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MECHANISMS OF RESISTANCE

Conservation of Resistance-Nodulation-Cell Division Efflux Pump-Mediated Antibiotic Resistance in *Burkholderia cepacia* Complex and *Burkholderia pseudomallei* Complex Species

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ABSTRACT *Burkholderia cepacia* complex (Bcc) and *Burkholderia pseudomallei* complex (Bpc) species include pathogens that are typically multidrug resistant. Dominant intrinsic and acquired multidrug resistance mechanisms are efflux mediated by pumps of the resistance-nodulation-cell division (RND) family. From comparative bioinformatic and, in many instances, functional studies, we infer that RND pump-based resistance mechanisms are conserved in *Burkholderia*. We propose to use these findings as a foundation for adoption of a uniform RND efflux pump nomenclature.

KEYWORDS *Burkholderia*, multidrug resistance, RND efflux pumps

The genus *Burkholderia* encompasses a diverse group of beneficial and pathogenic bacteria (1–3). The genus is separated into two main groups; the *Burkholderia cepacia* complex (Bcc; 22 members) (1, 4, 5) and the *Burkholderia pseudomallei* complex (Bpc; 6 members) (6). Both complexes contain potent opportunistic pathogens of plants, animals, and humans (1, 5, 7). In Bcc, *Burkholderia cenocepacia* and *Burkholderia multivorans* are pathogens that frequently afflict compromised individuals, most notably people with cystic fibrosis (7). In Bpc, *B. pseudomallei* and its close relative *Burkholderia mallei* are the etiologic agents of melioidosis and glanders, respectively (8, 9). A common hallmark of *Burkholderia* infections is that they are often recalcitrant to antibiotic therapy (10–12).

Efflux via pumps belonging to the resistance-nodulation-cell division (RND) family are the dominant intrinsic and acquired multidrug resistance mechanism in *Burkholderia* species (13). Despite the fact that Bpc species have been intensively studied for only a short time compared to Bcc species, the landscape of RND pumps in Bpc bacteria such as *B. pseudomallei* (14–20) and *Burkholderia thailandensis* (21, 22) has been well defined. RND pumps in Bcc have been studied to a significant extent in *B. cenocepacia* (12, 23–29) and more sporadically in other Bcc members, such as *Burkholderia vietnamiensis* (30) and *Burkholderia ubonensis* (31). We previously noted that the RND efflux pump nomenclature in Bcc species is nonuniform and confusing (13). Bioinformatic and, in many instances, functional analyses predict that RND efflux pump-based resistance mechanisms are conserved in Bcc and Bpc species. We propose to use these findings as a foundation for adoption of a more uniform RND efflux pump nomenclature.

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We focused on three Bcc and Bpc RND efflux pumps for which published and unpublished experimental data were available, as well as evidence of potential clinical significance with regard to the resistance profiles bestowed by these pumps (Table 1).

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TABLE 1 Established RND efflux pumps in *Burkholderia* species[a]

Species	Efflux pump	Gene names	Gene annotation	Major antibiotic substrate(s)	Reference(s)
B. pseudomallei	AmrAB-OprA	*amrAB-oprA*	BPSL1804–1802	AG, MAC, TET, CET	14, 46
	BpeAB-OprB	*bpeAB-oprB*	BPSL0814–0816	CHL, FQ, MAC, TET[b]	15, 17
	BpeEF-OprC	*bpeEF-oprC*	BPSS0292–0294	CHL, FQ, TET, TMP, SMX[c]	18, 19, 20
B. thailandensis	AmrAB-OprA	*amrAB-oprA*	BTH_I2445–I2443	AG, MAC, TET	22
	BpeAB-OprB	*bpeAB-oprB*	BTH_I0680–I0682	TET	22
	BpeEF-OprC	*bpeEF-oprC*	BTH_II2106–II2104	CHL, FQ, TET, TMP, SMX	21, 22
B. cenocepacia	RND-3	NA	BCAL1674–1676	AG, FQ, NAL, TET	25, 28
	RND-4	NA	BCAL2822–2820	ATM, CHL, FQ, TOB, TET	26, 28
	RND-10	*ceoAB-opcM*	BCAM2551–2549	CHL, FQ, TET, TMP[c]	23, 47

