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Improved Electrotransformation and Decreased Antibiotic Resistance of the Cystic Fibrosis Pathogen *Burkholderia cenocepacia* Strain J2315[▽]

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The bacterium *Burkholderia cenocepacia* is pathogenic for sufferers from cystic fibrosis (CF) and certain immunocompromised conditions. The *B. cenocepacia* strain most frequently isolated from CF patients, and which serves as the reference for CF epidemiology, is J2315. The J2315 genome is split into three chromosomes and one plasmid. The strain was sequenced several years ago, and its annotation has been released recently. This information should allow genetic experimentation with J2315, but two major impediments appear: the poor potential of J2315 to act as a recipient in transformation and conjugation and the high level of resistance it mounts to nearly all antibiotics. Here, we describe modifications to the standard electroporation procedure that allow routine transformation of J2315 by DNA. In addition, we show that deletion of an efflux pump gene and addition of spermine to the medium enhance the sensitivity of J2315 to certain commonly used antibiotics and so allow a wider range of antibiotic resistance genes to be used for selection.

Burkholderia cenocepacia is part of the *Burkholderia cepacia* complex (Bcc), a group of closely related bacteria of soil, water, and roots (41) recently updated to at least 15 related species (42). Bcc displays many interesting features (see reference 27 for a review). Originally discovered as responsible for soft onion rot (3), Bcc species also interact beneficently with plants (see reference 34 for a review) and may degrade pollutants such as phthalate or the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5,-T) (25, 33). But it is the emergence of Bcc as an opportunistic pathogen of people suffering from cystic fibrosis (CF) (19) and immunocompromizing conditions that has drawn most attention to these bacteria. Among Bcc species, *Burkholderia multivorans* and *B. cenocepacia* are the most prevalent in the epidemiology of CF. In particular, strains of the ET12 lineage of *B. cenocepacia* were responsible for a major transcontinental epidemic among CF patients in the 1990s (20), an outbreak aggravated by the high levels of resistance to nearly all antibiotics that characterizes Bcc. Species of the Bcc have large genomes (7 to 9 Mb) composed of two or three chromosomes and one or more plasmids, an unusual genomic organization among bacteria. The first Bcc genome to be sequenced was that of *B. cenocepacia* J2315 (also known as LMG16656), the type strain of the ET12 lineage and the reference strain for CF epidemiology; the sequence was completed and made available by the Wellcome Trust Sanger Institute in 2003. It revealed three chromosomes of 3.9, 3.2, and 0.9 Mb and a plasmid of 93 kb. The annotation of this genome was released recently (15).

The pathogenicity and multipartite genome of *B. cenocepacia* make it an important subject for both practical and fundamental study. Genetic modification is essential to the success of many such investigations. Unfortunately, J2315 throws up major barriers to genetic manipulation. Standard electrotransformation techniques are ineffective with this strain, as also found elsewhere (26). Conjugal introduction of DNA has proved unreliable despite adaptations (7) that have enabled occasional successes with *B. cenocepacia* species (9, 40) including J2315 (39) (see also Results below). Besides, the natural resistance of J2315 to antibiotics, high even on the scale of the generally extensive resistance of *B. cenocepacia* species (31), severely restricts the use of antibiotic resistance in genetic selections. Circumventing these problems by resorting to a proxy strain, *B. cenocepacia* K56-2, that has not been sequenced and is more permissive to gene transfer (26, 17, 32, 9) runs the risk that results will be of uncertain relevance to J2315.

In the context of our general aim to decipher the role of the four replicon-specific ParABS systems of J2315 (6), we have sought to overcome these obstacles. We report here the reproducible electrotransformation of J2315, and we analyze factors that improve its efficiency. We report also our isolation of a J2315 derivative with reduced antibiotic resistance and the broadened selection possibilities this offers. Detailed protocols are provided which should facilitate studies of this pathogen.

MATERIALS AND METHODS

Plasmids, strains, and growth media. Plasmids and strains are described in Table 1. Luria Bertani (LB) medium was used for routine growth of both *Escherichia coli* and *B. cenocepacia*, and super optimal broth (SOB) medium was used for preparation of electrocompetent cells.

Electrocompetent cells and electrotransformation of J2315. The medium for overnight growth was SOB containing 10 mM $MgSO_4$ and 10 mM $MgCl_2$, supplemented with 10 μg/ml gentamicin to minimize growth of contaminants. Five milliliters of this medium was inoculated with 50 μl of a culture of *B. cenocepacia* J2315 (optical density at 600 nm [OD_{600}] of ~1; freshly prepared from a frozen stock) and incubated overnight with aeration at 37°C. The overnight culture reached an OD_{600} of 3. (Excessively long incubation, recognized by browning of the medium due to production of melanin, should be avoided

