JOURNAL OF BACTERIOLOGY, Oct. 1989, p. 5542–5550
0021-9193/89/105542-09$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 171, No. 10

Cloning, Sequencing, and Expression of the Gene for NADH-Sensitive Citrate Synthase of *Pseudomonas aeruginosa*

LYNDA J. DONALD, GILLES F. MOLGAT, AND HARRY W. DUCKWORTH*
Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Received 13 March 1989/Accepted 27 June 1989

The structural gene for the allosteric citrate synthase of *Pseudomonas aeruginosa* has been cloned from a genomic library by using the *Escherichia coli* citrate synthase gene as a hybridization probe under conditions of reduced stringency. Subcloning of portions of the original 10-kilobase-pair (kbp) clone led to isolation of the structural gene, with its promoter, within a 2,083-bp length of DNA flanked by sites for *Kpn*I and *Bam*HI. The nucleotide sequence of this fragment is presented; the inferred amino acid sequence was 70 and 76% identical, respectively, with the citrate synthase sequences from *E. coli* and *Acinetobacter anitratum*, two other gram-negative bacteria. DEAE-cellulose chromatography of *P. aeruginosa* citrate synthase from an *E. coli* host harboring the cloned *P. aeruginosa* gene gave three peaks of activity. All three enzyme peaks had subunit molecular weights of 48,000; the proteins were identical by immunological criteria and very similar in kinetics of substrate saturation and NADH inhibition. Because the cloned gene contained only one open reading frame large enough to encode a polypeptide of such a size, the three peaks must represent different forms of the same protein. A portion of the cloned *P. aeruginosa* gene was used as a hybridization probe under stringent conditions to identify highly homologous sequences in genomic DNA of a second strain classified as *P. aeruginosa* and isolates of *P. putida*, *P. stutzeri*, and *P. alcaligenes*. When crude extracts of each of these four isolates were mixed with antiserum raised against purified *P. aeruginosa* citrate synthase, however, only the *P. alcaligenes* extract cross-reacted.

The citrate synthases of gram-negative bacteria are allosteric enzymes, whose activity is inhibited strongly and specifically by NADH (35). *Escherichia coli* and *Acinetobacter anitratum* citrate synthases are strongly homologous in amino acid sequence and more distantly resemble the nonallosteric citrate synthase of eucaryotes (2, 8, 24). *Pseudomonas* citrate synthases are also allosteric, and studies of partially purified preparations suggest that they have kinetic properties intermediate between those of the *E. coli* and *A. anitratum* enzymes (11, 14, 15, 20). Until now, no one has succeeded in obtaining a pure sample of a *Pseudomonas* citrate synthase which is still inhibited by NADH, perhaps because the enzyme is sensitive to attack by proteases present in crude extracts of this organism. The picture has been further complicated by the demonstration that two peaks of citrate synthase activity are obtained when crude extracts of various pseudomonads are chromatographed; it has been argued that these peaks represent two distinct citrate synthases, one allosteric and the other not (22, 31).

In this paper we report the molecular cloning and DNA sequencing of the structural gene for the NADH-sensitive citrate synthase of an isolate of *Pseudomonas aeruginosa*. A plasmid subclone, harbored in an *E. coli* host which itself lacks citrate synthase protein, produces moderate amounts of *Pseudomonas* enzyme, which upon purification retains much of its sensitivity to NADH. Unexpectedly, three peaks of enzyme activity were obtained from cation-exchange chromatography, which were similar in kinetic properties and must all have arisen from the same cloned gene.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. All cloning experiments were performed with *P. aeruginosa* UM74 from the Culture Collection of the Microbiology Department, University of Manitoba; this is an isolate of strain ATCC 7700. DNA was also prepared from a second strain of *P. aeruginosa* (UM211 [ATCC 13525]) and from *P. putida* (UM76 [ATCC 12633]), *P. stutzeri* (UM430 [NCTC 10475]), and *P. alcaligenes* (UM127 [ATCC 12815]). The organisms were grown aerobically in LB medium (19), and genomic DNA was prepared from cells (28). Plasmid clones containing citrate synthase genes were grown in *E. coli* MOB154, which is a glutamate auxotroph because it lacks citrate synthase (7).

