Strains for production of RcoM$_{Bx}$-1 proteins and assay of in vivo activity.

The following plasmids and strains are components of the protein production and *in vivo* assay system for the CO-dependent "RcoM-1" regulatory protein cloned from *Burkholderia xenovorans* LB400 [2]. All plasmid (pUX) and host strain (UQ) designations indicated below refer to strains created and used in the laboratory of Dr. Gary Roberts, Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706. For further details and references see [2, 3, and 5].

1. Routine procedures. Routine cultivation of *Escherichia coli* strains utilized 2×LC medium (per liter medium: 20 g tryptone, 10 g yeast extract, 10 g NaCl). Antibiotics used (μg/ml) and their resistance designations included kanamycin sulfate, 20, Km^r; ampicillin (Na salt), 75, Ap^r; spectinomycin dihydrochloride, 20, Sp^r; chloramphenicol, 10, Cm^r. As noted below, derivatives of the UQ5853 or VJS6737 host strains grew rapidly and were routinely cultured at approximately 28°C. Plasmid isolation used cultures grown at 35 - 37°C.

2. Production system for protein accumulation. Production of the heme-containing protein (+C-terminal 6×His) utilized plasmid pUX2410 (also encodes resistance to ampicillin) in host strain UQ4803 (see Table 1). As detailed below, the synthesis of protein containing a high level of heme cofactor and a low level of the FeII-CO species required cultivation with a low level of IPTG inducer and a high degree of aeration. This strain grows quickly and routine cultivation for protein accumulation should be done at approximately 28°C.

Specifically, the growth medium contained (per liter tap-distilled water): 20 g tryptone (Fisher Scientific, Fair Lawn, NJ), 2 g yeast extract (Fisher), 3 g Difco™ nutrient broth (Becton, Dickinson and Co., Sparks, MD), and 5 g NaCl. The medium pH was adjusted to 6.8, 5 ml of a 2g/l iron(III)-citrate (Sigma-Aldrich, St. Louis, MO) stock solution was slowly mixed in, and 400 ml was dispensed per 2.8-l flask (Pyrex® #4420, Corning Life Sciences, Lowell, MA). These were covered with gauze and autoclaved for 30 min at 121°C. Flasks were amended with a low level of IPTG (to 6 μM) and ampicillin (Na salt, 75 μg/ml), then inoculated at an OD_{550} = 0.060 with a log-phase culture growing in the same medium (without IPTG). Cultivation

occurred at 28°C with vigorous agitation (220 rpm/2.5-cm throw) for 19 – 20 hours, then cultures were placed on ice and harvested by centrifugation, yielding ~10 g cells (wet weight) per liter of medium.

Cell pellets were resuspended at a ratio of ~1 g wet weight/3 ml buffer "A" [50 mM MOPS, 500 mM KCl, 0.5 mM dithiothreitol, pH 7.4] then lysed by two passages at 18,000 psi through a French press. Supernatants prepared by centrifugation (4°C, 60 min, r_{max} 20,800 × *g*) were immediately frozen on crushed dry ice and stored at −80°C. For RcoM purification the supernatant was amended with imidazole (pH 8.0) to 10 mM and settled Ni-NTA agarose resin (Qiagen) was gently mixed in at a ratio of 4 ml/10 ml extract. This suspension was held on ice and periodically remixed for 30 min, centrifuged (5 min, ~150 × *g*) and the supernatant discarded, and then the resin was resuspended in 5 volumes of buffer "A" containing 10 mM imidazole. This suspension was similarly processed and a second buffer "A" + 10 mM imidazole wash was performed, except that this time the slurry was poured into a column and the buffer eluted. The matrix was next rinsed with 5 to 7 volumes of room-temperature buffer "B" (50 mM imidazole, 50 mM MOPS, 500 mM KCl, pH 7.7) and the RcoM protein eluted in this buffer containing 220 mM imidazole. The protein solution was adjusted to 40% of saturation in $(NH_4)_2SO_4$ and the protein pelleted by a brief centrifugation. After removing the supernatant the protein pellet was dissolved in a minimal amount of sample buffer "C" (40 mM MOPS, 500 mM KCl, 5% glycerol, pH 7.4), applied to a buffer "C"-equilibrated Sephadex™ G-25 column (GE Healthcare, Piscataway, NJ), and eluted. The desalted protein was distributed into aliquots, frozen, and stored at −80°C. Sample protein concentration was determined with a bicinchoninic acid assay (Pierce, Rockford, IL) using bovine serum albumin as the standard and the heme content was measured by the formation of a reduced pyridine hemochrome [7]. Protein purities were >90% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, with ~10% in the CO-bound form and an A_{423} (heme Soret peak after reduction and CO-binding) to A_{280} (protein, as isolated) ratio of ≥ 4.5.

