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areCBA Is an Operon in *Acinetobacter* sp. Strain ADP1 and Is Controlled by AreR, a σ^{54}-Dependent Regulator

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The *areCBA* genes in *Acinetobacter* sp. strain ADP1, determining growth on benzyl alkanoates, are shown to be transcribed as a single operon and regulated by *areR*, which encodes a regulatory protein of the NtrC/XylR family. Assays of the Are enzymes and of two insertions of *lacZ* as a reporter gene have shown that the operon is induced by benzyl acetate, benzyl alcohol, and benzaldehyde, as well as 2- and 4-hydroxybenzyl acetates and benzyl propionate and butyrate. Two adjacent sites of transcriptional initiation were 97 and 96 bp upstream of the start codon for *areC*, near a σ^{54}-dependent −12, −24 promoter. Inactivation of *areR* and *rpoN* (for RNA polymerase σ^{54}) drastically reduced growth rates on the Are substrates and induction of the operon.

The *areC*, *-B*, and *-A* genes in *Acinetobacter* sp. strain ADP1 are located (9, 10) in a 30-kbp supraoperonic cluster of catabolic genes which includes the *ben* and *cat* genes encoding the enzymes of the catechol branch of the β-ketoadipate pathway (5, 6, 7, 17, 19). The *are* genes (Fig. 1) encode an esterase and two dehydrogenases, responsible for the sequential catabolism of benzyl alkanoates to benzoate, salicylate, or 4-hydroxybenzoate, respectively (Fig. 2), which feed into the β-ketoadipate pathway (9, 10). Sequence analysis of *areC*, *-B*, and *-A* and the promoter region suggests that they may be cotranscribed and expressed as an operon (9), upstream of which is a gene designated *areR* (homologous to genes encoding transcriptional activators of the NtrC/XylR family [16, 18] and a possible regulator of the putative operon).

Table 1 lists the plasmids and bacterial strains used in this study. The latter were cultivated as previously described (9). Aromatic substrates were obtained as previously described (9). Standard methods for DNA manipulations and plasmid preparation (20) or previously described methods (9) were used. Growth of cells was monitored, and preparation of cell extracts and assays of Are enzymes was conducted, as described previously (9). β-Galactosidase in lysed cells was determined using the method described by Miller (15). The promoterless *lacZ*-Km cassette was removed from pKOK6.1 (12) and inserted into the *Nsi*I site within *areA* on pADPW36 to create pADPW38 (Fig. 1). For its insertion into *areC*, genomic DNA from ADPW56 (*areC*::Km) (Table 1) was digested with *Xba*I and *Sac*I and ligated into pUC18, selecting for Ap^r Km^r transformants: the plasmid obtained (pADPW45) contained a 6.2-kb insert with the whole of *areR* and an *areC*::Km insert (Fig. 1). The *Xba*I-*Sac*I fragment from pADPW45 was then cloned into pUC18NP to create pADPW46 (Table 1). The Km cassette in *areC* was then replaced by the *lacZ*-Km on a *Pst*I fragment to create pADPW47 (Fig. 1). In both pADPW38 and pADPW47, *lacZ* was transcribed in the same direction as *areA* and *areC*, respectively. Both plasmids were linearized and separately transformed into ADP1 to create ADPW61 (*areA*::*lacZ*-Km) and ADPW63 (*areC*::*lacZ*-Km), respectively, and integration into the chromosomes was confirmed by Southern blot analysis (data not shown).

The Km cassette from pUC4K (26) was inserted into the sole *Bgl*II restriction site in the *areR* gene of pADPW71, a *Hin*dIII subclone of pADPW45 (Fig. 1), to form pADPW90 (Fig. 1). It was linearized and transformed into ADP1 to create ADPW79 (*areR*::Km), as was confirmed by Southern blot analysis.

Total RNA was prepared from *Acinetobacter* sp. using an RNeasy kit (Qiagen). The primer, for primer extension reactions (5′-ATCAAGTAATGT**CAT**ATAGACCTCGTA-3′) complementary to nucleotides on either side of the *areC* translational start (boldface), was end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase and was annealed to the total RNA at 45°C for 3 h. Reactions were carried out with avian myeloblastosis virus reverse transcriptase (RT) (Promega, Madison, Wis.) at 37°C for 1 h. No sequence ladder was obtained with the same primer, but one was created instead from the cloning vector M13mp18 (28) using the M13 forward primer.