[a]For details, see Table S1. This is an updated and condensed version of a previously published table (13). Abbreviations: AG, aminoglycosides; ATM, azithromycin; CET, cethromycin; CHL, chloramphenicol; FQ, fluoroquinolones; MAC, macrolides; MEM, meropenem; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline(s); TMP, trimethoprim; TOB, tobramycin; NA, not applicable.
[b]Low-level resistance in BpeR repressor-deficient mutant (15, 17).
[c]Resistance only in induced strains or *bpeT* or *bpeS* regulatory mutants (18, 19, 20, 47).

Since these criteria are best defined in Bpc bacteria, especially *B. pseudomallei* (14–20) and the closely related *B. thailandensis* (21, 22), we chose these species' well-characterized AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC efflux pumps as models and queries for identification and naming of the corresponding efflux pumps in Bcc species. Lesser-known Bcc members were included either as an example of a potential environmental reservoir of novel antimicrobial resistance determinants (*B. ubonensis*) (31, 32) or as an emerging opportunistic pathogen whose biology and antimicrobial resistance potential are not yet well understood (*Burkholderia latens*) (33).

DIAMOND BLASTP (34) within the *Burkholderia* Genome Database (www.burkholderia.com) (35) was employed for identification of candidate efflux pump proteins in other *Burkholderia* species. These candidates were further scrutinized by using them in DIAMOND BLASTP, performing protein sequence alignments and similarity predictions using MUSCLE on the EMBL-EBI server (https://www.ebi.ac.uk/Tools/msa/muscle/) (36), and by genomic content analyses (e.g., chromosome localization and presence of predicted cognate transcriptional regulators). In addition to similarity predictions, transcriptional regulators were analyzed for conserved helix-turn-helix (HTH) DNA-binding domains by the Rhone-Alpes Bioinformatic Pole Gerland Site (https://npsa-prabi.ibcp.fr) (37). In four instances, DNA sequence alignments were used to reexamine and revise translational start sites (see Fig. S1 in the supplemental material).

By using these methods, we identified gene clusters encoding AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC in the four Bcc species that we studied, namely, *B. ubonensis*, *B. cenocepacia*, *B. multivorans*, and *B. latens* (Fig. 1). In all instances, the architecture of the predicted structural and regulatory gene clusters was identical in Bcc and Bpc: (i) the genes encoding the AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC efflux system components exhibit the same operon structure; (ii) the locations and transcriptional orientation of the operon-associated regulatory genes are the same; (iii) transcriptional regulator protein sequences and their predicted DNA-binding domains are highly conserved; and (iv) the gene clusters are located on the same chromosomes (*amrAB-oprA* and *bpeAB-oprB* on chromosome 1 and *bpeEF-oprC* on chromosome 2). These conclusions are supported by published data and new experimental findings presented below. Most previously published experimental data about RND pumps in Bcc were obtained from studies of *B. cenocepacia*. These and more recent studies lend credence to correct efflux gene assignments in *B. cenocepacia* and other Bcc species.

Studies with *B. cenocepacia* strain J2315 found that AmrAB-OprA (also known as RND-3) (see Table S1) plays a role in antimicrobial resistance (Table 1). Increased expression of AmrAB-OprA encoded by the genes BCAL1674 to BCAL1676 on chromosome 1 led to increased antimicrobial resistance (29). Conversely, deletion of AmrAB-OprA led to decreased antibiotic resistance (38). The *amrAB-oprA*-associated AmrR repressors are highly conserved in the examined Bcc and Bpc species (Fig. 1A), with the Bcc proteins exhibiting 72.1 to 72.7% identity to *B. pseudomallei* K96243 AmrR (Fig.