Library construction and screening. A partial *Sau*3AI digest of DNA from *P. aeruginosa* UM74 was fractionated on a 10 to 40% sucrose density gradient (19), and fractions of 15 to 20 kilobase pairs (kbp) in size were selected. Lambda phage vector EMBL3 DNA was prepared from phage lysates (6) and digested first with *Bam*HI and then with *Eco*RI (18). The size-fractionated *Sau*3AI fragments were ligated with this, packaged by using a kit (Amersham), and plated on LB agar. Plaques were screened by blotting phage DNA onto Plaque-Lift (NEN DuPont) and probing with the nick-translated pHS*gltA* or its smaller *Bam*HI-*Sal*I fragment, which contains the entire coding region of the *E. coli* citrate synthase structural gene (10). Hybridizations were carried out at 55°C with 6× SSC (19) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) because Southern blotting experiments had shown that a hybridizing band of *P. aeruginosa* genomic DNA may be detected under these conditions. Positive plaques were purified by replating and rescreening, and one clone, designated λP4-2, was selected for further study. DNA from plaques was then prepared (16).

Isolation of the citrate synthase gene. The restriction map of λP4-2 is shown in Fig. 1. The cloned DNA contained many *Sal*I sites; two *Sal*I restriction fragments, of about 950 and 360 bp, hybridized with the *gltA* DNA under conditions of reduced stringency. Further Southern blotting experiments showed that this hybridizing region was contained in a

* Corresponding author.

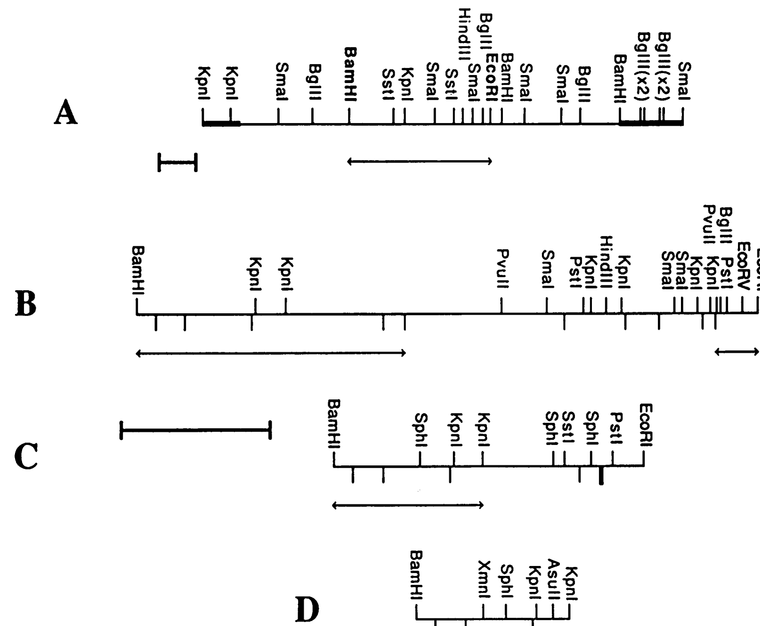

FIG. 1. Restriction maps of *P. aeruginosa* clone λP4-2 and of plasmids pSS2, pS34, and pPKB derived from it. Restriction sites are shown only if they have been verified by restriction mapping in the particular clone (and sequencing, in the case of pPKB). (A) Cloned portion of λP4-2, with flanking regions (heavy lines) as far as sites for *Kpn*I (in the λ left arm) and *Sma*I (in the λ right arm). The heavy horizontal bar just to the left and below this map represents 2 kbp, and the double-headed arrow indicates the portion subcloned into pSS2. No attempt was made to map the many *Sal*I restriction sites in this clone. (B) pSS2; the heavy horizontal bar below to the left represents 2 kbp, while the double-headed arrows show the two portions subcloned into pS34 after partial digestion with *Sal*I. The unlabeled lines drawn below the map are sites for *Sal*I. (C) pS34, on the same scale and with the same conventions as in panel B; the double-headed arrow shows the portion subcloned to make pPKB, and the heavy vertical line near the right of the map marks the point where two noncontiguous *Sal*I fragments were ligated in making the clone. (D) pPKB, on the same scale and with the same conventions as in panel C.