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli strains		
DH10B	*endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* ϕ80*dlacZ*ΔM15 *araD139* Δ(*ara-leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$	10
SCS110	*rpsL thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q} *Z*Δ*M15*]	Stratagene
B. cenocepacia strains		
J2315	ET12 lineage; index for the epidemiology of CF	BCCM
Mex1 strain	J2315 but with a deletion of the *mex1* locus	This study
Plasmids		
RSF1010	Broad host-range oriV; *mob*$^+$ Str^r	37
pMMB190	RSF1010 ori; *lacI*^{q} P_{taclac} *lacZ*α *bla*(Amp^r)	30
pMMB206	pMMB190 Δ*bla* Ω*cat*(Cm^r)	30
pBBR1MCS2	pBBR1MCS ori; *mob*$^+$ Kan^r	21
pBBR1MCS5	pBBR1MCS ori; *mob*$^+$ Gen^r	21
pMLBAD	pBBR1MCS ori; *mob*$^+$ araC-P_{BAD} Tp^r	24
pEX18tc	pUC ori; *mob*$^+$ *sacB* Tet^r	14
pCM157	oriV ColE1 ori; Tet^r *cre*$^+$	29
pCM351	ColE1 ori; Tet^r Amp^r Gen^r	29
pCM351-cat	pCM351; Cm^r instead of Gen^r	This study
pRF91	pCM351-cat derivative; each 1.3 kb flank from *B. cenocepacia parA* c1 on each side of *cat*	This study
pDAG824	pCM351-cat derivative; 2.4 kb from *B. cenocepacia* ori c3; 3.4-kb *parS* P1 fragment from pDAG394 (laboratory stock); Spc^r	This study
pBR325	ColE1 ori; Amp^r Tet^r Cm^r	2
pBL2	pBR325 with BCAL0028 on a 1.4-kb fragment; Tet^s	This study
pRK2013	RK2 derivative; ColE1 ori; *mob*$^+$ *tra*$^+$ Kan^r	8

[a] Str^r, Amp^r, Cm^r, Kan^r, Tet^r, Gen^r, Tp^r, and Spc^r indicate resistance to streptomycin, ampicillin, chloramphenicol, kanamycin, tetracycline, gentamicin, trimethoprim, and spectinomycin, respectively.

because it results in slow and unpredictable growth.) The overnight culture was then diluted to an OD_{600} of 0.01 in 50 ml of SOB medium containing 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 10 μg/ml gentamicin and supplemented with 0.8% glycine from a filter-sterilized 20% glycine stock solution. Next, the culture was incubated with aeration for 2 to 3 generations to an OD_{600} of 0.04 to 0.08, which takes 4 to 6 h. The culture was then chilled for 5 min and centrifuged at 4°C for 8 min at 5,000 × *g*. The cells were washed twice by gentle resuspension in 20 ml of ice-cold 0.5 M sucrose, followed by centrifugation as above. The washed cells were resuspended in 0.5 ml of 0.5 M sucrose–10% glycerol and either used directly for electrotransformation or aliquoted and frozen at −80°C. The loss of electrotransformation frequency after freezing and thawing was minor, and for convenience we usually employed frozen cells. Electrotransformation followed the standard procedure, with the following specific measures: DNA was added as ~0.1 μg of replicative plasmid or ~5 μg of nonreplicating DNA (for chromosomal recombination) in 1 to 15 μl of water (a volume variation with no effect on standard transformation frequency [TF]) to 55 μl of thawed competent cells. The DNA-bacteria mixture was subjected to a pulse of 2,500 V and 200 μF (using an Eppendorf 2510 electroporator); 950 μl of SOC medium (SOB medium supplemented with $MgSO_4$ [10 mM] and glucose [2%]) was added, and the cells were incubated, standing, at 37°C for at least 4 h before being spread on selective LB or SOB medium, followed by incubation at 37°C for two (SOB) or three (LB) days.

Triparental mating. The mating procedure was adapted from Engledow et al. (7). Overnight cultures of the donor, helper, and recipient strains were grown in LB medium supplemented with appropriate antibiotics (kanamycin for the *E. coli* helper carrying pRK2013; chloramphenicol, trimethoprim, or tetracycline for the *E. coli* donor; gentamicin for *B. cenocepacia*). Fresh overnight cultures of the *E. coli* strains and J2315 were diluted 200-fold and 100-fold, respectively, into LB medium without antibiotics, and the cultures were grown to an OD_{600} of ~0.5; *E. coli* cultures that reached this density early were maintained at 37°C without shaking. A total of 400 μl of each of the three cultures was mixed in 10-ml plastic tubes and left to stand at 37°C without shaking for 1 h. One milliliter of the mixture was poured onto a positively charged nylon membrane (Membrane Biodyne B; 0.45-μm pore size, nylon >0; VWR), placed on an LB agar plate, and incubated for 15 h at 37°C. Bacteria were then scraped from the membrane and suspended in 1 ml of LB medium. Samples of 100 μl and 900 μl were spread on LB agar medium containing antibiotics to select for the donor plasmid and counter-select (gentamicin) for *E. coli*, and samples were incubated 2 to 3 days at 37°C.

Antibiotics and chemicals. Antibiotic discs for the antibiogram diffusion test were from Sanofi Pasteur Diagnostics. Spermine and all antibiotics except nalidixic acid were from Sigma Aldrich. Nalidixic acid was from Calbiochem. Stock solutions of spermine (1 M) and of ampicillin, gentamicin, kanamycin, streptomycin, and spectinomycin (100 mg/ml) were made in distilled water and filter sterilized. Stocks of trimethoprim (100 mg/ml) and tetracycline (200 mg/ml) were made in dimethyl sulfoxide (Merck). Stocks of erythromycin and chloramphenicol were made at 100 mg/ml in absolute ethyl alcohol (VWR). A stock of nalidixic acid was made at 20 mg/ml in 0.1 M NaOH and filter sterilized.

