Published in final edited form as:

Gene. 2007 April 15; 391(1-2): 53–62.

Bacteriophage-based Vectors for Site-specific Insertion of DNA in the Chromosome of *Corynebacteria*

Mark Oram^{1,§}, Joelle E. Woolston¹, Andrew D. Jacobson², Randall K. Holmes², and Diana M. Oram^{1,*}

¹ Department of Biomedical Sciences, University of Maryland Baltimore Baltimore MD 21201, USA

² University of Colorado School of Medicine, Department of Microbiology, Aurora, CO 80045, USA

Abstract

In *Corynebacterium diphtheriae*, diphtheria toxin is encoded by the *tox* gene of some temperate coryneophages such as β . β -like coryneophages are capable of inserting into the *C. diphtheriae* chromosome at two specific sites, *attB1* and *attB2*. Transcription of the phage-encoded *tox* gene, and many chromosomally-encoded genes, is regulated by the DtxR protein in response to Fe^{2+} levels. Characterizing DtxR-dependent gene regulation is pivotal in understanding diphtheria pathogenesis and mechanisms of iron-dependent gene expression; although this has been hampered by a lack of molecular genetic tools in *C. diphtheriae* and related Coryneform species. To expand the systems for genetic manipulation of *C. diphtheriae*, we constructed plasmid vectors capable of integrating into the chromosome. These plasmids contain the β -encoded *attP* site and the DIP0182 integrase gene of *C. diphtheriae* NCTC13129. When these vectors were delivered to the cytoplasm of non-lysogenic *C. diphtheriae*, they integrated into either the *attB1* or *attB2* sites with comparable frequency. Lysogens were also transformed with these vectors, by virtue of the second *attB* site. An integrated vector carrying an intact *dtxR* gene complemented the mutant phenotypes of a *C. diphtheriae* ΔdtxR strain. Additionally, strains of β -susceptible *C. ulcerans*, and *C. glutamicum*, a species non-permissive for β , were each transformed with these vectors. This work significantly extends the tools available for targeted transformation of both pathogenic and non-pathogenic *Corynebacterium* species.

Keywords

Corynebacterium; Integrating Vector; Lambda Integrase Family; Attachment Sites; Diphtheria

1. Introduction

Corynebacterium diphtheriae is the causative agent of respiratory diphtheria. While all *C. diphtheriae* strains are capable of colonizing humans, only those that produce diphtheria toxin (DT) cause the life-threatening toxin-mediated manifestations of the disease (Holmes, 2000). Immunization with formaldehyde-treated DT leads to induction of a protective antitoxic immune response, although periodic re-immunization is required to maintain immunity.

*Corresponding author: Department of Biomedical Sciences, Rm 4E-04, University of Maryland Dental School, 666 W. Baltimore St., Baltimore, MD 21201, Phone: 00 1 410 706 8705, Fax: 00 1 410 706 0865, email: doram@umaryland.edu

§Current address: Biochemistry and Molecular Biology Department, University of Maryland Baltimore, 108 N. Greene St, Baltimore MD 21201, USA

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Diphtheria continues to pose a significant health threat where *C. diphtheriae* is endemic and in areas where a significant proportion of the population lacks or has failed to maintain full immunity. Indeed, loss of protective levels of antitoxin in the adult population was a major contributing factor in the diphtheria epidemic that occurred in the 1990's in states of the former Soviet Union (Golaz et al., 2000; Mattos-Guaraldi et al., 2003).

The *tox* gene encoding DT is carried on various temperate corynebacteriophages, such as β , which integrate into the *C. diphtheriae* chromosome during the lysogenic phase of the infective cycle (Holmes, 2000). The expression of *tox* in lysogens is regulated by the chromosomally-encoded *dtxR* gene product in response to Fe^{2+} levels (Boyd et al., 1990; Schmitt and Holmes, 1991). The DtxR protein, when complexed with Fe^{2+} , binds to the *tox* promoter and represses transcription of *tox*. Conversely, when iron is limiting, the un-complexed form of DtxR is unable to bind DNA, leading to induction of DT as a consequence of iron starvation. In addition to its role in DT production, DtxR is a global regulator of transcription, regulating the expression of multiple genes involved in iron metabolism, protection against oxidative stress and pathogenesis (Kunkle and Schmitt, 2003; Lee et al., 1997; Schmitt, 1997; Schmitt and Holmes, 1994; Schmitt et al., 1997). DtxR is also the prototype for a large family of bacterial gene regulators found in numerous bacterial species, including several that are medically relevant; and the proteins of this family share structural and functional similarities (Feese et al., 2001). Characterizing DtxR-dependent pathways at the molecular level is pivotal in understanding the (linked) phenomena of diphtheria pathogenesis, mechanisms of iron-dependent gene expression, and responses to oxidative stress.