*Bam*HI-*Eco*RI fragment of 8.0 kbp, which was subcloned into pBR322 to produce plasmid pSS2 (Fig. 1). The transformation host for all subcloning experiments was *E. coli* MOB154 (7), and selection was on defined medium lacking glutamate, on which MOB154 cannot grow. Plasmid pSS2 still contained many *Sal*I sites, making the construction of an accurate restriction map for this enzyme difficult. In order to isolate the citrate synthase gene with flanking regions that were as short as possible, we constructed two further plasmids. The first, pS34, was created from a partial *Sal*I digest of pSS2, which was then religated and selected on minimal plates in transformed MOB154. Although pS34 was the smallest plasmid obtained from this experiment, it still contained six *Sal*I fragments, including two (of 950 and 360 bp) which hybridized under stringent conditions (65°C, 6× SSC) with Southern blots of *P. aeruginosa* genomic DNA.

The second plasmid was constructed after preliminary sequencing had located the citrate synthase coding region. First, it was shown that the 363-bp *Kpn*I fragment of pS34 contains the entire promoter region of the gene by cloning it (after blunt-ending with the Klenow fragment of DNA polymerase I) into the *Sma*I site of plasmid pKK232-8, which contains a promoterless gene for chloramphenicol transacetylase (4). Some of the resulting plasmids permitted growth of *E. coli* HB101 on as much as 60 μg of chloramphenicol per ml, indicating the presence of a complete promoter sequence in the small *Kpn*I fragment. A plasmid containing only this 363-bp *Kpn*I fragment plus the adjacent *Kpn*I-*Bam*HI fragment was then constructed as follows. First, the 1.8-kbp *Kpn*I-*Bam*HI fragment of pS34 was cloned into the *Kpn*I and *Bam*HI sites in the polylinker region of phage M13mp18. The double-stranded form of this clone was then digested with *Eco*RI and *Bam*HI, giving the original *Kpn*I-*Bam*HI fragment with a portion of the M13mp18 polylinker, containing sites for *Sst*I and *Eco*RI, at the *Kpn*I end. The resulting *Eco*RI-*Bam*HI fragment was then cloned into *Eco*RI-*Bam*HI-digested pBR322, giving the plasmid pSKB1, which does not allow growth of MOB154 on minimal plates lacking glutamate. Finally, pS34 was digested with *Kpn*I, treated with alkaline phosphatase, and ligated with *Kpn*I-digested pSKB1, and the MOB154 transformants were selected for ability to grow on minimal medium without glutamate. One plasmid from this selection procedure, pPKB, contained the entire citrate synthase coding region plus its promoter as a 2,087-bp *Kpn*I-*Bam*HI insert. The restriction map of pPKB is shown in Fig. 1.

DNA sequencing. Sequencing was performed by the method of Sanger and co-workers (29), with M13-based sequencing vectors (25). Because *P. aeruginosa* DNA is so rich in G · C pairs, some regions of the DNA to be sequenced showed severe "band compression" on autoradiograms. To avoid this, we conducted all primer extension reactions at 50°C and used 7-deaza-dGTP (Pharmacia) in place of dGTP when compression had been encountered in a preliminary run. Most sequencing was performed with shotgun libraries of *Sau*3AI, *Rsa*I, and *Sal*I prepared in M13mp18 plus some specific fragments (data not shown). Both strands of the cloned portion of pPKB were fully sequenced, with overlaps of all restriction sites. Where necessary, clones were identified by hybridization of plaques with the smaller *Bam*HI-*Kpn*I restriction fragment of pS34 in 6× SSC at 65°C.

Enzyme purification. Methods for assaying and purifying citrate synthase have been described (8, 10). Briefly, *E. coli*

TABLE 1. Purification details for *Pseudomonas* citrate synthase from pPKB[a]

Fraction	Protein (mg)	Activity (U)	Sp act (U/mg)	Recovery (%)
Crude extract	7,480	817	0.11	(100)
DEAE-cellulose				
Peak A	235	342	1.46	42
Peak B	217	117	0.54	14
Peak C	223	92	0.41	11
Sephadex G-200				
Peak A	43	114	2.67	14
Peak B	45	41	0.91	5.0
Peak C	32	18	0.57	2.2

[a] The course of the purification from 50.7 g of cells, wet weight, is shown. Activities in the presence of 0.1 M KCl were 4.1 ± 0.5 times those shown here.

MOB154 containing pS34 or pPKB was grown in 1-liter batches in LB medium for 18 to 24 h, and the cells were harvested and broken by passage through a French press. The resulting suspension was clarified by centrifugation, and the supernatant was loaded on a column of Whatman DE-52 cation exchanger, which was eluted by increasing KCl concentration as described previously (8). Three peaks of citrate synthase activity were obtained, which were pooled separately, concentrated, and further purified by gel filtration through Sephadex G200, again as described previously (8). The course of a typical purification experiment is shown in Table 1.