RT-PCR was carried out as described previously (10) by using the following primer pairs: across the intergenic regions between *areC* and *areB*, forward (primer CBf) 5′-TCAAAGC GCGTGTAATCGAAAAGGTCAAAC-3′ and reverse (primer CBr) 5′-ATGCCCATCTGGATCTCCACCACTGAAGT-3′; for the intergenic region between *areB* and *areA*, forward (primer BAf) 5′-CAGGCGGTGGTGTAAAGTTTGCTCTT GAAT-3′ and reverse (primer BAr) 5′-ATTGCCCCCTGCG CTGTCTCCTG-3′. Sequence determinations and analysis were performed as described previously (9, 10).

Induction of the *areCBA* genes in ADP1. Induced AreA, AreB, and AreC activities were found in extracts of cells grown on all three pathway substrates that were compared with uninduced (succinate-grown) cells (Table 2), indicating that growth on each induces all three *are* genes.

Induction of *areA* and *areC* expression by *are* operon substrates in strains ADPW61 and ADPW63. β-Galactosidase activities of whole cells of ADPW61 (*areA*::*lacZ*-Km) and ADPW63 (*areC*::*lacZ*-Km) cultures were determined after

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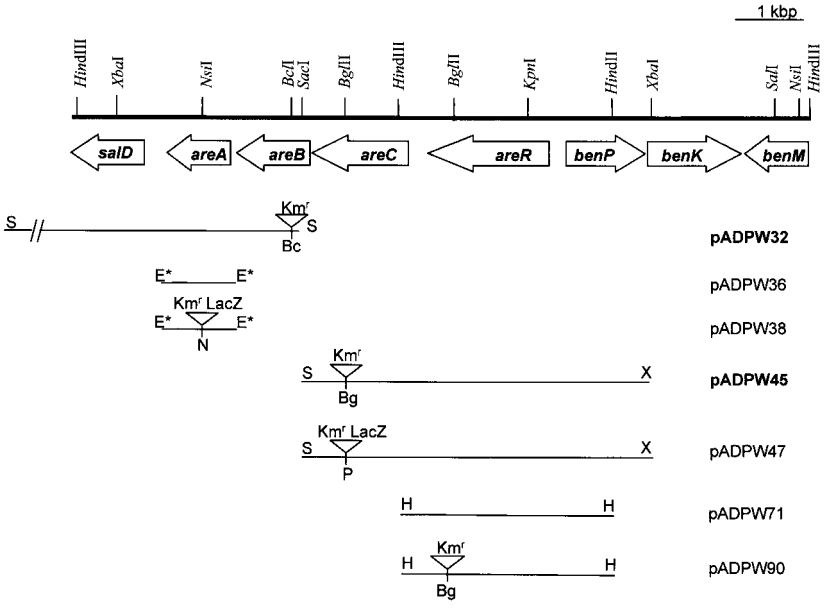

FIG. 1. Physical map of the DNA adjacent to the *ben* genes in ADP1. Physical map of the *areCBA* genes and their locations relative to one end of the supraoperonic *ben-cat* cluster. The various inserts of the plasmids produced from cloning genomic DNA into vectors are specified in Table 1. Plasmids named in boldface contain inserts that were cloned directly from genomic DNA. All other plasmids were produced by PCR from genomic DNA or by subcloning from plasmids containing genomic DNA. Sites at the termini of the inserts marked with an asterisk were incorporated via PCR primers. The Km cassette or *lacZ*-Km cassette insertions are not to scale. The abbreviations for the restriction sites are Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; S, *Sac*I; X, *Xba*I.

growth to late log phase on succinate in the presence of aromatic inducers (15).

Benzyl acetate, benzyl alcohol, and benzaldehyde induced transcription of β-galactosidase in ADPW61 and ADPW63 (Table 3), and in the latter, the activities were approximately 8- to 10-fold higher than in ADPW61. Benzyl acetate, 2-hydroxybenzyl (salicyl) acetate, and 4-hydroxybenzyl acetate were equally effective as inducers, but the level of induction decreased as the length of the aliphatic chain of benzyl esters increased from acetate through propionate to butyrate (Table 3).

