release of GM symbionts

The potential release of GM symbionts for control of Chagas disease transmission introduces many concerns (Beard et al., 1998; Aultman et al., 2000). Currently, significant controversy surrounds the production and consumption of GM food products. Similar types of concerns arise with respect to using genetically modified organisms (GMOs) for public health applications. These include environmental concerns, human health and safety-related concerns, and public/political concerns.

7.1. Environmental concerns

These concerns relate primarily to the potential for (1) unanticipated effects of GM symbionts either on triatomines or on other animals that may come in contact with them, or (2) horizontal transfer of the released transgene or transgenes into other bacterial species. A number of studies are currently planned that are aimed at addressing these concerns. One set of studies will examine native bacterial flora in other insects that are known to inhabit homes in the rural tropics where GM symbionts could potentially be used for Chagas disease control. Once native bacterial flora have been characterised in ants, crickets, and cockroaches, these insects will be fed CRUZIGARD and analysed to determine if they can be colonised by GM symbionts, thereby posing a risk for unanticipated environmental dispersion.

Other studies are currently underway that are aimed at evaluating the possibility for horizontal transfer of the introduced genes between and among bacteria. In these studies, strains of GM *R. rhodnii* transformed with either shuttle plasmids or DNA integration elements, expressing different selectable marker genes, are co-cultivated and/or co-maintained in paratransgenic bugs. At various intervals, samples are taken and individual clones examined for dual expression of both markers, suggesting horizontal transfer. Similar studies are planned where mixtures of different soil actinomycetes are cultured together with GM *R. rhodnii*. Following a specified period of co-cultivation, colonies from the culture will be examined for transfer of the marker phenotype to other bacterial species. Many additional studies will no doubt be necessary before GM symbionts are likely to be released for pilot studies in the field.

7.2. Human health and safety concerns

These concerns focus on both short-term and long-term potential effects related to exposure of humans to GM symbionts. Currently, these bacteria are not known to be pathogens in mammals. The question has been raised, however, relating to the possibility that these agents could cause disease in immunosuppressed or immunocompromised hosts. For this reason, we recently tested groups of normal (CD1) and nude mice for signs and symptoms of disease when exposed orally to large numbers of bacteria (Dotson et al., unpublished data). The study design entailed inoculating mice, by gastric lavage, with approximately 10^7 live bacteria, and then examining the mice daily for persistence of bacteria in faeces, for symptoms of disease, and for any signs of pathology upon necropsy. The results were identical in both normal and immunocompromised mouse groups. Faecal shedding was observed generally only for the 2–3 days following inoculation. No signs of diarrhoea or other intestinal distress were observed, and no signs of pathology were seen at necropsy. These results suggest that the bacteria do not pose any serious health concerns as a consequence of oral exposure. Additional studies are planned to test the effects of aerosol inoculation and the potential for allergic reactions as a result of chronic exposure.

7.3. Public and political concerns

Chagas disease is primarily a disease of the developing world; consequently, if a control program based on the release of GMOs is to be piloted, it will presumably take place somewhere in rural South or Central America. This suggestion immediately evokes concern regarding political appropriateness and public perception. If such studies are ever to move forward to the stage of field-testing, they must be carried out by local scientists in conjunction with ministries of health in the particular countries where the work is being conducted. Furthermore, the public must be informed and brought into discussions at the earliest possible time. These measures are critical to insure the greatest opportunity for successful completion of the work and to reduce the risk for potential adverse effects.

8. Summary and conclusions

The potential use of GM symbionts for paratransgenic control of Chagas disease transmission by triatomines is based on the following concepts: (1) triatomines harbour bacterial symbionts that are essential for survival and reproduction in these insects; (2) it is possible to culture these symbionts, genetically transform them, and express a gene whose product kills trypanosomes, thus rendering the insect incapable of transmitting Chagas disease; and (3) normal insect symbionts can be replaced with GM symbionts, resulting in a population of vectors that can no longer transmit Chagas disease. Two primary mechanisms are currently being used for transforming microbial symbionts of Chagas disease vectors. These include shuttle plasmids and DNA integration elements. Two groups of potential anti-trypanosomal genes have been expressed in paratransgenic insects. These include the anti-microbial peptide cecropin A and an active single chain antibody fragment which can be directed against a specific target. Bacterial symbionts of several important Chagas disease vectors have been transformed, and methods have been developed to potentially introduce GM symbionts into natural populations of insects. This tech-

nology may someday be used to supplement insecticide-based vector control approaches.

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