Routine procedures. Restriction and DNA-modifying enzymes were obtained from Boehringer Mannheim Canada, Pharmacia, Gibco-BRL, and New England BioLabs. Restriction digests, nick translations, Southern blots, and ligations were performed as described before (19). Antiserum to citrate synthase peak A (see Results) was raised in rabbits as described previously (30). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and automated Edman degradation, were done as described before (10). Steady-state kinetic data were subjected to nonlinear least squares analysis as described before (1).

RESULTS

Nucleotide sequence of the *P. aeruginosa* citrate synthase gene. The nucleotide sequence of the cloned region of plasmid pPKB is shown in Fig. 2, together with the amino acid sequence inferred from the single long open reading frame within this sequence. As noted above, the DNA sequence was completely determined on both strands. The 950-bp *Sal*I fragment originally noted as hybridizing with the *E. coli* citrate synthase gene in Southern blots was actually 942 bp long and was bounded by *Sal*I sites beginning at base numbers 443 and 1383 in Fig. 2; this fragment lay entirely within the coding region of the cloned gene. The reading frame consisted of 428 codons, including the initiator methionine; as will be shown below, this methionine is not present in the mature protein, as isolated either from *P. aeruginosa* or from *E. coli*. The amino acid sequence of the protein specified by the cloned gene had a high homology throughout with the citrate synthases of other gram-negative bacteria (see below).

At the 5′ end of the gene, there was a typical ribosome attachment site, AGGAGG, located 5 bases from the initiator codon. Potential promoter sites resembling the *E. coli* consensus sequence TATAAT (underlined in Fig. 2) occurred at bases 74 to 79 (TATAGA), 140 to 145 (TATAAA), and 238 to 243 (TATAGT), but in the absence of a direct determination of the transcription start site, it is not possible to determine which sequence, if any, forms part of the natural promoter. Nineteen bases beyond the 3′ end of the open reading frame (at base 1607) began a hyphenated dyad sequence of 22 bases, similar to an *E. coli* rho-independent terminator; this is underlined in Fig. 2. The free energy of stabilization of this dyad structure, calculated as described before (33), was 19.0 kcal/mol at 25°C. A second apparent hyphenated dyad, which began with the cytosine at position 1656 in Fig. 2, had a calculated free energy of stabilization of no more than 3.0 kcal/mol, and we do not regard this as significant. Although the regulatory importance of these sequences in *P. aeruginosa* cannot be established without direct studies on the transcript synthesized in that organism, structural features of this kind have been found in other cloned pseudomonad genes (see, for instance, references 17, 21, 34, and 38), and their existence in this particular gene probably explains why it is expressed in *E. coli* (see below).

The codon usage within the *P. aeruginosa* citrate synthase reading frame is shown in Table 2. Codon choices were quite different from those in *E. coli* (12, 13) but consistent with the 67.2% G+C content of *P. aeruginosa* DNA (26). The codon differences evidently do not prevent the gene from being expressed moderately well in an *E. coli* host (see below), although the expression of the gene was noticeably less than for the *E. coli* citrate synthase gene (10) or for *Acinetobacter* citrate synthase, whose codon preferences are similar to those normally found for strongly expressed *E. coli* genes (8). From the data in Table 2, it may be calculated that, where a choice between A and T on one hand and G and C on the other is possible, G or C is chosen 88% of the time—this bias was evident for all amino acids except glutamic acid, for which the two codons, GAA and GAC, were used equally (Table 2). The same lack of bias in use of glutamic acid codons has been noted in several other *Pseudomonas* genes (34, and references therein). The overall G+C content of the DNA sequence shown in Fig. 2 was 60%.

Purification of *P. aeruginosa* citrate synthase from MOB154 (pPKB). Although partially purified samples of citrate synthases have been prepared from various pseudomonads in the past and used for functional studies on the enzyme (11, 14, 15, 20), no completely pure sample of the enzyme has yet been reported. Previous experience in our laboratory was that the enzyme could be purified to homogeneity only with the loss of sensitivity to NADH (V. Bhayana, Ph.D. thesis, University of Manitoba, 1983). By starting with a crude extract of *E. coli* MOB154 harboring pPKB or pS34, however, we have now found that the enzyme can be purified to