The transcription initiation site of the *are* genes. Primer extension reactions using RNA purified from induced ADP1 cells grown on either benzyl acetate or benzyl alcohol (Fig. 3) showed two products. The strongest corresponded to 97 bases upstream of the *areC* ATG, showing the transcription start site at an A residue at position +1 (Fig. 4), but there was also a second, weaker extension product corresponding to the adjacent T residue at position −1. No primer extension products were found in succinate-grown cells. Upstream of the start of transcription is a −12, −24 consensus element for a σ^{54} promoter binding site. The genetic organization of the *areR*-to-*areC* intragenic region, including the start codon and the putative ribosome binding site of *areC*, can be deduced (Fig. 4).

RT-PCR of induced ADP1 total RNA. RT-PCR conducted on RNA that was purified from induced ADP1 cells amplified products across the boundaries between *areC* to *areB* (953 bp) and *areB* to *areA* (946 bp), which had both the expected sizes and restriction sites in the expected positions (Fig. 5). No RT-PCR products using primers whose products would span the *areR*-to-*areC* and *areA*-to-*salD* intergenic regions were obtained from the same RNA preparations, indicating the absence of transcription across these regions (data not shown).

Analysis of ADPW79 (*areR*::Km) growth phenotype. Growth of ADPW79 on minimal medium plates containing benzyl acetate, benzyl alcohol, and benzaldehyde was drastically reduced, taking 2 to 3 days to grow to the same colony size that ADP1 attained overnight. Thus, as with the *areCBA* gene disruptions (9), insertional inactivation of the gene did not result

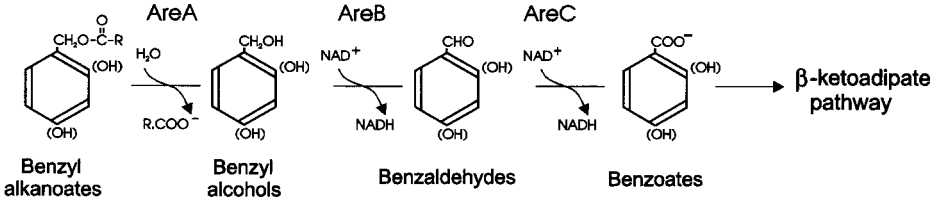

FIG. 2. Proposed pathway for catabolism of benzyl alkanoates by *Acinetobacter* sp. strain ADP1.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Reference or source
Acinetobacter strains		
ADP1 (BD413)	Wild type	11
ADPW56	*areC*::Km; transformation of ADP1 with pADPW22	9
ADPW61	*areA*::*lacZ*-Km; transformation of ADP1 with pADPW38	This study
ADPW63	*areC*::*lacZ*-Km; transformation of ADP1 with pADPW47	This study
ADPW79	*areR*::Km; transformation of ADP1 with pADPW90	This study
ACN274	*rpoN*::Km derivative of ADP1	E. L. Neidle
E. coli strains		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Gibco BRL
XL1-Blue MRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173′ endA1′ supE44′ thi-1′ recA1′ gyrA96′ relA1′ lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
Plasmids		
pUC18	Ap^r, cloning vector	28
pUC18NP	pUC18 without *Pst*I in the MCS	27
pUC4K	Ap^r Km^r; source plasmid for Km^r cassette	26
pKOK6.1	Ap^r Km^r *lacZ*; source plasmid for promoterless *lacZ*-Km cassette	12
pADPW32	8.0-kbp *Sac*I fragment cloned from ADPW57 containing Km^r cassette and the whole of *areA* in pUC18	This study
pADPW36	1.2-kbp *Eco*RI[a] fragment containing *areA* in pUC18	This study
pADPW38	pADPW36 with *lacZ*-Km cassette from pKOK6.1 cloned into *Nsi*I site in *areA*	This study
pADPW45	6.2-kbp *Hin*dIII fragment cloned from ADPW56 containing *areR* and *areC*::Km in pUC18	This study
pADPW46	6.2-kbp *Hin*dIII fragment cloned from ADPW56 containing *areR* and *areC*::Km in pUC18NP	This study
pADPW47	pADPW46 with *lacZ*-Km cassette from pKOK6.1 cloned into *Pst*I of the Km^r cassette	This study
pADPW71	3.5-kbp *Hin*dIII fragment from pADPW45 in pUC18	This study
pADPW90	pADPW71 with Km^r cassette from pUC4K cloned into the *Bgl*II site in *areR*	This study

[a] *, restriction site added by PCR.

in a complete loss of ability to grow on the substrates and is probably due to low levels of other enzymes with overlapping substrate specificities (9).