Direct transformation of *C. diphtheriae* with DNA is significantly hampered by both the relatively impermeable Coryneform cell wall, as well as host DNA restriction barriers (Puech et al., 2001). Nevertheless, some key results have recently extended the systems available for genetic manipulation of *C. diphtheriae*. First pNG2, a plasmid that replicates in *C. diphtheriae* has been isolated and characterized (Tauch et al., 2003). Second, conjugal transfer of DNA from an *E. coli* donor to a *C. diphtheriae* recipient (followed by integration of the transferred DNA into the chromosome *via* homologous recombination) has been reported (Ton-That et al., 2004); providing an alternative to electroporation as a mechanism for introducing foreign DNA into the *C. diphtheriae* cytoplasm. Third, the homologous recombination pathway has also been exploited in a targeted allelic exchange method; where plasmids carrying a portion of a gene, when transformed into *C. diphtheriae*, were capable of integrating into the chromosome (Schmitt and Drazek, 2001). Fourth, use of the Tn5 transpososome system for mutagenesis resulted in isolation of the first marked mutations in *C. diphtheriae*; the first characterization of a *dtxR* null mutant, and the demonstration that DtxR is not essential for cell survival (Oram et al., 2002). Following on from these advances, the recently determined genome sequence of a pathogenic *C. diphtheriae* clinical isolate, NCTC13129 (Cerdeno-Tarraga et al., 2003) will aid further genetic studies of *C. diphtheriae*.

In other bacterial systems, the availability of a genome sequence has facilitated the development of vector systems for the targeted insertion of DNA. Such systems typically exploit the integrase protein and *attP* site of temperate bacteriophages, or prophages identified by genomics studies (Groth and Calos, 2004). An integrase-dependent, site-specific recombination reaction between the vector-borne *attP* site and the chromosomal *attB* locus generates the recombinant *attL* and *attR* sites and causes integration of the vector (which can include virtually any foreign DNA sequence) into the chromosome. Some notable examples using this methodology include the stable transformation of *Mycobacterium tuberculosis*, *M. smegmatis* and BCG strains using the tyrosine integrase proteins of Φ Rv1 or L5 (Bibb and Hatfull, 2002; Lee et al., 1991). Most relevant to the current work is vectors utilizing the integrase protein and *attP* sites from the corynebacteriophages Φ 304L, Φ AAU2 or Φ 16 (Le Marrec et

al., 1998; Moreau et al., 1999a; Moreau et al., 1999b) that transformed *C. glutamicum* host strains. In the latter case, a strain of *Arthrobacter aureus* and other strains of *C. glutamicum* that are not normally permissive hosts for $\Phi 16$ could also be transformed with the same vector by virtue of the appropriate *attB* sequences present on the chromosome of these recipients.

To facilitate the molecular characterization of gene regulatory pathways in *C. diphtheriae*, we developed an integrating vector system that exploits the *attP* site and integrase protein of phage β . The vector also included a conjugal transfer origin to facilitate delivery of DNA to the *C. diphtheriae* cytoplasm via conjugal mating with an *E. coli* donor; thus circumventing transformation and restriction barriers. As shown in this work, there are two particularly attractive features of using the β recombination system in this manner. First, since *C. diphtheriae* carries two closely-spaced *attB* sites designated *attB1* and *attB2* (Rappuoli and Ratti, 1984), single β lysogens can serve as recipients for the integrating vector. Second, since both *C. glutamicum* and *C. ulcerans* possess the β *attB* site(s) (Cianciotto et al., 1990), these two Coryneform species could also be transformed with the β phage-based vectors, thus establishing the utility of this vector system for species other than *C. diphtheriae*.

2. Materials and Methods

2.1. Cultivation conditions and media

C. diphtheriae and *C. ulcerans* were routinely grown in heart infusion broth (Difco) supplemented with 0.2% Tween 80. For doubling time analyses and siderophore assays *C. diphtheriae* strains were grown at 37°C with shaking in the deferrated casein hydrolysate medium of Mueller and Miller (Mueller and Miller, 1941) as modified by Barksdale and Pappenheimer (Barksdale and Pappenheimer, 1954): that is PGT with (high iron) or without (low iron) 10 μ M FeCl₃. Stocks of deferrated PGT were prepared by Chelex-100 treatment for 2 hrs as described (Tai et al., 1990) followed by filter sterilization.

C. glutamicum was grown in Luria-Bertani (LB) broth (Sambrook and Russell, 2001). *E. coli* strains DH5 α (Hanahan, 1983) and TE1 (Jobling and Holmes, 2000) were used as hosts for cloning and were cultivated in LB broth. The *E. coli* strain B327 which lacks functional *dam* and *dcm* type methylases (M. G. Jobling, personal communication) was used to isolate DNA lacking these modifications. Antibiotics were used at the following concentrations: for *E. coli* kanamycin at 25 μ g per ml, for *Corynebacterium* kanamycin at 10 μ g per ml and nalidixic acid at 20 μ g per ml.

2.2. Introduction of genetic material into *Corynebacterium* species

Electroporation was used to introduce DNA into *C. diphtheriae* (Oram et al., 2002); with pKN2.6 (Schmitt and Holmes, 1991) used as a positive control. Matings were performed as previously described (Oram et al., 2006) using *E. coli* S17-1 containing an RP4 mobilizable plasmid as the donor and strains of *C. diphtheriae*, *C. glutamicum*, or *C. ulcerans* as the recipients. Resistance to nalidixic acid was used to select for the Coryneform species.