Antibiotic sensitivity testing. An antibiogram diffusion test (Sanofi Pasteur Diagnostics) was carried out to determine the approximate sensitivities of J2315 and its *mex1* derivative. Colonies grown on Mueller-Hinton (MH) medium were suspended in physiological saline at ~10^6 bacteria per ml. Petri dishes containing solid MH medium were flooded with 3 ml of suspension; excess liquid was discarded, and the plates were allowed to dry for 15 min at room temperature. Antibiotic-carrying discs were applied, and the plates were incubated at 37°C for 20 h. The diameter of the complete growth inhibition zone, including the central antibiotic-carrying disc and the clear halo surrounding it, was measured with a graduated ruler put directly on the petri dish. The growth inhibition was defined as the width of the clear halo, which was calculated by subtracting the disc diameter (7 mm) from the measured zone and dividing by two. The values obtained allowed us to classify the bacteria as resistant, intermediate, or sensitive, according to supplier and clinical recommendations.

We also tested antibiotic sensitivity by broth dilution. Briefly, fresh LB cultures of *B. cenocepacia* grown to an OD_{600} of 0.1 to 0.3 were diluted in LB broth to ~5 × 10^6 bacteria/ml. Aliquots of the dilute suspension were added to 5-ml sterile capped tubes. Antibiotic was added to one tube, and the mixture was transferred from this into the others to make serial 2-fold dilutions. Tubes were incubated 18 h at 37°C with shaking. The MIC in LB medium (MIC_{LB}) is the lowest concentration of each antibiotic that completely inhibits growth under these conditions.

DNA and genetic manipulations. Plasmids were extracted from *E. coli* and *B. cenocepacia* using a miniprep kit (Qiagen). PCRs were carried out with Phusion high-fidelity DNA polymerase (Finnzymes). Oligonucleotides were made by Sigma and Eurogentec. Oligonucleotide sequences are given in Table 2. Restric-

TABLE 2. Oligonucleotides used in this study

Primer function and name	DNA sequence (5′–3′)[a]	Localization[b]
mex deletion		
mex-A	gtgtgaagcttAGCCGCTTCGTGTGTGCGACG	c1 3094985
mex-B	AGCATCAGCATGTCGCGGAAGTGTCGGATGGTCT	c1 3096138
mex-C	AGACCATCCGACACTTCCGCGACATGCTGATGCT	c1 3102727
mex-D	tgctctagaTCGGCGAGCGAATCGACG	c1 3104447
pCM351-cat		
Fop-106	accgctcgagCCGAACAGGCTTATG	pCM351
Fop-107	aagctgcagTCAAGCCGAGATCG	pCM351
Cat-up	accgctcgagTGAGACGTTGATCGG	pBR325
Cat-dwn	aagctgcagATTACCCGGCATTCG	pBR325
BCAL0028 (*ctg*)		
ctg-E5	caagatatcGCCGCGATACACGTCGTCTC	c1 31225
ctg-B1	gttcggatccGGCGGCAGCACTTATCAACA	c1 32630
P1-f	ATGGACGAAGGCAGCCTG	c1 30657
P1-r	GATAATGGCCTGCTTCTC	pBR325
P8-f	TGCCTGAGGCCAGTTTGC	pBR325
P8-r	TGATTCAAGGCTTTAGAC	c1 30000
ori c3 fragment		
tic-A	aaggatccGGAGGTCACCATGAAGTC	c3 858085
tic-B	aaggatccTGATCGAGATCGCGCTGA	c3 860763
vrf-A	GAGCGAAGCGCGAAATCAACAGAAC	c3 858042
vrf-B	CAGGCGGGCAAGAATGTGAATAAAGGC	pCM351-cat
parA c1 deletion		
dpa-1	ccggaattcCGGAAACGCGACGCTG	c1 28389
dpa-2	ctaagtcatatgGTTGGTGTGTTCCCTT	c1 29673
dpa-3	gtcagggcccGAGCGAAGCGAGGAAG	c1 30479
dpa-4	atgcacgcgtAGCGGTGTCAGCGCG	c1 31764
F-34	CGTCTAGAGCGGCATCTGTTCGAAGTG	c1 29599
F-35	TGTAGCATGCAAGCGCCAGCACCGACAGTA	c1 31485

[a] Lowercase letters are heterologous 5′ extensions. Restriction sites used for this study are underlined: HindIII, XbaI, EcoRV, BamHI, EcoRI, NdeI, and ApaI for mexA, mexD, ctg-E5, ctg-B1, dpa-1, dpa-2, and dpa-3, respectively; XhoI for Fop-106 and Cat-up; and PstI for Fop-107 and Cat-dwn.
[b] For oligonucleotides matching large chromosomes c1 and c3, the indicated number is the chromosomal position of the 5′ base.

tion enzymes were from New England Biolabs; 2-log and 1-kb DNA ladders were from New England Biolabs; a 1-kb Plus DNA ladder was from Gibco BRL. To delete the *mex1* locus (6.6 kb) two flanking amplicons of 1,152 bp (primers mexA and mexB) and 1,720 bp (primers mexC and mexD), sharing 34 bp of homology on their respective B and C ends, were mixed and used as the template for a third PCR primed with oligonucleotides A and D. The resulting amplicon of 2.8 kb, corresponding to the fusion of the left and right flanks of the *mex1* locus, was digested by HindIII and XbaI and inserted between the corresponding sites of pEX18Tc. The resulting plasmid was introduced into J2315 by triparental mating. Tetracycline resistant (Tet^r) exconjugants were selected, and integration of the plasmid was confirmed by PCR. Plasmid excision was detected by replica plate screening for loss of Tet^r following nonselective growth. Tet^s clones were analyzed by PCR to identify those from which *mex1* had been deleted.