Induction of *areCBA* in ADPW79 and ACN274. By comparison with ADP1, AreA, -B, and -C in extracts of ADPW79 (*areR*::Km) grown on minimal media containing succinate plus benzyl alcohol (Table 2) exhibited no induced activities. In the *rpoN* mutant ACN274, which has the same reduced growth on the Are substrates as does ADPW79, the activity levels of AreA and AreC were partially elevated by growth on succinate plus benzyl alcohol above those in uninduced ADP1 but were still significantly below those in induced cells of ADP1.

TABLE 2. Specific activities of the Are enzymes in extracts of ADP1 and its derivatives

Strain and growth substrate	Sp act[a] (U/mg of protein) against benzyl substrates		
	Benzaldehyde dehydrogenase	Benzyl alcohol dehydrogenase	Benzyl esterase
ADP1			
Succinate	<0.04	<0.04	<0.04
Benzaldehyde	0.5	0.6	11
Benzyl alcohol	0.4	0.9	9
Benzyl acetate	0.26	0.7	8
Succinate + benzyl alcohol	0.24	0.33	6
ADPW79			
Succinate + benzyl alcohol	<0.04	<0.04	0.14
ACN274			
Succinate + benzyl alcohol	<0.04	0.15	0.6

[a] Specific activities were determined from three determinations at two protein concentrations on each of two cell extracts prepared from independent cultures.

All the evidence presented indicates that *areCBA* is an operon which is coordinately induced by AreR. The three genes are transcribed in the same direction, with only short intragenic regions. At the mRNA level, we have shown by RT-PCR that transcription is continuous across both intragenic regions *areC* to *areB* and *areB* to *areA* but that continuity of transcription does not extend to the two flanking genes, *areR* and *salD*. The only hint of an additional regulatory element is the presence of a small, inverted repeat within the *areB*-to-*areA* intragenic region, which might act as a partial transcription terminator and could account for the difference in activities beween ADPW61 and ADPW63. Downstream, 11 bp from the stop codon of *areA*, is a 17-bp perfect inverted repeat sequence, 5′-AATTA AAAAGGTTCTTAATAAGAACCTTTTTAATT-3′, which is the likely candidate to be a transcriptional terminator for the operon.

Biochemical analysis also points to AreA, -B, and -C being coordinately induced. We demonstrated that AreB and AreC activities were induced by growth of ADP1 on benzyl acetate and benzyl alcohol (9) and have now extended the assays of ADP1 to include the esterase (AreA). Growth on benzyl acetate, benzyl alcohol, or benzaldehyde causes induction of all three enzymes (Table 2). This indicates coordinate rather than sequential expression, since in the case of growth on benzyl alcohol and benzaldehyde, the esterase AreA (and also the alcohol dehydrogenase AreB for growth on benzaldehyde) is