3. Features of the low-copy number compatible reporter system. The basic reporter system utilizes a low-copy number plasmid that is compatible with the RcoM-1 protein expression plasmid [3]. Specifically, the reporter plasmid constructs were derived from pEXT21 [1] in a

series of steps that deleted the SalI site, *lacI*^{q}, and P_{tac} (see pUX2970, Table 1), then a cassette bearing a *coxM*$_1$ promoter fused to a promoterless *lacZ* reporter was introduced. This reporter was fused to the 5′-end of *coxM*$_1$ and as such was under the regulation of the *coxM*$_1$ promoter, which was flanked by unique *Bam*HI and *Sal*I sites, as illustrated in Fig. 1.

Various *coxM*$_1$ promoter regions were also introduced into *Bam*HI / *Sal*I-cut pEXT20 (see pUX3025, 3056, 3057, Table 1) to provide suitable templates for mutagenesis. The mutagenized promoter regions could be excised by *Bam*HI / *Sal*I digestion, isolated and introduced into the *coxM*$_1$ reporter system (*e.g.*, pUX3007) by standard directional cloning procedures, resulting in a series of reporter systems bearing different promoter regions (Fig. 1).

All activity assays are performed in the UQ5853 background that lacks both endogenous LytTR-domain regulatory systems (encoded by *yehTU* and *ypdAB*). This strain bears chromosomal resistance to chloramphenicol, grows quickly, and cultivation for assays and plate cultures were routinely done at approximately 28°C.

4. Verification of constructs. Maximal plasmid yields are obtained using rich medium and cultivation at 35 - 37°C. The RcoM-1 expression and reporter plasmids were readily isolated using standard miniprep procedures (QIAprep spin miniprep, Qiagen). Sequencing reactions for the *rcoM* insert were primed using oligonucleotide "pEXT20-F2" (5′-GCATAATTCGTGTCGC-3′; see Fig. 1) and ~100 ng plasmid DNA (see Fig. 3).

Sequencing the low copy number reporter system plasmids (*e.g.* pUX3010) required a larger DNA preparation: pellet cells from a total of ~6 ml of culture and combine the lysed supernatants onto one QIAprep spin column. Columns are washed per the manufacturer's instructions using both "PB" and "PE" washes, and the DNA is eluted with ~40 µl of buffer (10 mM Tris-HCl, pH 8.0, 0.01 mM EDTA). Note that in the dual-plasmid reporter strains most of the plasmid DNA is the RcoM-1 expression system (*e.g.* pUX2410) and therefore the level of DNA used in sequencing reactions needs to be increased to 1000 ng/reaction so that the level of low copy reporter plasmid is sufficient. The *coxM*$_1$ promoter sequence was obtained using a

primer that bound near the 5′-end of the *lacZ* gene: “LacZ-R1” (5′-GACGTTGTAAAACGACG-3′; see Figs. 1 and 4).