The plasmid pCM351-cat was constructed by PCR amplification (with oligonucleotides Fop106 and Fop-107) of all but the Gen^r cassette of pCM351, followed by XhoI-PstI digestion and ligation of the resulting amplicon to the chloramphenicol acetyltransferase (CAT) cassette amplified from pBR325 (oligonucleotides cat-up and cat-dwn) and similarly digested. DNA flanking the sequence to be deleted can be cloned on both sides of CAT, allowing the selection of exchange events that replace the sequence with CAT.

The pBL2 plasmid was obtained by cloning into pBR325 (digested with EcoRV and BamHI) a 1.4-kb fragment spanning BCAL0028 amplified with oligonucleotides ctg-E5 and ctg-B1. Plasmid pDAG824 is a pCM351-cat derivative, carrying both the *parS* locus of phage P1 and a 2.4-kb fragment of *B. cenocepacia* chromosome 3 (c3) origin (obtained by PCR with oligonucleotides tic-A and tic-B and then digested with XmnI and AatII and inserted into pCM351-cat). Plasmid pRF91 is a pCM351-cat derivative carrying on each side of CAT the left and right 1.3-kb flanks of *parA* chromosome 1 ([c1] amplified with dpa-1/dpa-2 and dpa-3/dpa-4, respectively) (Table 2).

RESULTS AND DISCUSSION

Setting up an electrotransformation protocol. The standard *E. coli* electrotransformation procedure yielded no transformants when it was applied to *B. cenocepacia*. Because Gram-positive bacteria are more refractory to electrotransformation than Gram-negative bacteria, we adapted a protocol designed for the Gram-positive *Leuconostoc carnosum* 4010 (13). The main differences of this protocol from the *E. coli* procedure are as follows: (i) inoculation of the culture at a lower cell density, (ii) fewer generations of growth before harvesting, (iii) addition of glycine to the culture medium, (iv) gentle centrifugation and resuspension during cell washing, and (iv) longer phenotypic expression. Using the protocol described in Materials and Methods, we were able to transform J2315 with plasmid DNA extracted from wild-type *E. coli*. As with *L. carnosum* (13), the duration of phenotypic expression before selection is important. Few or no transformants were obtained if electroporated cells were incubated for times shorter than 2 h; the number of transformants per viable cell was 2.5- to 5-fold higher at 4 h than at 2 h, and extending incubation to 6 h or more did not further increase this ratio. By routinely allowing 4 to 5 h of phenotypic expression, we obtained up to 10^4 transformants per μg of plasmid DNA.