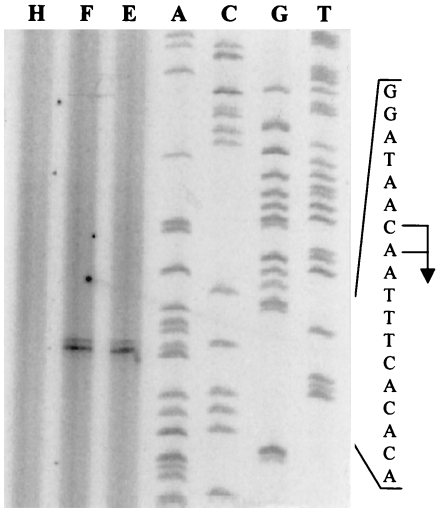

FIG. 3. Primer extension of mRNA from *Acinetobacter* sp. strain ADP1. The experiment was performed with total RNA and a primer which overlaps the putative start codon of *areC*. Lanes A, C, G, and T show the respective products from M13mp18 that were sequenced using the M13 forward primer to size the extension products. The bases on the left are from the corresponding M13 sequence. Lanes E, F, and H represent the signal obtained from the experiment using 10 μg of total RNA from cells of ADP1 grown with benzyl acetate, benzyl alcohol, or succinate, respectively, as the sole carbon sources. The locations corresponding to both transcriptional starts are designated by the arrow and are positions 112 and 111 from the 5′ end of the universal primer. The corresponding bases on the ADP1 sequence are shown in Fig. 4.

not required for growth and yet is induced. It cannot be deduced from these data for ADP1 which compound or compounds can induce, since active metabolism is occurring. However, the use of a promoterless *lacZ* as a reporter gene to monitor *are* gene expression clarifies this (Table 3), since the *lacZ*-Km cassette has its two genes in opposite orientations with a central transcriptional terminator and therefore exerts a polar effect on downstream genes (12). With *lacZ* inserted into *areA* (ADPW61), β-galactosidase activity was induced by benzyl acetate, confirming that benzyl acetate must be an inducer, since it cannot be metabolized because of the *areA* insertional inactivation. Additionally in ADPW63, with *lacZ* in *areC*, thus blocking transcription of all three genes, β-galactosidase was induced by all three aromatic metabolites, indicating nonspecificity in the control of expression. In addition, a variety of esters with modifications in the aromatic alcohol moiety (2- and 4-hydroxybenzyl acetates) and in the alkanoic acid moieties (benzyl propionate and butyrate), all of which act as substrates, also induce β-galactosidase in both ADPW61 and -63.

TABLE 3. Induction by benzyl acetate and its metabolites of β-galactosidase activity expressed from *are*::*lacZ* chromosomal fusions

Inducer[b]	β-Galactosidase activity[a] (Miller units)	
	ADPW61 (*areA*::*lacZ*-Km)	ADPW63 (*areC*::*lacZ*-Km)
None	44 (±20)	660 (±220)
Benzaldehyde	1,050 (±145)	9,200 (±2,200)
Benzyl alcohol	950 (±125)	8,450 (±1,900)
Benzyl acetate	1,000 (±150)	8,200 (±1,600)
Benzyl propionate	730 (±65)	4,150 (±700)
Benzyl butyrate	380 (±50)	2,100 (±350)
2-Hydroxybenzyl acetate	1,000 (±50)	8,040 (±820)
4-Hydroxybenzyl acetate	1,100 (±200)	8,400 (±1,000)

[a] All values are the average of three independent duplicated experiments. Figures in parentheses are the standard deviations of the means.
[b] Inducers were added at 1 mM.

The regulator protein for *areCBA* appears to be AreR. Upstream, 74 bp from the −12, −24 promoter is an 18-bp inverted repeat with only two mismatches. Each half of the inverted repeat contains two direct repeats of 7 bp (Fig. 4), which could provide the binding site for a regulator protein, since an inverted repeat sequence has also been postulated to act as the regulator binding site in the related DmpR system (22). It could also serve as a transcription terminator for *areR*, since it is located only 10 bp downstream of its termination codon.

The sequence of *areR* has all the characteristics of the σ^{54}-dependent NtrC/XylR family of regulatory proteins (16, 18, 21). The two most homologous proteins in the databanks are the AcoR proteins from *Clostridium magnum* (13) and *Pseudomonas putida* (GenBank accession no. CAA72019). Alignment of amino acid sequences of AreR with sequences of these proteins (data not shown), as well as with the two related regulators of aromatic catabolism in *Pseudomonas*, DmpR (24) and XylR (8), shows the three domains characteristic of the bacterial enhancer binding protein family (16, 18). The amino-terminal A domain, which is responsive to the inducers (23), is the least conserved and the most variable in length, but the sequence from residues 159 and 174 of AreR (PVFNG QGKILGALDIT) is completely conserved between AreR and both AcoR proteins. The central C domain, carrying the ATPase activity, is well conserved between the five aligned sequences, and the lengths of the C domains of the genes are very similar.