Table 1. Plasmids and Strains

Plasmid (Strain I.D.)	Derivation and Relevant Characteristics[a]	Reference
--- (UQ5853)	Cm^r; *E. coli* BW29858; *ΔlacZ4787*(::*rrnB*-3) *Δ(yehT-yehU)1324* with Δ*ypdAB*::*cat* [For reference to BW29858 see [8]]	[3]
pUX2396 (UQ4802)	/Ap^r; 831-bp *Eco*RI-*Hin*dIII fragment bearing *Burkholderia xenovorans* LB400 *rcoM*$_1$ cloned into pEXT20. Suitable for expression of $RcoM_{Bx}$-1 protein (untagged). Host strain is *E. coli* VJS6737 [see [6]; for information about pEXT20 see [1].	[2]
pUX2397 (UQ4884)	/Ap^r; 831-bp *Eco*RI-*Hin*dIII fragment bearing *Burkholderia xenovorans* LB400 *rcoM*$_2$ cloned into pEXT20. Suitable for expression of $RcoM_{Bx}$-2 protein (untagged). Host strain is *E. coli* VJS6737 [see [6]]; for information about pEXT20 see [1].	[2]
pUX2410 (UQ4803)	/Ap^r; pUX2396 derivative suitable for expression of $RcoM_{Bx}$-1 protein + C-terminal 6×His tag. Host strain is *E. coli* VJS6737 [see [6]]; for information about pEXT20 see [1].	[2]
pUX2424 (UQ4861)	/Ap^r; ~1200-bp fragment from *Burkholderia xenovorans* LB400 with introduced terminal *Hin*dIII and *Sal*I sites [such that *Hin*dIII – (←*rcoM*$_1$ – intergenic region – *coxM*$_1$′→) – *Sal*I] cloned into pEXT20. Suitable template for amplification and mutagenesis. See [1] for information about pEXT20.	[2]
pUX2442 (UQ4876)	/Ap^r + Km^r; the *lacZ* + Km^r cassette from pKOK6 cloned at the *Sal*I site of pUX2424 such that *coxM*$_1$′::*lacZ*→ *t*$_{fd}$ ←Km^r. Suitable template for amplification of the $RcoM_{Bx}$-1 binding region + reporter cassette. For information about pKOK6 see [4].	[2]
pUX2850 (UQ5457)	/Ap^r; pUX2396 derivative suitable for expression of $RcoM_{Bx}$-1 protein + N-terminal 6×His tag. Host strain is *E. coli* VJS6737 [see [6]].	[3]
pUX2970 (UQ5708)	/Sp^r; pEXT21 with Δ*Sal*I + Δ(*lacI*^{q} – P*tac*).	[3]
pUX2996 + pUX2410 (UQ5875)	Cm^r /Ap^r /Km^r + Sp^r; pUX2996 = pUX2970 with a *coxM*$_1$′::*lacZ* reporter system such that ...vector DNA – *Bam*HI (unique site) – "e + f" binding region – *coxM*$_1$′(with unique *Sal*I site)::*lacZ*→ *t*$_{fd}$ ←Km^r (with 5′-end unique *Xho*I site) – vector DNA ←Sp^r...; plus pUX2410; UQ5853 host.	[3]
pUX3007 (UQ5869)	Cm^r /Km^r + Sp^r; *coxM*$_1$′::*lacZ* reporter system (see pUX2996) with 0 RcoM binding sites; UQ5853 host.	[3]
pUX3008 + pUX2410 (UQ5874)	Cm^r /Ap^r /Km^r + Sp^r; pUX3008 = *coxM*$_1$′::*lacZ* reporter system (see pUX2996) with binding site "f"; plus pUX2410; UQ5853 host.	[3]
pUX3009 (UQ5871)	Cm^r /Km^r + Sp^r; *coxM*$_1$′::*lacZ* reporter system (see pUX2996) with binding sites "d + e + f"; UQ5853 host.	[3]