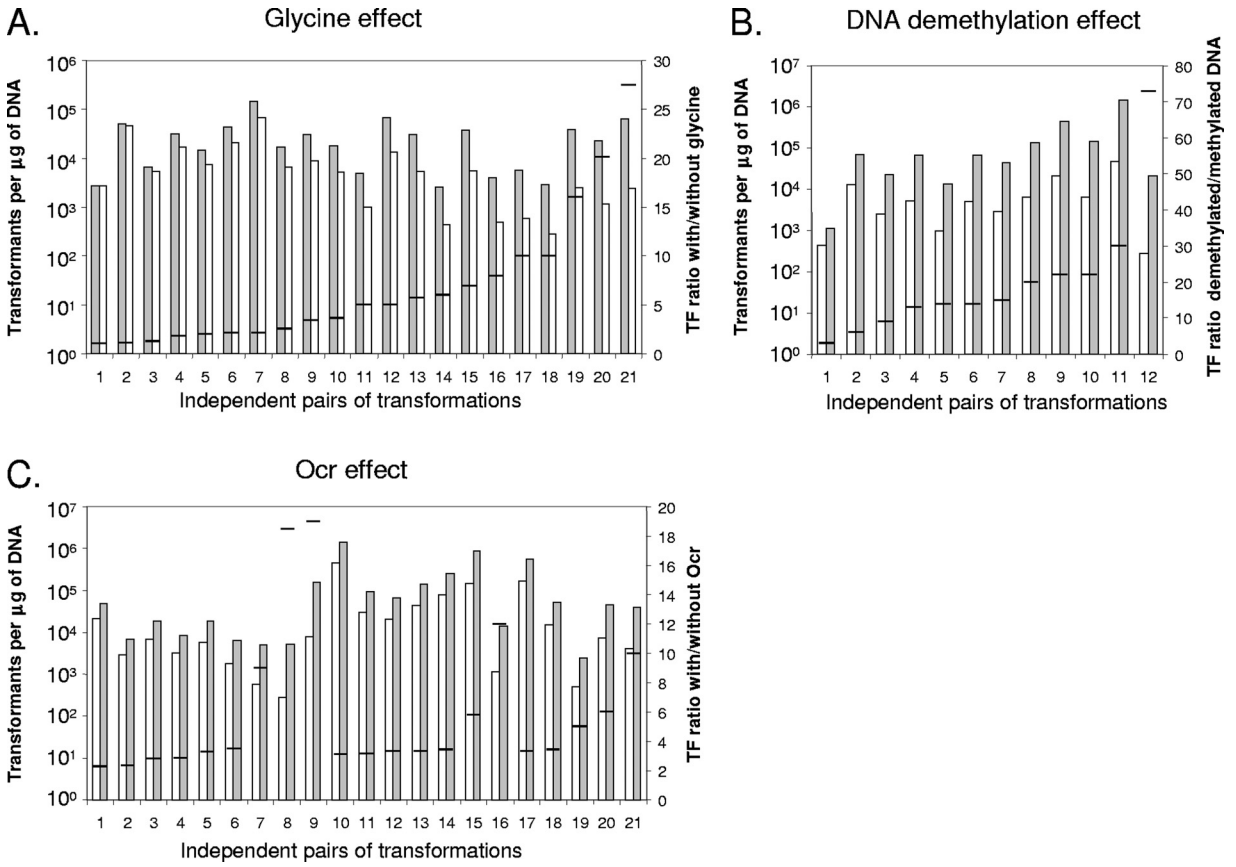

FIG. 1. Improvement of electrotransformation. (A) Transformants obtained with cells grown with added glycine (gray bars) or without (white bars) and the ratios of TFs with/without glycine (horizontal lines). (B) Transformants obtained with the donor plasmid unmethylated (gray bars) or methylated (white bars) and the ratios of TFs unmethylated/methylated (horizontal lines). (C) Transformants obtained with Ocr added during electrotransformation (gray bars) or without Ocr (white bars) and the TF ratios with/without Ocr (horizontal lines). In panel C, pairs of electrotransformations 1 to 9 are with donor DNA extracted from *E. coli dam*$^+$ *dcm*$^+$, transformants 10 to 16 are from *E. coli dam dcm*, and transformants 17 to 21 are from *B. cenocepacia*.

To improve TF further, we examined additional factors. In each case the data (Fig. 1) are presented as ensembles of all the independent experiments for which valid comparisons of TF can be made.

Addition of glycine to growth medium. Inclusion of glycine in the growth medium of Gram-positive bacteria has been observed to weaken the thick cell wall and to increase transformation efficiency (16). We therefore tested its effect on the TF of J2315. Glycine inhibited growth in LB and SOB media modestly at 0.8% but strongly at higher concentrations. Cells grown with 0.8% glycine were transformed with plasmid DNA 2- to 6-fold (and up to 25-fold) more efficiently than cells grown in parallel without it (Fig. 1A). The improvement in TF conferred by glycine suggests that cell wall structure could contribute to the poor competence of J2315.

DNA methylation. Burns and Hedin (4) reported that strain 249-2 of *Pseudomonas cepacia* (the original name of the Bcc) could be electrotransformed by plasmid DNA if it had been extracted from an *E. coli dam dcm* strain or from *Pseudomonas aeruginosa* and was thus unmethylated on GATC sites. We found electrotransformation of J2315 with plasmid DNA extracted from the *dam dcm* strain SCS110 to be 10 to 20 times more efficient than with methylated DNA (Fig. 1B). These data suggest that J2315 possesses a restriction system specific for methylated GATC sites.

Ocr protein. The bacteriophage T7 protein, Ocr, acts as a decoy to inhibit an attack by a type I restriction endonuclease on entering DNA by mimicking DNA structure (28, 1). Commercially available Ocr (Type One Restriction Inhibitor; Epicentre) may improve up to 100-fold the TF of restricting bacteria by unmodified DNA. We tested the effect of including 5 μg of Ocr protein with the DNA of our tester plasmid pMMB206 on electrotransformation of J2315. Ocr improved the TF by up to 20-fold but on average only 3-fold (Fig. 1C). This is far from the 100-fold factor expected for inhibition of a type I endonuclease. Increasing the amount of Ocr did not help; ≥10 μg impairs transformation in *E. coli* or *Salmonella enterica* serovar Typhimurium according to the supplier, and we observed it to reduce (by ~0.2 ms) the time constant of the electric pulse. Interestingly, we found the same 3-fold Ocr-mediated increase whether DNA was extracted from DH10B (*dam*$^+$ *dcm*$^+$), SCS110 (*dam dcm*) or J2315 (Fig. 1C), suggesting that Ocr can act beyond the type I restriction system to block nonspecific DNase activity. Our data suggest that homologues of type I restriction/modification systems identified in