2.3. Construction of integrating vectors

Restriction and other DNA modification enzymes were obtained through the BIORESCO freezer program of the University of Maryland Baltimore. Standard cloning procedures were followed throughout (Sambrook and Russell, 2001). PCR was performed with Taq polymerase (Fermentas Life Sciences), and restriction-digested fragments were purified from agarose gels with the UltraClean kit (Mo Bio Laboratories). The correct assembly of each construct was verified by restriction mapping and DNA sequencing (performed by the University of Maryland Biopolymer and Genomics Core Facility). Figure 1 shows the maps of the integrating vectors. The plasmid pK903 (the parent of the vectors constructed here) is a pUC19 (Yanisch-

Perron et al., 1985) derivative that confers kanamycin resistance; constructed by ligating together the 1.75 kb SspI-DraI fragment of pUC19 and a 0.93 kb fragment of EZ-Tn5 <KAN-2> (Epicentre) containing the *aph* gene. pKMO3W was assembled from the following three DNA fragments. The DIP0182/*int* gene was obtained by PCR on NCTC13129 genomic DNA, with primers Φ-INT-PstI (5'GTG TAA AGT GGG CTG CAG CTA ACC) and Φ-INT-BglII (5' CGT GAG ATC TCT GCA TAA GCA ATA G). The *attP* site was obtained by PCR on β DNA, with primers ATTP-UP-SphI (5'AGG TGC ATG CTA AGC TAT CGC TAT TTT TTG AAA) and ATTP-DN-PstI (5'TTC TAA CTG CAG GTC AGC TGT GTC GAG TTC). SphI/BamHI-cut pK903, BglII/PstI-cut DIP0182/*int* DNA and PstI/SphI-cut β *attP* DNA fragments were ligated together to yield pKMO3W (4.43 kb). The RP4 transfer origin was obtained from the 2.42 kb KpnI fragment of pK18mobsacB (Schafer et al., 1994); this was ligated into KpnI-cut, phosphatase-treated pKMO3W to yield pKMO3W+mob (6.85 kb). pKMO3W+mob was reduced in size by digesting with SacI: the largest 5.09 kb fragment was gel-purified and re-circularized to yield pK-AIM (5.09 kb). To make pK-AIMdtxR (6.04 kb), a copy of the *dtxR* gene was inserted in the SacI site of pK-AIM. The *dtxR* gene was obtained by PCR on NCTC13129 DNA with oligos dtxR-SacI-UP (5'GCC GAA AAA CTT GAG CTC TAC GCA CAA TAA AGC G) and dtxR-SacI-DN (5'CAT CTA ATT TCG AGC TCT TTA ATA TTT AGA G). We selected and subsequently used a clone of pK-AIMdtxR where the *dtxR* gene was transcribed in the same direction as the *int* gene. To construct pK-PIM, pK-AIM was first partially digested with EcoRI, and then with SacI. A 5.09 kb fragment was gel-purified and used for ligation with linker DNA. The linker was obtained by mixing the oligos polylinker1 (5'CTT AAT TAA CGT TAA CTA GTA GAT CTG GGC CCC GCG GCG GCC GCA CGT G) and polylinker2 (5'AAT TCA CGT GCG GCC GCC GCG GGG CCC AGA TCT ACT AGT TAA CGT TAA TTA AGA GCT), together in equimolar (40 μM) amounts in NEB#2 buffer (New England Biolabs), heating to 85°C for 5 min and then cooling slowly to room temperature. The DNA linker was ligated with SacI/partial EcoRI-cut pK-AIM to yield pK-PIM (5.14 kb).

2.4. Detection of integrants

Kanamycin-resistant colonies obtained from transformation of *C. diphtheriae* strains with an integrating vector were screened for insertion events at *attB1* or *attB2* by PCR. A sample of each colony was re-suspended in 35 μl water and heated at 95°C for 5 min: the cellular debris was then pelleted by centrifugation. Six 5 μl aliquots of the supernatants were used in separate PCR reactions. The primers used were ATTP-UP (5'AGG TGC ATG CTA AGC TAT CGC TAT TTT TTG AAA); ATTP-DN (5'TTC TAA CTG CAG GTC AGC TGT GTC GAG TTC); ATTB1-UP (5'GGC TCA ATC TGA TCG GCG TGG TGC T); ATTB1-DN (5'GGC GAG TAG GCA CGC AGC AAG AAA AA); ATTB2-UP (5'CGT ACG TCG GGA TCT GGG AAA GGT GGT CT), and ATTB2-DN (5'CGA AGA CTC TAG TGT AAT CGG TGT A). The combinations of these primers used to detect each *att* site are listed in Table 1. PCR was performed with a cycle profile of 94°C, 30 sec; 55°C, 30 sec; 72°C, 60 sec (22 cycles). Integration events in *C. glutamicum attB1* or *attB2* sites were detected with oligos ATTP-UP; ATTP-DN; ATTB1g-UP (5'CTG AAC ATC ATC GCA GTC ATC CTC ATT ACG); ATTB1g-DN (5'CGG CGC ACG GAT CGA AGT GTT C), ATTB2g-UP (5'CAT AAG TAG GGA TAG TTG CCA AAT CTG CTC) or ATTB2g-DN (5'TGT CGA GAA ACG AAT GCC CCA GTT TCA CCC). Integration of vector DNA at *C. ulcerans attB* sites was detected using oligos ATTBu-UP (5'CCA CCT ATG CGC CCG TAG CTC) and ATTBu-DN (5'CAA CAA TCC ACC AAC CAA ACA CAC).