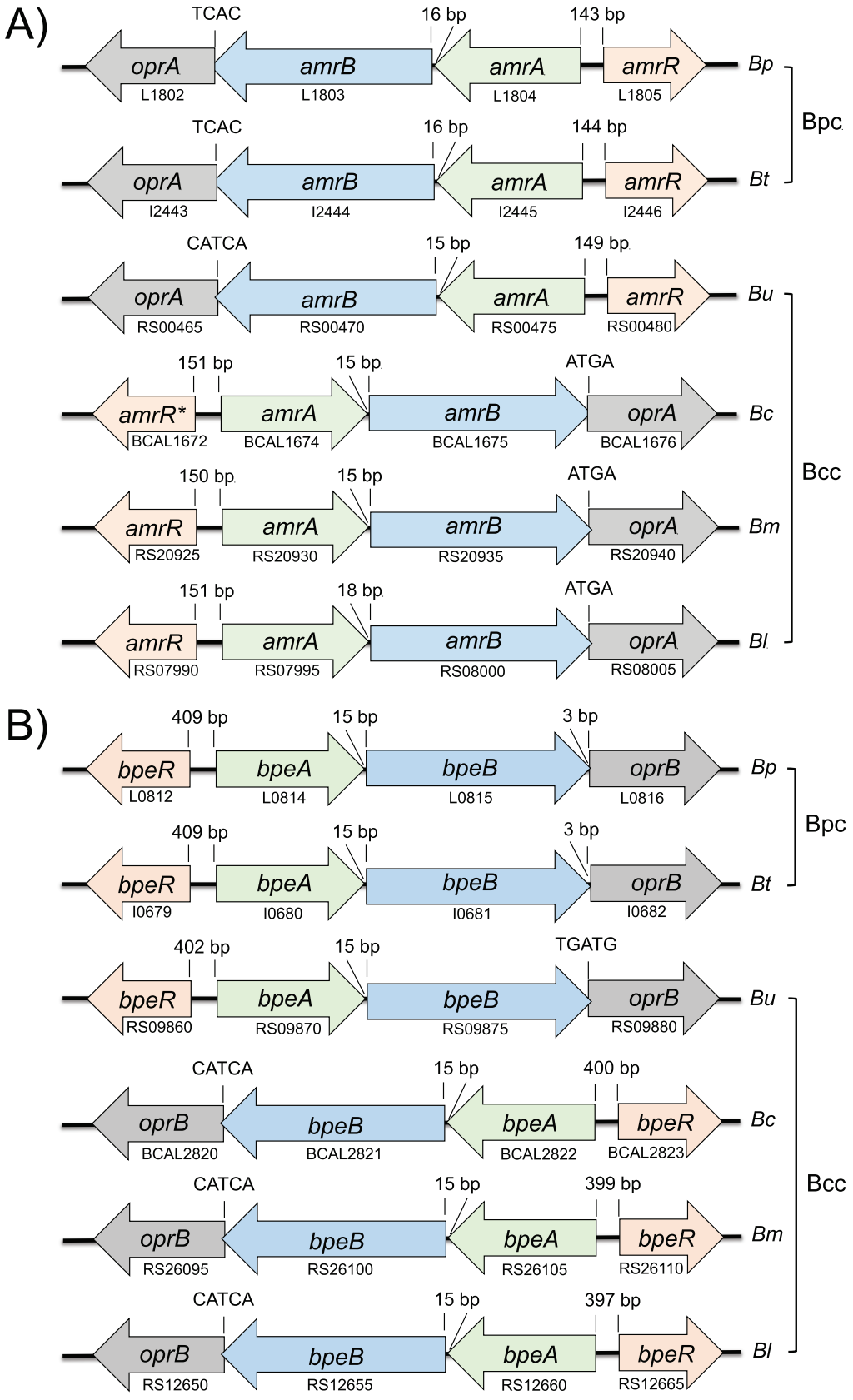

FIG 1 Continued.