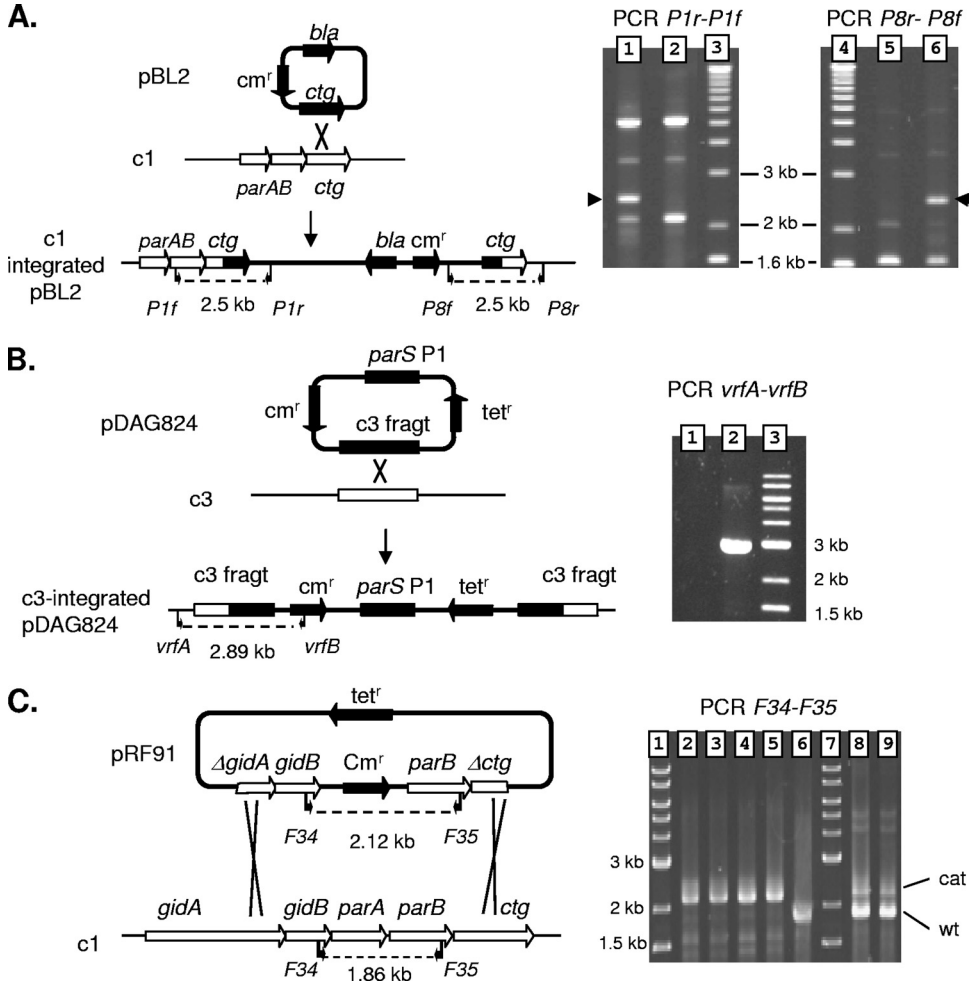

FIG. 2. Examples of *B. cenocepacia* genomic recombinants following electrotransformation (A). A single crossover (×) within *ctg* between the plasmid and c1 integrates pBL2 to give the structure drawn. Integration was confirmed on one Cm^r transformant by PCR amplification across the chromosome-plasmid junctions. Lanes 3 and 4, 1 kb Plus DNA ladder; the relevant sizes are indicated. Lanes 1 and 6, PCRs on the Cm^r transformant primed with P1r-P1f and P8r-P8f, respectively. A specific 2.5-kb amplicon appears (▶). Lanes 2 and 5, control PCRs on the untransformed J2315 strain. (B) A Cm^r Tet^r transformant obtained with pDAG824 was analyzed by PCR with oligonucleotides vrf-A and vrf-B flanking the plasmid-chromosome recombination junction. Lane 1, untransformed control strain; lane 2, Cm^r Tet^r transformant (the expected 2.89 kb amplicon is obtained); lane 3, 2-log DNA ladder. (C) A double crossover between pRF91 and c1, via the regions flanking *parA*, should exchange *parA* for Cm^r, so that the PCR primed with F34-F35 should yield an amplicon of 2.12 kb instead of 1.86 kb. Single crossovers integrate the whole plasmid so that the F34-F35-primed PCR should yield both amplicons. Lanes 1 and 7, 1-kb ladder; lanes 2 to 5, PCR on four Cm^r Tet^s transformants, yielding the 2.12-kb amplicon; lane 6, control PCR on the untransformed strain, yielding a wild-type 1.86-kb amplicon; lanes 8 and 9, PCRs on two Cm^r Tet^r clones (plasmid integration), yielding both 2.12 and 1.86-kb amplicons (*cat* and wild type [wt], respectively).

J2315 (e.g., BCAL0418 and BCAL0420 on chromosome 1) do not act on our tester plasmid DNA.

Even though the Ocr effect is weaker than expected, it adds to the effect produced by demethylation of DNA. The highest TF we have observed, 1.2 $\times 10^5$ per μg, was with cells from cultures grown with glycine and transformed with unmethylated pMMB206 DNA in the presence of Ocr.

Integration and deletion events following electrotransformation. The electrotransformation protocol established (see Materials and Methods) allows the routine introduction of replicative plasmids into J2315. We tested whether it was efficient enough to enable the isolation of J2315 genome recombinants. In one test, an amplicon of 1.4 kb spanning the locus BCAL0028, a putative citrate transporter gene (*ctg*) located downstream of the operon *gidAB-parAB* in chromosome 1, was inserted into the vector pBR325 (Cm^r; nonreplicative in *B. cenocepacia*). The resulting plasmid, pBL2 (see Materials and Methods), was demethylated by passage through SCS110 and introduced by electrotransformation into J2315. Integration was confirmed by PCR using oligonucleotide pairs P1r/P1f and P8r/P8f (Table 2 and Fig. 2A). Both chromosome-plasmid junctions were detected as amplicons of 2.5 kb (Fig. 2A.). In a second test, pDAG824, a derivative of pCM351 carrying a 2.4-kb fragment of the replication origin region of chromosome 3 (see Materials and Methods), was introduced by electrotransformation into J2315 *mex1* (see below). Integration of pDAG824 at the expected site of a Cm^r Tet^r transformant in chromosome 3 was confirmed by the generation of an amplicon of 2.89 kb using PCR with oligonucleotides vrfA/vrfB (Fig. 2B). In a third trial, the *parA* gene of chromosome 1 was

TABLE 3. Antibiogram diffusion test

Antibiotic (μg per disc)	Growth inhibition (halo width in mm) in[a]:	
	J2315	Mex1 strain
Rifampin (30)	0.15	0.3
Ticarcillin (75)	0	0
Streptomycin (500)	0	+/−
Kanamycin (1,000)	0	0
Gentamicin (500)	0	0
Nalidixic acid (30)	0.4	1
Trimethoprim/sulfamethoxazole (1.25/23.75)	0	0
Ticarcillin-clavulanic acid (75/10)	0	0
Ampicillin (10)	0	0
Amoxicillin (25)	0	0
Tetracyclin (30)	0	+/−

[a] No significant variation in growth inhibition was discerned in two separate tests. +/−, halo obvious but too small (i.e., width of <0.05 mm) to be correctly measured.