For Southern blots, DNA was isolated from each strain (Oram et al., 2002) and digested with either SacI or NotI. Digested DNA was separated on a 0.8 % agarose gel and then was transferred to nylon membranes (Sambrook and Russell, 2001). A DNA probe was generated using pKMO3W as a template in PCR with the primer oligos KanFor (5' GGA AAT GTG

CGC GGA ACC C) and KanRev (5' GTG GAA CGA AAA CTC ACG TTA AGG G). The probe was labeled using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Hybridization and detection were performed as recommended in the DIG- High Prime DNA Labeling and Detection Starter Kit II.

2.5. Siderophore assays

Siderophore assays were performed as described previously (Oram et al., 2002). A standard curve was constructed by performing assays with ethylenediamine-N,N'-diacetic acid (EDDA) at concentrations from 50 μ M to 1 mM. One siderophore unit was defined as the A_{630} of an assay performed with a 0.5-ml sample of 1 mM EDDA.

3. Results

3.1. Corynebacteriophage β integration functions and construction of pKMO3W

The two *attB* sites for phage β (and closely related phages) in the *C. diphtheriae* chromosome are each partly contained within a duplicated tRNA^{ARG} gene (anticodon ACG) (Ratti et al., 1997); a property shared with many other integrating phage systems (Campbell, 1992). The sites, which are named *attB1* and *attB2*, flank gene DIP0179 [numbering based on the genome annotation of strain NCTC13129 (Cerdano-Tarraga et al., 2003)] such that the genetic loci in this region map in the order DIP0178-*attB2*-DIP0179-*attB1*. The acquisition of *tox* (DIP0222) responsible for the toxigenic nature of NCTC13129 likely occurred from the integration of a 36.5 kb β -like phage at the *attB1* site in a progenitor strain, with the concomitant formation of the *attL1* and *attR1* sites flanking the prophage sequence.

The entire sequence of the NCTC13129 β -like prophage is present in the genome sequence. The annotation designates the prophage integrase gene as DIP0182, with two small open reading frames (DIP0180 and DIP0181) lying between it and the *attL1* site immediately adjacent to DIP0179. To learn more about the integrase and *attP* function in phage β itself we first cloned and sequenced a 2.5 kb region of β that spanned the equivalent *int-attP* region. This work revealed that in β an extra adenine insertion caused the DIP0180 and DIP0181 ORFs to be fused into a single ORF: the possible significance of this was not investigated further. The sequence also showed that the β integrase was highly similar to that encoded by DIP0182 from the sequenced strain. More specifically, each gene encoded a recombinase protein comprising 408 amino acids, with 11 (highly conservative) amino acid differences over their total lengths. The *attB* and *attP* regions from NCTC13129 and β are identical over the first 50 bp and in total share a 92 bp common 'core' region (Cianciotto et al., 1986): in addition, the core regions of β *attP* and the NCTC13129 prophage *attP* (deduced from *attL1* and *attR1*) were identical over this region.

We initially constructed vectors using PCR-generated DNA from either NCTC13129 or C7 (β) (Bardsdale and Pappenheimer, 1954), a separate *C. diphtheriae* toxigenic isolate (the progenitor strain C7(-) (Freeman, 1951) was also used as a recipient for some of the vectors constructed here). The first construct which we used extensively was termed pKMO3W (see Figure 1 and Section 2.3 for descriptions of plasmid constructions). This plasmid and subsequent derivatives carried the entire DIP0182/*int* gene from NCTC13129 along with 200 bp upstream of the open reading frame. pKMO3W also contained a 452 bp fragment of the β *attP* region, which included the 92 bp core along with 115 bp of the upstream and 245 bp of the downstream regions. A gene encoding resistance to kanamycin was included on the vector to enable selection of transformants, as well the *colE1*-derived replication origin of pUC19, which permits episomal propagation in *E. coli* but not *C. diphtheriae*. The absence of a replication origin capable of functioning in *C. diphtheriae* ensured that kanamycin-resistant

C. diphtheriae isolates would be indicative of a recombination event between the vector and chromosome resulting in integration.

3.2. Electroporation of pKMO3W into *C. diphtheriae*

We first delivered pKMO3W to the cytoplasm of C7(−), C7(β), or NCTC13129 by electroporation. The efficiency of transformation was very low (<1 transformant per μg DNA), although a clear increase of approximately 5-fold was observed when using DNA that had been previously passaged through a *dam dcm E. coli* host prior to electroporation. Integration events at the *attB1* or *attB2* sites in these strains were detected with a PCR-based screening approach. The basis of this screen was that convergent oligo primers designed to hybridize in the ORFs immediately flanking the *attB1*, *attB2* or *attP* sites (Figure 2) would yield products, but only while these sites remained intact. Conversely, suitable permutations of these oligos (Table 1) could be used to reveal the formation of the recombinant *attL* and *attR* sites following an integration event. The DNA of transformant colonies was screened in six PCRs performed in parallel: each with a combination of oligos designed to reveal the presence or the absence of the appropriate *att* site(s). The PCR profiles of the three host strains using this assay are shown in Figures 3A–3C. C7(−) had both *attB1* and *attB2* sites intact, while both C7(β) and NCTC13129 possessed *attL1* and *attR1* in place of *attB1*. This was as expected, since the respective β or the β-like phages have been previously mapped to the *attB1* site in both cases (Cerdeno-Tarraga et al., 2003; Rappuoli et al., 1983). Colonies arising from electroporation of C7(−) with pKMO3W were screened in exactly the same way, and some illustrative examples are shown in Figures 3D and 3E. One experiment in which 40 μg of pKMO3W DNA was electroporated into C7(−) gave two colonies on kanamycin plates: one of these showed evidence of an integration event at *attB1* (Figure 3D) while the incoming DNA in the other isolate had recombined at *attB2* (Figure 3E). This result established that the vector was capable of integrating into either *attB* site. In a separate experiment using 100 μg of the same DNA to transform C7(−), 23 colonies were obtained: of these 12 had recombination occurring at *attB1*, and the remaining 11 had recombination at *attB2*. This latter result strongly implies the vector has no preference for insertion into either *attB* site.