pUX3009 + pUX2410 (UQ5876)	Cm^r /Ap^r /Km^r + Sp^r; pUX3009 = *coxM*$_1$′::*lacZ* reporter system (see pUX2996) with binding sites "d + e + f"; plus pUX2410; UQ5853 host.	[3]
pUX3010 + pUX2410 (UQ5877)	Cm^r /Ap^r /Km^r + Sp^r; pUX3010 = *coxM*$_1$′::*lacZ* reporter system (see pUX2996) with binding sites "a – f"; plus pUX2410; UQ5853 host.	[3]
pUX3025 (UQ5784)	/Ap^r; ~5-kb *Bam*HI + *Xho*I fragment from pUX2996 cloned into *Bam*HI + *Sal*I-cut pEXT20. Useful for mutagenesis and excision of the "e + f" binding region. For information about pEXT20 see [1].	[3]
pUX3056 (UQ5827)	/Ap^r; ~5-kb *Bam*HI + *Xho*I fragment from pUX3009 cloned into *Bam*HI + *Sal*I-cut pEXT20. Useful for mutagenesis and excision of the "d + e + f" binding region. For information about pEXT20 see [1].	[3]
pUX3057 (UQ5828)	/Ap^r; ~5-kb *Bam*HI + *Xho*I fragment from pUX3010 cloned into *Bam*HI + *Sal*I-cut pEXT20. Useful for mutagenesis and excision of the "a – f" binding region. For information about pEXT20 see [1].	[3]
pUX3236 (UQ6106)	/Ap^r; pUX2424 but with a higher-affinity "e↑" binding site (5′-TCCTACAGTTCACGCACGT-3′).	[3]
pUX3242 + pUX2410 (UQ6139)	Cm^r /Ap^r /Km^r + Sp^r; pUX2996 reporter system with higher-affinity $RcoM_{Bx}$-1 binding site at the "e" position ("e↑" binding site = 5′-TCCTACAGTTCACGCACGT-3′); plus pUX2410; UQ5853 host.	[3]
pUX3254 + pUX2410 (UQ6148)	Cm^r /Ap^r /Km^r + Sp^r; pUX3010 reporter system with a deletion between the "c" and "d" binding motifs such that the individual binding motifs "a," "b," "c," "d," "e," and "f" are spaced at 21-bp intervals. The "c – d" sequence is 5′-TTCGGGAAACCG*GGTTCGGGTTTCATG-3′ where the underlined bases indicate the "c" and "d" "TTnnnG" binding motifs and the asterisk represents the junction; plus pUX2410; UQ5853 host.	[3]
pUX3272 + pUX2410 (UQ6177)	Cm^r /Ap^r /Km^r + Sp^r; pUX3009 reporter system with an improved −35/−10 RNAP binding region (5′-TTGACA-(18N)-TATCCT-3′); plus pUX2410; UQ5853 host. High activity independent of RcoM accumulation or the addition of CO.	[3]
pUX3297 + pUX2410 (UQ6211)	Cm^r /Ap^r /Km^r + Sp^r; pUX3009 reporter system with "e" binding site "TTnnnG" motif changed to "GGnnnG;" plus pUX2410; UQ5853 host.	[3]

[a] Drug resistances (or the encoding resistance genes when in a genetic context) are denoted as follows: Ap^r (ampicillin), Cm^r (chloramphenicol), Km^r (kanamycin), Sp^r (spectinomycin). Cm^r is chromosomally-encoded. Plasmid-encoded Ap^r, Km^r and Sp^r are preceded by a "/." When appropriate, arrows indicate the transcriptional orientation of the associated gene or drug resistance marker. Double-underlined positions indicate differences from the wild-type sequence. If not specified, the host strain is *E. coli* DH5α.