TABLE 4. Effect of spermine on antibiotic sensitivity of J2315 and Mex1 strains

Antibiotic	MIC_{LB} (μg/ml)[a]					
	B. cenocepacia J2315			*B. cenocepacia* Mex1 strain		
	−Sp	+Sp	Ratio (−Sp/+Sp)	−Sp	+Sp	Ratio (−Sp/+Sp)
Rifampin	128	128	1	64	64	1
Tetracycline	64*	64*	1	16*	16*	1
Ampicillin	8,192	512	16	1,024	32	32
Gentamicin	2,048	64	32	512	32	16
Kanamycin	2,048	128	16	512	32	16
Trimethoprim	512	512	1	128	128	1
Streptomycin	4,096	256	16	2,048	128	16
Spectinomycin	1,024	128	8	512	64	8
Chloramphenicol	32	16	2	4	4	1
Erythromycin	256	64	4	256	64	4
Nalidixic acid	32	64	0.5	32	16	2

[a] MICs were measured in LB medium by serial 2-fold dilution with (+Sp) and without (−Sp) 1 mM spermine. MICs were found to be identical in three independent experiments. *, growth occurred even at tetracycline concentrations from 512 μg/ml (256 μg/ml for Mex1 strain +Sp) up to the highest tetracycline concentration that we tested (4,096 μg/ml) (see text).

deleted. The pRF91, a pCM351-cat dervivative, carries two amplicons of 1.3 kb corresponding to the flanks of chromosome 1 *parA* (see Materials and Methods) on either side of *cat*. Each of three independent electrotransformations of a Δ*mex1* derivative of J2315 *mex1* with pRF91 yielded ~10 Cm^r Tet^r transformants, with the plasmid integrated via a single crossover at the *parA* locus, as confirmed by PCR with oligonucleotides F34/F35 (Fig. 2C, lanes 8 and 9). In addition, one of the transformations gave a Cm^r Tet^s clone with *parA* replaced via a double crossover by *cat*, while another gave three such clones. Double crossover events were confirmed by PCR analysis with oligonucleotides F34/F35 (Fig. 2C, lanes 2 to 5). Thus, double crossovers are sufficiently frequent, ~10% in this case, to be obtainable by electrotransformation of J2315 with plasmid DNA.

Construction of an antibiotic-sensitive mutant. The high level of intrinsic resistance to most antibiotics is another factor that limits our ability to obtain transformants and exconjugants of J2315. It appeared likely that tripartite multidrug efflux pumps of the resistance-nodulation-division (RND) family (for reviews, see references 35 and 38) would be responsible for this since using the Mex/Opr peptides that perform this function in *P. aeruginosa* as *in silico* probes of the translated J2315 genome revealed 14 homologous systems: five on chromosome 1, six on chromosome 2, and three on chromosome 3. Most of them correspond to those found in a similar search by Guglierame et al. (11) and were recently reported in the genome analysis of J2315 (15). In an attempt to increase antibiotic sensitivity of J2315, we chose to delete the genes encoding the highest-scoring homologue of *P. aeruginosa mexAB-oprM*, a locus on chromosome 1 that we called *mex1* (BCAL2820-2822 in reference 15), as described in Materials and Methods. The antibiotic sensitivity of the Δ*mex1* strain (Mex1 strain) was first assessed by an antibiogram diffusion test (Table 3). Although according to the clinical recommendation the Mex1 strain is still resistant to all the tested antibiotics, slight reductions in resistance to streptomycin, nalidixic acid, rifampin, and tetracycline were evident (Table 3). These indications were extended using serial dilution to determine MICs (Table 4). Deletion of *mex1* causes significant increases in sensitivity to chloramphenicol, tetracycline, trimethoprim, kanamycin, gentamicin, and ampicillin and slightly higher sensitivity to rifampin, streptomycin, and spectinomycin. Deletion of other *mex* loci has been initiated with the aim of increasing the spectrum of sensitivity to antibiotics although it happens that this has been achieved in part by another approach, which we now describe.

Effects of spermine on growth and antibiotic sensitivity. The natural polyamines, putrescine, spermine, and spermidine, are cationic compounds essential for eukaryotic and prokaryotic cells and are involved in many cellular processes (for reviews, see references 12 and 18). Exogenous polyamines promote closure of the OmpF and OmpC porin channels in *E. coli* and possibly of OprD in *P. aeruginosa* (5, 23). They thus modify membrane permeability and, as a possible consequence, impair growth and/or enhance antibiotic sensitivity. In particular, clinical isolates of *P. aeruginosa* and strains of *E. coli*, *S.* Typhimurium, and the Gram-positive *Staphylococcus aureus* are sensitized to some antibiotics by spermine (23). Enhancement of antimicrobial susceptibility by chemical cationic compounds was reported for strains of *B. cepacia* while spermidine and cadaverine had no effect (spermine was not tested) (36). We have tested the effects of spermine on the growth and antibiotic sensitivity of J2315. Growth was unaffected at spermine concentrations below 2 mM, impaired at 2 to 3 mM, and effectively abolished at 3.5 mM (the MIC). The Mex1 strain is slightly more sensitive (MIC of 3 mM). It was at first surprising that the multidrug-resistant J2315 is inhibited by such low concentrations of spermine, especially as 16 mM spermine does not impair growth of *P. aeruginosa* (23).