FIG. 2. Nucleotide sequence of the cloned portion of pPKB, and inferred amino acid sequence of *P. aeruginosa* citrate synthase. The amino acid sequence specified by the single long open reading frame is given, with the standard one-letter abbreviations, above the nucleotide sequence. Certain restriction sites are labeled below the sequence. Underlined sequences are three potential TATAA sequences; the presumed ribosome-binding site AGGAGG; and a hyphenated dyad sequence which resembles a rho-independent terminator. For further description, see the text.

```
                                                                            GGTACCCAT CACCAGGGAG   19
                                                                             KpnI
TGGCGACCGT CTATGAAAGC GGGCCAGGAA TACCCCTTGC GGTCGGACGC CGCGTATAGA CAGTTAGGCT ACTAATGACA   99

ACGAAAACAC CCATCGCCAA ATGGTTGATT GCGGGCCTTT TATAAAAGGC GTAAATAGCC CCCTTTTTCT GCGAAAACCC  179

CGCAACGAAA GGCTCTACGG CATGGATTCG CGCAAATTGA CTTTCGAATT TATCCCTCTA TAGTGGTGCG GGCCCTGCGT  259
                                             AsuII
                                            M   A   D   K   K   A   Q   L   I   I   E
GGGGGGTACT GATGATTTCA AGCATAAATA GGAGGCCATC ATG GCT GAC AAA AAA GCG CAG TTG ATC ATC GAG  332

 G   S   A   P   V   E   L   P   V   L   S   G   T   M   G   P   D   V   V   D   V   R
GGC TCA GCC CCC GTC GAA CTG CCC GTC CTA TCC GGT ACC ATG GGT CCC GAT GTA GTG GAT GTA CGG  398
                                             KpnI
 G   L   T   A   T   G   H   F   T   F   D   P   G   F   M   S   T   A   S   C   E   S
GGC CTC ACC GCC ACG GGC CAC TTC ACC TTC GAT CCT GGC TTC ATG TCG ACC GCC TCC TGC GAG TCG  464
                                                         SalI
 K   I   T   Y   I   D   G   D   K   G   V   L   L   H   R   G   Y   P   I   E   Q   L
AAG ATC ACC TAT ATC GAC GGC GAC AAA GGC GTC CTC CTC CAT CGC GGC TAC CCC ATC GAG CAA CTG  520

 A   E   K   S   D   Y   L   E   T   C   Y   L   L   L   N   G   E   L   P   T   A   A
GCG GAG AAA TCC GAC TAC CTG GAA ACC TGC TAC CTG CTG CTG AAC GGC GAG CTG CCC ACC GCC GCG  586

 Q   K   E   Q   F   V   G   T   I   K   N   H   T   M   V   H   E   Q   L   K   T   F
CAG AAG GAA CAG TTC GTC GGC ACC ATC AAG AAC CAC ACC ATG GTT CAC GAG CAG TTG AAG ACC TTC  652
                                                                         XmnI
 F   N   G   F   R   R   D   A   H   P   M   A   V   M   C   G   V   I   G   A   L   S
TTC AAC GGC TTC CGC CGC GAC GCC CAT CCG ATG GCC GTG ATG TGC GGC GTG ATC GGC GCC CTC TCG  718

 A   F   Y   H   D   S   L   D   I   T   N   P   K   H   R   E   V   S   A   H   R   L
GCC TTC TAC CAC GAC TCC CTG GAC ATC ACT AAC CCG AAG CAT CGC GAA GTC TCC GCG CAT CGC CTG  784

 I   A   K   M   P   T   I   A   A   M   V   Y   K   Y   S   K   G   E   P   M   M   Y
ATC GCC AAG ATG CCG ACC ATC GCC GCC ATG GTG TAC AAG TAC TCC AAG GGC GAG CCG ATG ATG TAT  860

 P   R   N   D   L   N   Y   A   E   N   F   L   H   M   M   F   N   T   P   C   E   T
CCG CGT AAC GAC CTG AAC TAC GCG GAA AAC TTC CTG CAC ATG ATG TTC AAC ACC CCC TGC GAG ACC  926