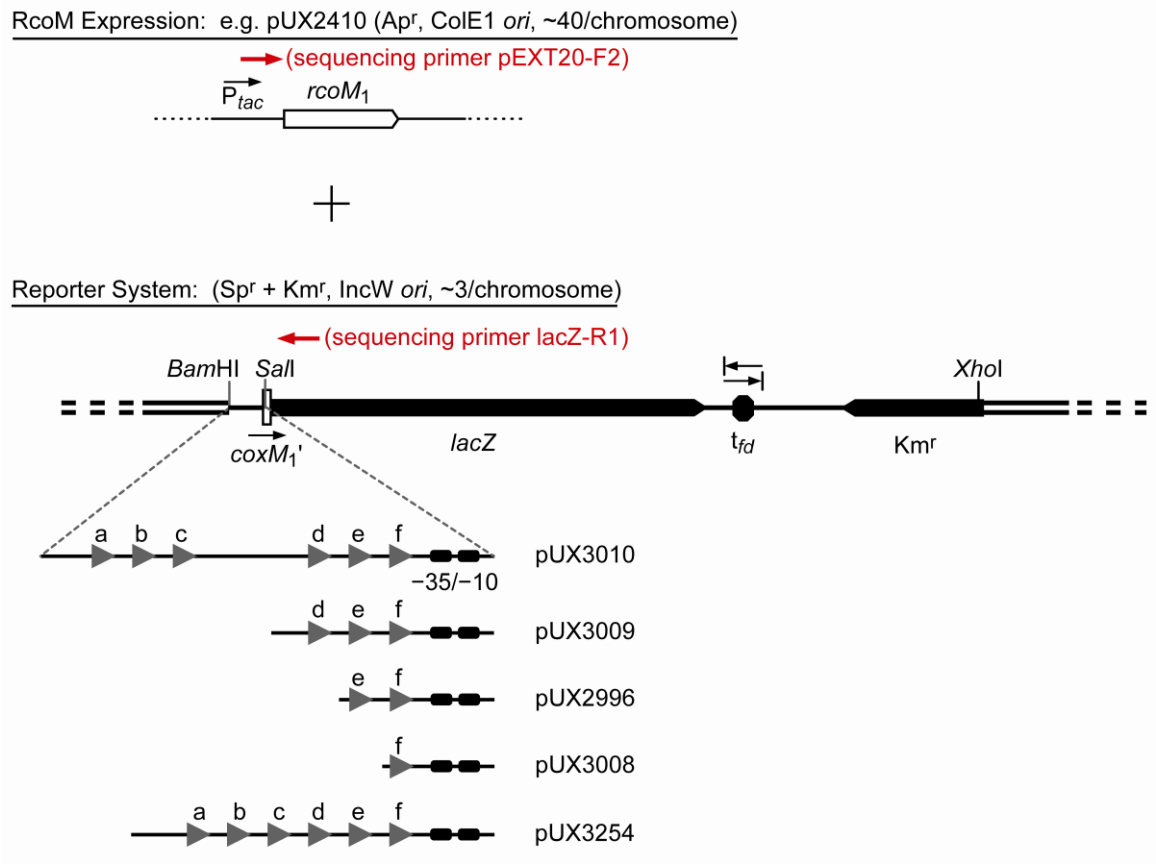


Figure 1. Schematic representation of the in vivo activity system. RcoM$_{Bx}$-1function was assessed in vivo using a compatible two-plasmid system in host strain UQ5853 (Table 1). IPTG-regulated accumulation of RcoM$_{Bx}$-1 occurred from pUX2410 (Table 1). When CO-bound, this protein recognized promoter direct-repeat binding sites (designated "a" through "f," see also Fig. 2, below) and regulated transcription of *coxM*$_1$ fused to a promoterless *lacZ* cassette carried on a low-copy number reporter plasmid, such as pUX3010 (Table 1). Unique restriction sites in the reporter plasmid permitted the directional replacement of the reporter system (*e.g.* using BamHI and XhoI) or the *coxM*$_1$ promoter region (*e.g.* using BamHI and SalI), as indicated by representative binding site inserts shown for plasmids pUX2996, 3008, 3009, 3010, and 3254.

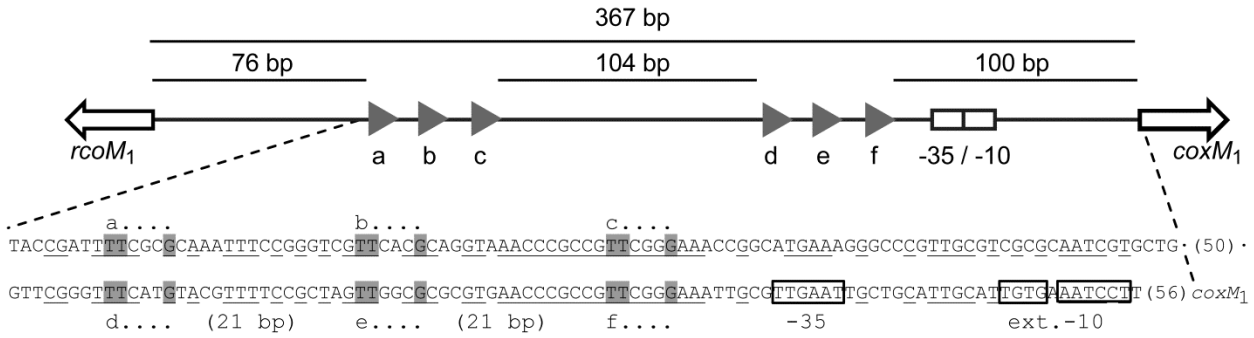


Figure 2. Detailed organization of the *B. xenovorans rcoM*$_1$/*coxM*$_1$ (chromosome 1) intergenic DNA. Sequence and footprinting analyses indicate two parallel RcoM binding regions, each comprised of triplet sets of "TTnnnG" direct-repeat motifs (shaded) designated "a + b + c" and "d + e + f" (showing the "forward" strand). The two binding regions are separated by a 104-bp interval, and they align with 71% identity (residues underlined). The "f" binding motif is adjacent to a putative RNAP σ^{70} −35/extended −10 region (residues boxed) located upstream of *coxM*$_1$.