3.3. Mobilization of integrating vectors from *E. coli* to *C. diphtheriae*

To provide an alternative method to electroporation for delivery of an integrating vector to *C. diphtheriae*, we utilized the RP4 origin of transfer, obtained from plasmid pK18mobsacB (Schafer et al., 1994). This origin was added to pKMO3W to produce vector pKMO3W+mob. [The episomally replicating, positive control plasmid pCB303 was constructed by inserting the pNG2 *C. diphtheriae* origin of replication (Schmitt and Holmes, 1991), carried on a 2.6 kb pCM2.6 EcoRI/ClaI fragment (ClaI end made blunt with T4 polymerase), into pK19mobsacB digested with EcoRI and SmaI.] Mating reactions were set up with S17-1/pKMO3W+mob as the donor and C7(−) as the recipient. Kanamycin-resistant colonies were obtained in this manner, at a frequency comparable to that of the pCB303 control (Table 2). Significantly more colonies were obtained with this method compared with the delivery by electroporation, and once again the PCR screen showed that integration had occurred in either of the *attB1* or *attB2* sites at near identical frequencies (data not shown). In addition we observed some rarer double integration events where insertions occurred both at *attB1* and *attB2* in the same cell (Figure 3F).

Finally, to confirm that the insertions occurred solely at the *attB1* and/or *attB2* sites, we isolated DNA from strains containing single and double insertions (as detected by the PCR assays). We performed Southern blots on this genomic DNA using a probe identical to the *aph* gene common to all of the vectors described here. The DNA samples were digested with restriction enzymes that do not have recognition sites in the *aph* probe. A single band was detected in DNA from those strains containing insertions at *attB1* or *attB2*; while in samples from strains

that contained insertions at both *attB1* and *attB2* sites two and only two bands were detected (data not shown). Based on these results, we infer that insertion of pKMO3W and its derivatives occurred solely at the *attB* sites.

3.4. Utility and further development of the integrating vector system

The above analyses with pKMO3W and pKMO3W+mob established the integrating vector as an efficient means to construct targeted DNA insertions in *C. diphtheriae*. Following on from this, two other vector derivatives were constructed. Firstly we deleted sequences from pKMO3W+mob which were not necessary for its function as a mobilizable integration vector (and which contained extraneous copies of otherwise useful restriction sites) to create pK-AIM (Figure 1). We then added to pK-AIM a short region of DNA containing recognition sites for several novel restriction enzymes, to construct pK-PIM (Figure 1). The presence of the polylinker in pK-PIM expands the versatility of the plasmid integration vector for insertion of novel DNA sequences that encode genes of interest. These plasmids were constructed to improve the utility of the system; and two further experimental approaches to this end, namely the complementation of a single gene deletion in *C. diphtheriae*, and the use of the vector in other species of *Corynebacterium*, are described below.

3.5. Complementation of an inactivated *C. diphtheriae* gene in single copy

The *dtxR* gene in *C. diphtheriae* is non-essential, since a C7(β) Δ *dtxR* deletion strain is viable. Nevertheless, the Δ *dtxR* mutant has a growth defect compared with C7(β) in high iron medium and is unable to regulate siderophore production in response to iron availability (Oram et al., 2006). We thus determined if *dtxR* expressed from a novel chromosomal location (namely *attB2*) could complement these two DtxR-dependent phenotypes of C7(β) Δ *dtxR*. A copy of the *dtxR* gene was inserted into pK-AIM to create pK-AIM*dtxR*, and both C7(β) Δ *dtxR* and C7(β) were transformed with these plasmids. Southern blot and PCR analyses were used to confirm that C7(β) Δ *dtxR*::pK-AIM*dtxR* carried a full-length copy of *dtxR* at the *attB2* locus and the deleted Δ *dtxR* (data not shown).

We performed the growth rate determinations on three separate cultures grown under high iron or low iron conditions (Section 2.1.). Using this method, the doubling times $\pm$ standard deviation of the test strains during log phase growth in low iron medium were as follows: C7(β)::pK-AIM = 57 ± 4 min, C7(β)::pK-AIM*dtxR* = 56 ± 5 min, C7(β) Δ *dtxR* = 72 ± 4 min, C7(β) Δ *dtxR*::pK-AIM = 71 ± 5 min and C7(β) Δ *dtxR*::pK-AIM*dtxR* = 59 ± 3 min. In high iron medium the doubling times of all strains expressing a wild-type copy of *dtxR* decreased by approximately 3 to 5 minutes indicating an increased rate of growth. In stark contrast the doubling times of C7(β) Δ *dtxR* and C7(β) Δ *dtxR*::pK-AIM *increased* by approximately 3 to 5 minutes. These data indicate that the presence of the pK-AIM integration vector alone has no effect on the growth rate of *C. diphtheriae* and that expression of *dtxR* from pK-AIM*dtxR* inserted at *attB2* is capable of restoring a wild-type growth rate to C7(β) Δ *dtxR* in both high and low iron media.