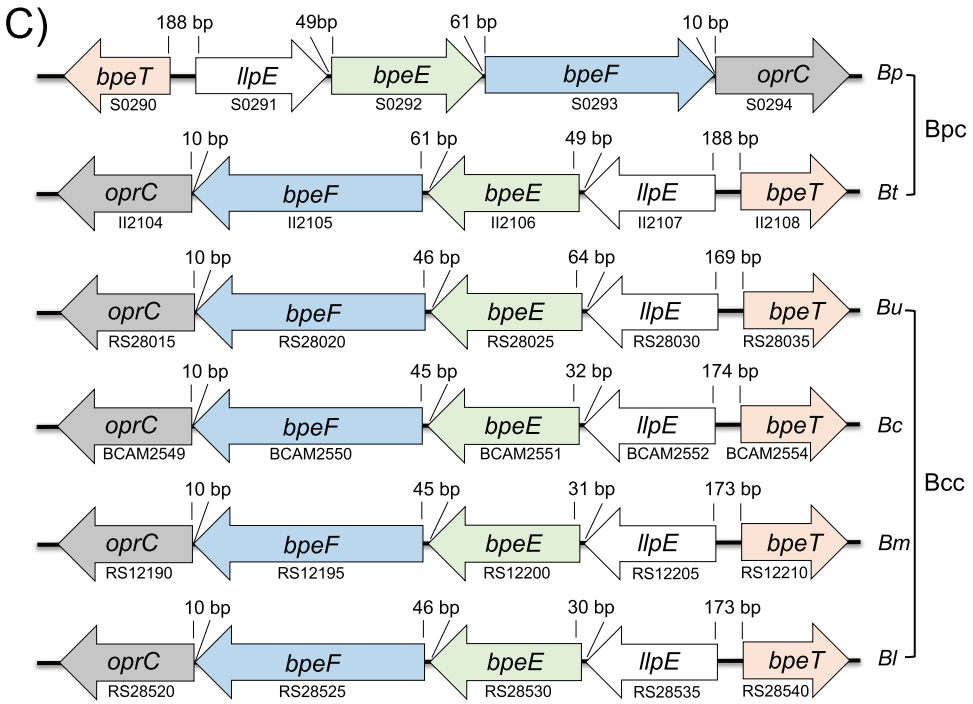

FIG 1 Conservation of genetic organization of RND efflux pump structural and regulatory genes in *Burkholderia* Bcc and Bpc. (A and B) *amrAB-oprA* and *bpeAB-oprB* and their respective cognate *amrR* and *bpeR* regulatory genes located on chromosome 1; (C) *bpeEF-oprC* and cognate *bpeT* regulatory genes located on chromosome 2. The efflux pump structural genes encode membrane fusion proteins (*amrA*, *bpeA*, and *bpeE*), RND transporters (*amrB*, *bpeB*, and *bpeF*), and outer membrane channel proteins (*oprA*, *oprB*, and *oprC*). The *bpeE-bpeF-oprC* genes are cotranscribed with *llpE*, which encodes a putative lipase/esterase of unknown function. Previous studies with *B. cenocepacia* and *B. pseudomallei* showed that LlpE is not required for antibiotic efflux. Expression of the *amrAB-oprA* and *bpeAB-oprB* operons is regulated by the TetR family proteins AmrR and BpeR, respectively. In contrast, expression of the *llpE-bpeE-bpeF-oprC* operon is governed by the closely regulated LysR-type transcriptional regulators, BpeT (adjacent to *llpE-bpeE-bpeF-oprC*) and BpeS (located on chromosome 1 [Fig. S4]). The illustrated examples are *B. cenocepacia* J2315 (*Bc*), *B. latens* AU17928 (*Bl*), *B. multivorans* BAA-247 (*Bm*), *B. pseudomallei* K96243 (*Bp*), *B. thailandensis* E264 (*Bt*), and *B. ubonensis* Bp8955 (*Bu*). Distances between genes are indicated in base pairs. Overlaps of the *amrB* and *oprA* start and stop codons are indicated with TCAC (*Bp* and *Bp*), CATCA (*Bu*), and ATGA (*Bc*, *Bm*, and *Bl*). Similarly, overlapping *bpeB* and *oprB* start and stop codons in Bcc are indicated by TGATG (*Bu*) and its reverse CATCA (*Bc*, *Bm*, and *Bl*). Letters and/or numbers below genes indicate locus tags (Table S1). Of note is that *B. cenocepacia* strain J2315 *amrR* is a pseudogene (*amrR**) due to deletion of nucleotide G124 (29). This mutation causes a frameshift after amino acid residue 42 that results in expression of a truncated 107-amino-acid protein versus a 215-amino-acid