Sensitization to antibiotics was assessed by measuring MICs of J2315 and Mex1 strains with and without 1 mM spermine (Table 4). The results showed that spermine sensitizes *B. cenocepacia* to many antibiotics. Spermine and the Δ*mex1* mutation act in apparent synergy to reduce resistance to aminoglycosides and ampicillin. Sensitivity to rifampin, trimethoprim, and chloramphenicol is increased by Δ*mex1* but virtually unaffected

TABLE 5. Antibiotic concentrations used for the selection of transformants

Antibiotic (vector)	Concn for selection (μg/ml) in[a]:			
	J2315		Mex1 strain	
	−Sp	+Sp	−Sp	+Sp
Chloramphenicol (pMMB206)	80	25	25	NT
Tetracycline (pCM157 or pEX18Tc)	300	NT	300	NT
Trimethoprim (pMLBAD)	600	300	200	NT
Gentamicin (pBBR1MCS5)	3,000	150	NT	100
Kanamycin (pBBR1MCS2)	No (2,000)	250	1,000	125
Streptomycin (RSF1010)	No (4,000)	1,000	4,000	250
Ampicillin (pMMB190)	No (8,000)	No (600)	No (2,000)	No (2,000)

[a] Selections were carried out on LB agar without spermine (−Sp) or on LB agar supplemented with 1.5 mM spermine (+Sp). NT, not tested; No, counterselection of untransformed cells was ineffective even at the highest concentrations tested (shown in parentheses).

by spermine while the reverse (affected by spermine, not by Δ*mex1*) is seen for erythromycin. These data suggest that spermine inhibits at least one antibiotic resistance pathway other than Mex1 efflux. On the other hand, spermine might interact with the Mex1 pump: in the presence of spermine, resistance to nalidixic acid increases in J2315 but decreases in the Mex1 strain (Table 4). Possibly, spermine activates a slight efflux of nalidixate by the Mex1 pump, as has been proposed to explain polyamine-induced resistance to quinolones in *P. aeruginosa* (22).

Tetracycline resistance is a special case. Spermine has no effect, and Δ*mex1* has a weak one. Curiously, although the MIC of tetracycline measured by the serial dilution assay is 64 μg/ml, concentrations above 512 μg/ml allow growth (Table 4). Tetracycline at high levels appears to induce resistance to itself, at least in a fraction of the population. Such levels should be avoided in the selection of Tet^r transformants.

Broadened spectrum of selective antibiotics. The applicability of reduced antibiotic resistance levels to transformant selection was tested using plasmids with a variety of resistance genes. Plating of electroporated cells of the J2315 and Mex1 strains on LB agar plates with or without 1.5 mM spermine gave the results shown in Table 5. For chloramphenicol and trimethoprim, antibiotics previously used in experiments with J2315, the concentrations required to select transformants were significantly reduced by addition of spermine and/or the Δ*mex1* allele. More importantly, spermine and Δ*mex1*, alone or in combination, enabled selection of resistances that were not previously usable—to gentamicin, kanamycin, and streptomycin. Under the conditions used (Table 5) there is no or negligible background, as seen by lack of growth of cells electroporated with a plasmid carrying a different resistance gene and plated in parallel. In addition, transformation was verified by identifying plasmids extracted from resistant colonies. However, despite the strong reduction in the MIC of ampicillin achieved using spermine and Δ*mex1* (Table 4), we were unable to distinguish Amp^r transformants from the heavy background that grows after 3 days.

Conclusion. Electrotransformation of *B. cenocepacia* strain J2315 at frequencies high enough to allow selection of single and double crossover recombination events is now a routine procedure. Genetic manipulation of J2315 can thus be performed directly. The main factors contributing to this improvement are additional glycine in the growth medium, demethylation of transforming DNA by extraction from an *E. coli dam dcm* strain, and, to a lesser but useful extent, inclusion of the Ocr protein in the transformation mixture.

The additive effects of spermine and the Δ*mex1* allele on sensitivity to a number of antibiotics have proven to be remarkably efficient in decreasing the MICs of ampicillin, kanamycin, gentamicin, and streptomycin. As a consequence, we can now use streptomycin, kanamycin, and gentamicin to select for electrotransformation of *B. cenocepacia* J2315 by plasmids carrying the corresponding resistance genes. This should greatly facilitate genetic experimentation with this multichromosomic pathogen.

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AUTHOR'S CORRECTION

Improved Electrotransformation and Decreased Antibiotic Resistance of the Cystic Fibrosis Pathogen *Burkholderia cenocepacia* Strain J2315

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Volume 76, no. 4, p. 1095–1102, 2010. Page 1096, column 1, line 17: "a pulse of 2,500 V and 200 μF" should read "a pulse of 12.5 kV/cm and 10 μF."