We also assayed the ability of each strain to regulate production of siderophore in response to iron in the growth medium. The results of this assay are shown in Figure 4. C7(β)::pK-AIM was capable of suppressing production of siderophore in the presence of 10 μ M FeCl₃ by approximately 50-fold. C7(β) Δ *dtxR*::pK-AIM suppressed production of siderophore in the presence of FeCl₃ by approximately 6-fold but it produced approximately 10 times more siderophore under high-iron conditions than strains that expressed *dtxR*. This result is equivalent to that observed previously with C7(β) Δ *dtxR* (Oram et al., 2006). In contrast when pK-AIM*dtxR* was integrated into *attB2* in C7(β) Δ *dtxR* (to form C7(β) Δ *dtxR*::pK-AIM*dtxR*) the ability to repress siderophore production by approximately 50-fold in the presence of FeCl₃ was restored. Thus a single copy of pK-AIM*dtxR* inserted at *attB2* is sufficient to correct

the defects of $C7(\beta)\Delta dtxR$ in both the growth rate and the regulation of siderophore production in the presence of iron.

3.6. Use of the integration vectors in other species of *Corynebacterium*

The β *attB* site of *C. diphtheriae* is present in other Coryneform species (Cianciotto et al., 1986), opening up the possibility that the vectors developed here could be used to transform other species of *Corynebacterium*. To test this hypothesis we focused on two species: *C. glutamicum* and *C. ulcerans*. *C. glutamicum* strains, while possessing two phage β *attB* sites (Cianciotto et al., 1990), have not been shown to be susceptible to infection by β -like corynephages. *C. ulcerans* strains, by contrast, are permissive hosts for β -like phages (Groman et al., 1984). We used the strains *C. glutamicum* ATCC 13032, for which the genome sequence has been determined (Kalinowski et al., 2003); or *C. ulcerans* 712 which does not host a β -like prophage and instead possesses two intact *attB* sites (Groman et al., 1984).

We used *E. coli* S17-1 as a donor to mate pKMO3W+mob or pK-AIM into each of these hosts. In all cases the frequency of recipients that received and maintained the integrating vector (integrants) was equal or better than that observed with the replicating plasmid pCB303 (Table 2). To confirm the presence of the integrating vector in the chromosome of the integrants, we again utilized a PCR based method. We designed novel oligo primers based on the *C. glutamicum* ATCC 13032 genome sequence, whose binding sites lie in open reading frames adjacent to the *attB1* and *attB2* sites, and used these primers in combination with the original *attP*-specific primers to detect insertions (Table 1). [Note that to maintain the convention used in *C. diphtheriae* the *attB2* site in *C. glutamicum* is the first site encountered on the chromosome between Cgl0218 and Cgl0219 while *attB1* lies between Cgl0225 and Cgl0226, following the gene annotation established in (Kalinowski et al., 2003)]. The intact *attB1* and *attB2* sites were readily detectable in the recipient strain prior to transformation and we detected insertions in either *attB1* or *attB2* in all kanamycin-resistant *C. glutamicum* transformants (Figure 5A–C). This shows that pKMO3W can integrate into either of the *attB* sites present in the *C. glutamicum* chromosome.

For *C. ulcerans* 712, formation of lysogens with the phage sequences inserted at two different chromosomal sites (*attB1* and *attB2*) following infection with β has been described (Cianciotto et al., 1986). The genome sequence was not available, although the sequence of *attB1*, but not *attB2* had been determined previously (Cianciotto et al., 1990). With this in mind, we designed screening primers ATTBu-UP and ATTBu-DN based on the reported *attB1* sequence (Table 1). However, it is important to note that the primers designed to detect *attB1* in *C. ulcerans* may in addition bind at *attB2*. Kanamycin-resistant integrants from matings performed between S17-1/pK-AIM and *C. ulcerans* 712 were screened for *attB*, *attL* and *attR* as described for *C. diphtheriae* and *C. glutamicum*. In *C. ulcerans* 712 we detected a band of the predicted size for *attB1* using the primers ATTBu-UP and ATTBu-DN (data not shown). In addition, 6 out of 10 transformant strains in one screening experiment showed the presence of an *attR1* band when screened with ATTBu-DN and ATTP-UP primers (Figure 5D). Strains that lacked this band instead gave an *attB1* band when screened with ATTBu-UP and ATTBu-DN (data not shown). This data implies that 4 integration events occurred at *attB2*: however, all ten colonies also gave a positive PCR signal when screened for *attL1* (Figure 5D). This latter result would arise if ATTBu-UP also bound in *attB2*, as noted in the caveat above. In summary, we detected insertions of pK-AIM at either *attB1* or *attB2*, in approximately equal frequencies, in the chromosome of *C. ulcerans* 712.

4. Discussion

We describe here the development and use of novel vectors to integrate DNA into the chromosome of *C. diphtheriae*, *C. glutamicum* and *C. ulcerans*; by exploiting the DNA

integration functions of temperate coryneophage β . The success of this approach is significant, as relatively few methods have hitherto been described for chromosomal manipulation of *C. diphtheriae*. The system developed here also has great utility for characterizing the biochemical events giving rise to pathogenesis or other gene expression pathways in both medically and commercially relevant Coryneform species. Furthermore, a search of the sequenced genomes available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) revealed β *attB*-like sequences in many bacterial genera including *Tropheryma*, *Nocardia*, *Mycobacterium* and *Bacillus* (all with greater than 85% identity over 100 bp) raising the possibility that these vectors might also function in some of these species.