residue AmrR in wild-type strains such as K56-2 (39). Data used for generating the graphs were from the *Burkholderia* Genome Database versions 8.1 and 9.1 (www.burkholderia.com) (35) and Table S1. While this paper was under review, a manuscript was posted on the *Antimicrobial Agents and Chemotherapy* website (*AAC Accepted Manuscripts*) that shows, for *B. multivorans* BAA-247, schematic representations of (i) the chromosome 1 location of *bpeA-bpeB-oprB* (with NCBI accession tags and annotation) and the adjacent *bpeR* and (ii) the chromosome 2 location of *llpE-bpeE-bpeF-oprC* (with NCBI accession tags and annotation) and the adjacent *bpeT*, as well as the chromosome 1 location of *bpeS* (45).

S2A) and containing highly conserved predicted DNA-binding domains (Fig. S2B). In strain J2315, the *amrR* gene encoding the TetR family repressor AmrR is a frame-shifted pseudogene coding for a truncated protein (29). In its stead, the wild-type AmrR from K56-2 was therefore used for AmrR protein analyses (39). Several studies with environmental and clinical *B. pseudomallei* isolates showed that AmrAB-OprA is the major intrinsic aminoglycoside (AG) resistance determinant. AG-susceptible strains either lack a functional efflux pump due to deletion of the *amrAB-oprA* operon (16) or *amrB* point mutations (40, 41) or fail to express the efflux operon (16). Similar mutations are responsible for the AG susceptibility of at least some *B. mallei* strains (13). We recently confirmed that AmrAB-OprA is the intrinsic AG resistance determinant in *B. ubonensis* (32). A study with *B. vietnamiensis* showed that changes in AmrR-dependent expression

of what was called AmrAB-OprM led to acquisition of AG resistance *in vivo* and *in vitro* (30). Studies with *B. pseudomallei* (42) and *B. ubonensis* (31) revealed that AmrAB-OprA also plays a role in resistance to tetracycline (TET) and its derivatives (e.g., doxycycline). Lastly, we show here that in *B. latens* AmrAB-OprA is induced in response to chloramphenicol (CHL) exposure (Table S2).