In addition to the sequence analysis implicating AreR as the regulator of *areCBA*, we have confirmed its physiological role by insertion of a Km cassette into *areR*. This not only drasti-

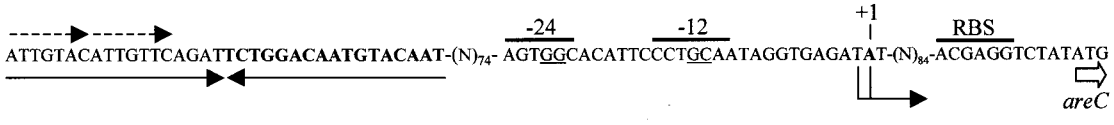

FIG. 4. Regulatory sequences upstream of *areC*. The start codon of *areC* is indicated, and the putative ribosome binding site (Shine-Dalgarno) of *areC* is indicated above the sequence (RBS). The nucleotides corresponding to both primer extension signals (Fig. 3) are indicated by the double-headed arrow with the strongest numbered as the +1 site. The putative −12, −24 promoter elements are indicated. Arrows below the sequence mark an inverted repeat that is hypothesized to act as the binding site for the regulator protein. Dashed arrows above the sequence mark a direct repeat.

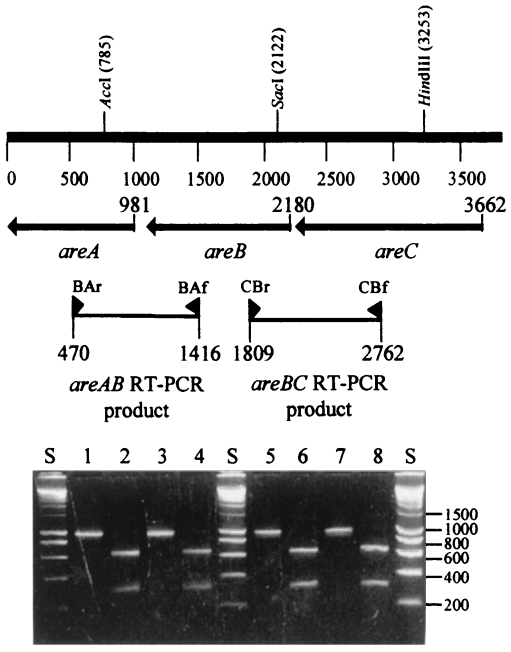

FIG. 5. Agarose gel electrophoresis of RT-PCR products amplified by primers from ADP1 grown on benzyl acetate and benzyl alcohol. The positions of the primers for spanning the *areAB* intergenic region (BAr, BAf) and the *areCB* intragenic region (CBr, CBf) are shown relative to the gene organization of *areCBA*. The values for molecular size markers (in base pairs) in lanes S (HyperLadder I; Bioline, London, United Kingdom) are indicated on the right side of the gel. Lanes: 1, *areCB*, benzyl acetate-grown cells (expected size, 953 bp); 2, *areCB*, benzyl acetate-grown cells digested with *Sac*I (640 and 313 bp); 3, *areCB*, benzyl alcohol-grown cells (expected size, 953 bp); 4, *areCB*, benzyl alcohol-grown cells digested with *Sac*I (640 and 313 bp); 5, *areBA*, benzyl acetate-grown cells (expected size, 946 bp); 6, *areBA*, benzyl acetate-grown cells digested with *Acc*I (631 and 315 bp); 7, *areBA*, benzyl alcohol-grown cells (expected size, 946 bp); 8, *areBA*, benzyl alcohol-grown cells digested with *Acc*I (631 and 315 bp). No detectable products were obtained in control reactions with each pair of primers, from which RT had been omitted, or in reactions carried out on succinate-grown cells (data not shown).

cally reduces the growth rate on the *are* substrates but also knocks out the ability of benzyl alcohol to induce all three Are protein activities (Table 2). Induction of the Are enzymes is also much reduced in an *rpoN* mutant of ADP1 lacking the σ^{54} subunit of RNA polymerase, completing the circle of evidence implicating the regulatory protein, promoter, and sigma factor in the induction of *areCBA*.

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