A number of systems for site-specific integration of DNA into bacterial chromosomes have been described that exploit one of two types of integrase: either a tyrosine- or serine recombinase enzyme. A great deal is known about the function of the tyrosine family of enzymes, due in large part to extensive studies on the archetypical lambda integrase protein. The β integrase enzyme utilized here is a member of this class, and the activity of the enzyme in this study was consistent with the known properties of the family. We used two integrase proteins in this work; since the NCTC13129 DIP0182 product and the actual β integrase protein differ in 11 positions over their 408 amino acid lengths. However, none of these changes affect residues that are highly conserved across the lambda family; and in addition vectors assembled with each form of the enzyme were functional in integration assays (data not shown). Thus the 11 changes can be regarded as polymorphisms with no affect on enzyme function. Perhaps most interestingly, in each of these two proteins, the histidine of the 'inviolat' RHR signature triad of the lambda integrase family (Argos et al., 1986) is replaced with tyrosine. This histidine to tyrosine substitution in a lambda recombinase is rare but not undocumented (Esposito and Scoocca, 1997; Nunes-Duby et al., 1998).

The *attB* and *attP* sites of the β system share a 92 bp core region (a large region compared with other examples of tyrosine integrase systems) with the first 50 bp of this alignment being 100 % conserved. In the lambda integrase systems the *attP* sites tend to be more extensive than *attB* sites, since they carry additional binding sites for host or phage factors that enhance the recombination reaction. In this study a fragment of β DNA 452 bp in length and which included the *attP* core was efficiently recombined with chromosomal *attB* sites in all three Coryneform hosts examined. Therefore all sequences required for recombination at the β *attP* site reside within this 452 bp region. However, the points of strand crossover in the core of the β *attP* and *attB* sites have yet to be mapped at the nucleotide level. Since exact sequence identity is usually demanded in site-specific recombination crossover regions, it is very likely that this region lies within the 50 bp of shared identity between the β *attB* and *attP* sites. Such a location is consistent with work (Michel et al., 1982) that mapped the point of strand exchange to within 50 bp of the EcoRI site near the *C. diphtheriae* *attB1* core.

The 'minimal' requirements to produce a vector for integrating DNA into Coryneform chromosomes thus seem to be the β integrase gene and some portion of the 452 bp region of *attP* contained in pKMO3W: no other phage sequences or functions were present on pK-AIM or the other vectors that catalyzed integration. The combination of these functions with the *oriT* mobilization system for DNA transfer yielded a system with many potential applications. For example, the construction of gene deletions in the chromosome of *C. diphtheriae* has been reported by several groups (Oram et al., 2006; Schmitt and Drazek, 2001; Ton-That et al., 2004). This approach, however, is not likely to be successful if an essential gene is to be deleted. One way to overcome this is to first integrate a second copy of the gene into a separate chromosomal locus. The native gene could then be deleted without depriving the cell of the essential function. Although such insertions could be accomplished by methods other than an integrating vector, the vectors described here have a significant advantage over these former approaches, in terms of speed and ease of use.

The vectors developed here also have advantages over methods that use pNG2-based replicating plasmids for gene manipulation; including obviating possible issues arising from the multiplicity and variations in copy number, and the usual requirement for some form of selection to maintain the episome. In fact, while we had previously demonstrated that an intact copy of *dtxR* was able to reverse the phenotype of the C7(β) $\Delta*dtxR* strain when introduced *in trans* on a replicating plasmid (Oram et al., 2006), this work is the first to establish that a single copy of *dtxR* integrated into *attB2* is capable of restoring DtxR functions to levels seen in a wild-type control. In addition, the inclusion of a transcriptional reporter gene to an integrating vector would permit characterization of the activity of a promoter in single copy from the *C. diphtheriae* chromosome. We are currently constructing such vectors.$

One particularly attractive feature of exploiting the β phage functions is that *C. diphtheriae* (and other Coryneform species) possess two β *attB* sites, allowing for a significant degree of flexibility with this system. Given the link between β lysogeny and diphtheria pathogenesis it is desirable to have a means to characterize the genetics and biochemical pathways of diphtheria progression. As shown here the C7(β) and NCTC13129 strains, both pathogenic isolates that possess only the *attB2* site, were transformed with high efficiency. Indeed, two separate integrating plasmids could be used (assuming the second one carried a different selection marker than the first) to create two differing and novel integrated sequences. Yet another possibility is that the vector(s) could be used in conjunction with replicating plasmids. Used in combination these approaches will facilitate the dissection and defined re-construction of complex regulatory and expression networks in *C. diphtheriae* and other Corynebacterial species.

Acknowledgements

We thank Jessica McGrattan for performing the Southern blots and Lindsay W. Black for helpful comments. This work was supported in the laboratory of D.M.O. by research grant NIH/NIAID K22 AI60882-01 and by research grant NIH/NIAID RO1 AI14107 in the laboratory of R.K.H.