Fig. 3. Typical sequence reaction obtained for pUX2410 using primer pEXT20-F2.

Note that the primers used for cloning introduced 4 silent changes at the 3′-end of *rcoM*$_1$ gene, as well as 6×His codons and a second stop codon.

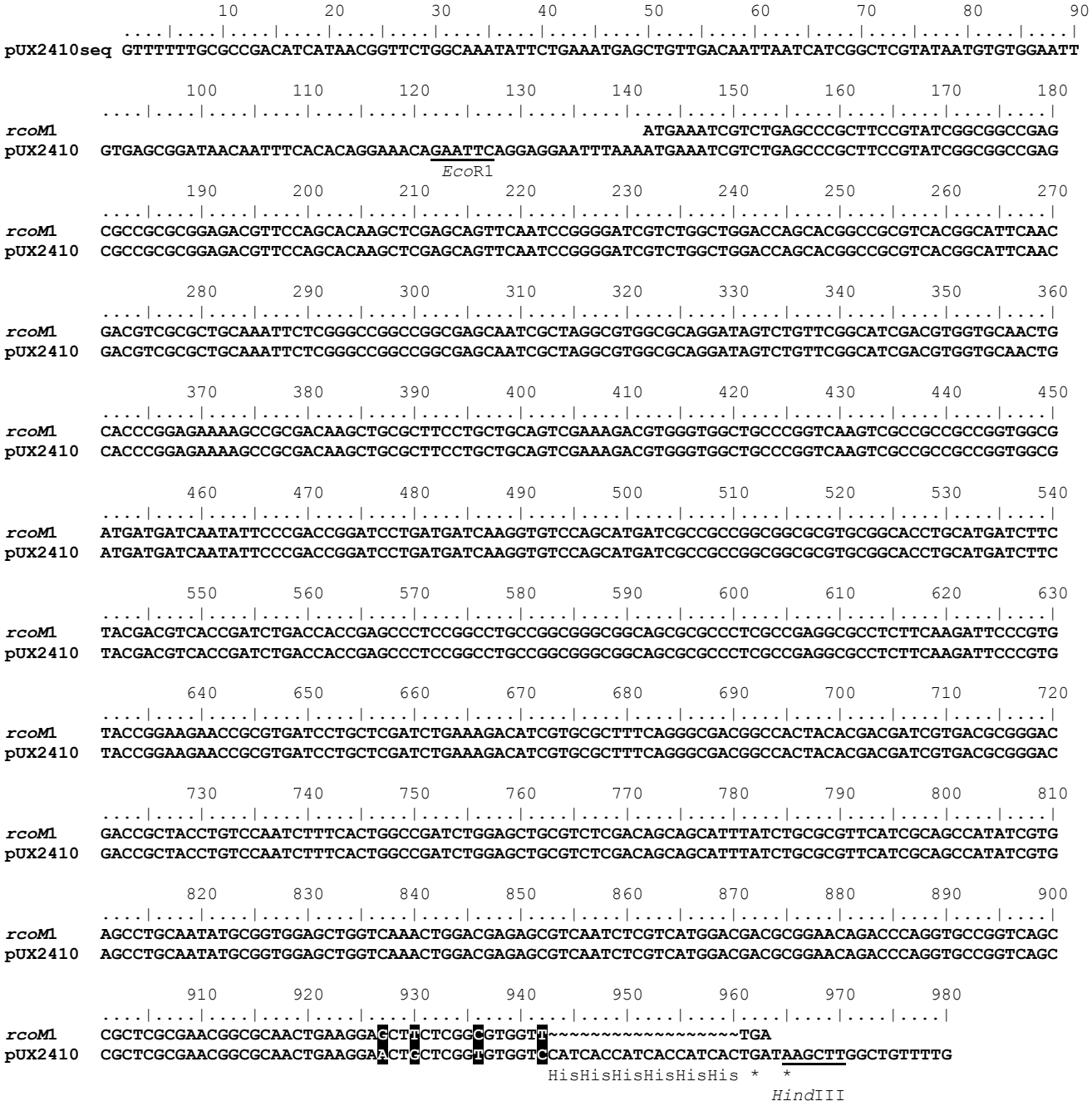


Fig. 4. Sequence data for various reporter constructs obtained with primer LacZ-R1 (reverse complement presented below).