In *B. cenocepacia* J2315, the BpeAB-OprB pump (also known as RND-4 [Table S1]) encoded by the genes BCAL2822 to BCAL2820 has previously been implicated in antibiotic resistance (Table 1). Deletion of this pump leads to decreased antimicrobial resistance (43). Like its *B. pseudomallei* counterpart, BpeAB-OprB is expressed at only low levels in strain J2315 (17, 29). Although J2315 has two potential *bpeAB-oprB* gene clusters annotated, RND-4 on chromosome 1 and RND-2 (BCAS0764 to BCAS0766) on chromosome 3 (Fig. S3), only RND-4 (or BpeAB-OprB) was shown to play a role in antibiotic resistance. Two lines of evidence support the notion that there is only one BpeR-regulated *bpeAB-oprB* operon on chromosome 1 as indicated in Fig. 1B. The first is the absence of the conserved *bpeR* gene upstream of the RND-2 genes (Fig. S3). The *bpeAB-oprB* associated BpeR repressors (confirmed in *B. pseudomallei* [17]) are highly conserved in the examined Bcc and Bpc species, with the Bcc proteins exhibiting 80.1 to 82% identity to *B. pseudomallei* K96243 BpeR (Fig. S2A) and containing highly conserved predicted DNA-binding domains (Fig. S2B). The second is the location of RND-2 genes on the dispensable chromosome 3 (44). In contrast to *B. pseudomallei* where BpeAB-OprB seems to play a minor role in antibiotic resistance (17), this efflux system apparently plays a more dominant role in Bcc species, at least in *B. cenocepacia* (28).

The BpeEF-OprC pump (also known as RND-10 or CeoAB-OpcM [Table S1]) encoded by the genes BCAM2551 to BCAM2549 in strain J2315 was the first RND efflux pump characterized in *B. cenocepacia* (23, 24). Its substrates include CHL, TET, trimethoprim (TMP), and ciprofloxacin. Expression of CeoAB-OpcM was shown to be inducible by CHL and sulfamethoxazole/trimethoprim (SXT), likely involving a divergently transcribed gene, *ceoR*, which encodes an uncharacterized LysR-type transcriptional regulator (LTTR) (23, 47). In *B. pseudomallei*, BpeEF-OprC expression is transcriptionally regulated by two closely related LTTRs: (i) BpeT, encoded by the divergently from the *llpE-bpeE-bpeF-oprC*-transcribed *bpeT* gene on chromosome 2 (Fig. 1C), and (ii) BpeS, encoded by a distant gene on chromosome 1 (19, 20). BpeT and BpeS mutations are the main causes of acquired CHL, fluoroquinolone, TMP, and SXT resistance (19, 20). Bcc BpeS homologs are also found on chromosome 1 in all species examined, although in a different genomic context (Fig. S4).

BpeT and BpeS are highly conserved in the examined Bcc and Bpc species, with the Bcc proteins exhibiting 91.0 to 93.4% (BpeT) and 82 to 85.2% (BpeS) identity to the corresponding *B. pseudomallei* K96243 proteins (Fig. S2A) and preservation of conserved predicted DNA-binding domains (Fig. S2B). A unique hallmark of the *ceoAB-opcM* operon is that its three genes are cotranscribed with *llpE*, which encodes a putative lipase/esterase of unknown function. LlpE is not required for antibiotic efflux (20, 23), and its presence in an operon with *bpeEF-oprC* is conserved in all Bcc, all Bpc, and other *Burkholderia* species (e.g., *Burkholderia gladioli* [Fig. S5]) examined to date. *Burkholderia cenocepacia* CeoAB-OpcM shares many traits with *B. pseudomallei* BpeEF-OprC (e.g., efflux pump components, substrate spectrum, transcriptional regulation by two LTTRs, inducibility with select pump substrates, organization in an operon with the unique *llpE*, and location on chromosome 2), and these traits are conserved in other Bcc and Bpc species (Fig. 1C). For instance, we show in this study that in *B. latens* BpeEF-OprC is constitutively expressed in a strain whose multidrug resistance (MDR) profile matches that of *B. pseudomallei* and *B. cenocepacia* BpeEF-OprC (Fig. S6).

Comparative bioinformatic analyses and mounting evidence from functional studies in Bcc and Bpc species support the notion that RND efflux pump-based resistance mechanisms are largely conserved across Bcc and Bpc. Based on the work and findings reported here we propose a uniform RND efflux pump nomenclature as shown in

Fig. 1. Its adoption by the research community would greatly facilitate following the literature and advancing the field.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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