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Abbreviations

DT	diphtheria toxin
orf	open reading frame

Figure 1. Maps of integration vectors

Features in each vector are shown to scale within each separate map (details are given in the Materials and Methods). pKMO3W was assembled from the BamHI-SphI fragment of pK903, a SphI-PstI fragment carrying the β attP and a PstI-BglII fragment carrying the DIP0182/int gene. A KpnI fragment of pK18mobsacB, carrying the RP4 oriT was inserted into the KpnI site of pKMO3W to yield pKMO3W+mob, and extraneous sequences between two SacI sites was removed to give pK-AIM. A SacI fragment carrying dtxR was added to form pK-AIMdtxR. In addition a novel polylinker, assembled by annealing two oligonucleotides, was added between the adjacent EcoRI and SacI sites of pK-AIM to create pK-PIM.

Figure 2. Site specific integration mediated by β -like corynebacteriophages

The episomal element (ie the phage or integrating plasmid vector) that includes *attP* (striped boxes) and *int* (black arrow) is shown as a dotted line. Additional genes may be included and in the case of a toxigenic corynebacteriophage *tox* (black and white vertical striped arrow) is present. The region of a (non-lysogen) *C. diphtheriae* host chromosome that includes *attB1* and *attB2* (grey boxes) as well as the orfs DIP0178, DIP0179 and DIP0225 (shown as white arrows) is shown below the circular element. Chromosome maps of strains in which insertions occur at *attB1*, *attB2* or both attachment sites (including the *attL* and *attR* sites that result following site-specific integration mediated by *Int* between *attP* and *attB*) are shown on the right. Figure is not drawn to scale.

Figure 3. Profiles of Strains in the PCR Screen

The presence or absence of the *attB1* or *attB2* sites was detected by PCR on recombinant clones, using oligos which hybridize in the immediately flanking open reading frames. The recombinant *attL* or *attR* sites were revealed by using the permutations of the bacterial- or phage-based oligo sequences given in Table 1. The PCRs were designed to detect the *att* site indicated above the lane. (A–C) the screen performed on the C7(–), C7(β) and NCTC13129 strains. (D–F) screens performed on isolates of C7(–) transformed with pKMO3W.

Figure 4. Siderophore assays

Total siderophore units (the average of at least three experiments) produced by each strain during overnight growth in high (black bars) and low (grey bars) iron PGT medium. Error bars indicate the standard deviations.

Figure 5. Site-specific integration into *C. glutamicum* and *C. ulcerans*

C. glutamicum and *C. ulcerans* genomic DNA was screened by PCR for *att* sites, with the combinations of primers, as well as the expected sizes of product bands, given in Table 1. The PCRs were designed to detect the *att* site indicated above the lane. (A). Screen of *C. glutamicum* ATCC 13032 DNA. (B) Screen of a *C. glutamicum* integrant at *attB2*. (C). Strain ATCC 13032 DNA and that of an integrant at *attB1* were screened for the *C. g. attB1*, *attB2*, *attL1* or *attR1* sites. The reactions for each site were loaded in pairs across the panel, with those from the kanamycin-resistant *attB1* integrant being the first reaction in each case. (D). Ten kanamycin-resistant *C. ulcerans* integrants screened for the *C.u. attL* or *attR* sites.

Table 1

Primers used to detect *att* sites

Site detected	Primer pair	Predicted size (bp)
<i>attP</i>	ATTP-UP & ATTP-DN	409
<i>attB1</i>	ATTB1-UP & ATTB1-DN	736
<i>attB2</i>	ATTB2-UP & ATTB2-DN	831
<i>attL1</i>	ATTB1-UP & ATTP-DN	475
<i>attR1</i>	ATTP-UP & ATTB1-DN	670
<i>attL2</i>	ATTB2-UP & ATTP-DN	599
<i>attR2</i>	ATTP-UP & ATTB2-DN	641
Cg <i>attB1</i>	ATTB1g-UP & ATTB1g-DN	464
Cg <i>attB2</i>	ATTB2g-UP & ATTB2g-DN	718
Cg <i>attL1</i>	ATTB1g-UP & ATTP-DN	511
Cg <i>attR1</i>	ATTP-UP & ATTB1g-DN	353
Cg <i>attL2</i>	ATTB2g-UP & ATTP-DN	591
Cg <i>attR2</i>	ATTP-UP & ATTB2g-DN	527
Cu <i>attB1</i>	ATTBu-UP & ATTBu-DN	150
Cu <i>attB2</i>	ATTBu-UP & ?	n/d ^a
Cu <i>attL1</i>	ATTBu-UP & ATTP-DN	329
Cu <i>attR1</i>	ATTP-UP & ATTBu-DN	243
Cu <i>attL2</i>	ATTBu-UP & ATTP-DN	(330) ^b
Cu <i>attR2</i>	ATTP-UP & ?	n/d ^a

^a Not determined and not detected

^b Size detected; it could not be predicted because the sequence of Cu *attB2* is not known.

Table 2

Mating Efficiency

Donor plasmid phenotype	Recipient	Mating frequency ^a
Replicating	<i>C. diphtheriae</i>	8.0×10^{-6}
Integrative	<i>C. diphtheriae</i>	5.1×10^{-6}
Replicating	<i>C. glutamicum</i>	3.3×10^{-9}
Integrative	<i>C. glutamicum</i>	1.2×10^{-9}
Replicating	<i>C. ulcerans</i>	8.2×10^{-5}
Integrative	<i>C. ulcerans</i>	1.2×10^{-4}

^a Number of transconjugants divided by number of donors