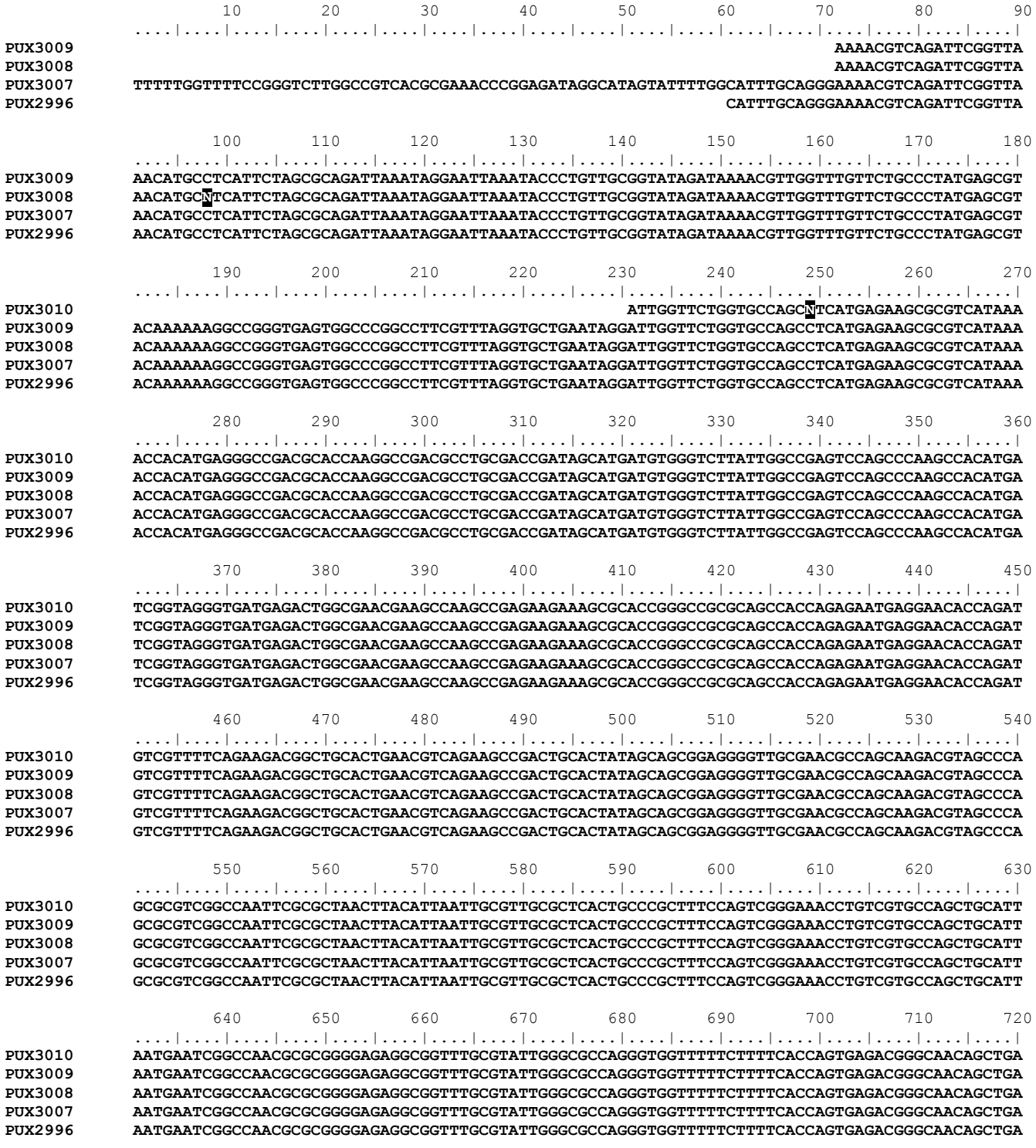
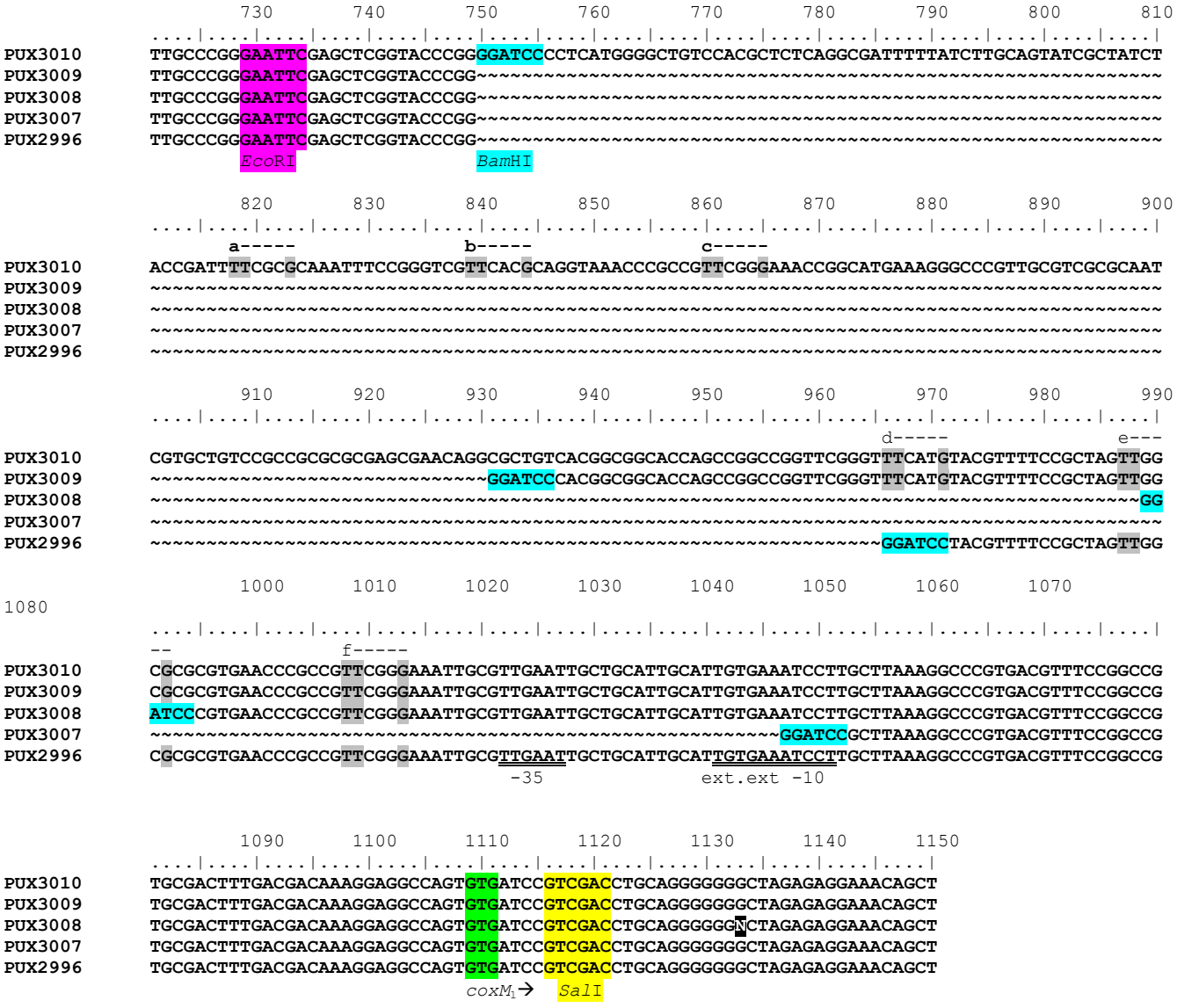


Fig. 4 (continued)



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