 K   P   I   S   P   V   L   A   K   A   M   D   R   I   F   I   L   H   A   D   H   E
AAG CCG ATC AGC CCC GTG CTG GCC AAG GCC ATG GAC CGC ATC TTC ATT CTC CAC GCC GAC CAC GAG  992

 Q   N   A   S   T   S   T   V   R   L   A   G   S   S   G   A   N   P   F   A   C   I
CAG AAC GCC TCC ACC TCC ACG GTG CGC CTG GCC GGC TCC TCC GGC GCC AAT CCG TTC GCC TGC ATC 1058

 A   S   G   I   A   A   L   W   G   P   A   H   G   G   A   N   E   A   V   L   R   M
GCC TCC GGC ATC GCC GCC CTG TGG GGA CCG GCC CAT GGG GGC GCG AAC GAA GCG GTG CTG CGC ATG 1124
                                                                             SphI
 L   D   E   I   G   D   V   S   N   I   D   K   F   V   E   K   A   K   D   K   N   D
CTC GAC GAG ATC GGC GAC GTG TCC AAC ATC GAC AAG TTC GTC GAG AAG GCC AAG GAC AAG AAC GAT 1190

 P   F   K   L   M   G   F   G   H   R   V   Y   K   N   F   D   P   R   A   K   V   M
CCG TTC AAG CTG ATG GGC TTC GGC CAT CGT GTC TAC AAG AAC TTC GAC CCG CGC GCC AAG GTC ATG 1256

 K   Q   T   C   D   E   V   L   Q   E   L   G   I   N   D   P   Q   L   E   L   A   M
AAG CAG ACC TGC GAC GAG GTC CTC CAG GAG CTG GGC ATC AAC GAC CCG CAA CTG GAA CTG GCG ATG 1322

 K   L   E   E   I   A   R   H   D   P   Y   F   V   E   R   N   L   Y   P   N   V   D
AAG CTG GAA GAA ATC GCC CGC CAC GAC CCC TAC TTC GTG GAA CGC AAC CTG TAC CCG AAC GTC GAC 1388
                                                                             SalI
 F   Y   S   G   I   I   L   K   A   I   G   I   P   T   S   M   F   T   V   I   F   A
TTC TAC TCG GGG ATC ATC CTC AAG GCG ATC GGC ATT CCG ACC AGC ATG TTC ACC GTG ATC TTC GCC 1454

 L   A   R   T   V   G   W   I   S   H   W   Q   E   M   L   S   G   P   Y   K   I   G
CTG GCG CGT ACC GTC GGC TGG ATC TCG CAC TGG CAG GAA ATG CTC TCC GGC CCC TAC AAG ATC GGC 1520

 R   P   R   Q   L   Y   T   G   H   T   Q   R   D   F   T   A   L   K   D   R   G   *
CGC CCG CGC CAG CTC TAT ACC GGC CAC ACC CAG CGC GAC TTC ACC GCC CTC AAG GAT CGC GGC TGA 1586

AACCCGTCAC GCCTGGCGAG AAAGGCTGCT TCGGCAGCCT TTTTTGTGCC CGCCCCTTTC CTCCGGACAC AAAAAAACGC 1666

CCCGGAGGCG GTCTTTTTGC AACGAAGGCG CGCTCAGCGC TTCTCGGCGG CGTACGCAGG CCCTTGAGGG TGTTCAGCGG 1746

CGCGTCGACC CACGAAGCTG TTGGCCAACC AGGAAGGAAT GCTGCCACCC GGCTCGGTTT CCACCTGGTA GGTCACTTCC 1826
   SalI
GTCAGGCCCT GCCCCTTCGG CTGCAACTTC CACTCGCCGA CCAGCTTGGG CACGCGGATC TGGCCCTTTT CCTCGGGGGA 1906

TATAGGTCGG ATCGGCCTTC AGGTGACGGG TCACGGTGCC ATCCGCGGTC TTCTCGGTGG TCACATGGAT GACCACGTCG 1986

CGACCGGTGA CCGGCCAGGG CAGTGTCGAT CTTCGAGTAG GTCCAGCGTC GGCGCCTTCC TGCTTCAGGA GTTTCATCTC 2066

GGCGCAGGCA TGGATCC                                                                     2083
      BamHI
```

TABLE 2. Codon usage in *P. aeruginosa* citrate synthase gene[a]

Amino acid	Codon	No. of times used	Amino acid	Codon	No. of times used	Amino acid	Codon	No. of times used	Amino acid	Codon	No. of times used
F	TTT	0	S	TCT	0	Y	TAT	3	C	TGT	0
	TTC	21		TCC	13		TAC	12		TGC	6
L	TTA	0		TTA	1	Term[b]	TAA	0	Term	TGA	1
	TTG	2		TCG	5		TAG	0	W	TGG	3
	CTT	0	P	CCT	1	H	CAT	6	R	CGT	3
	CTC	11		CCC	9		CAC	10		CGC	15
	CTA	1		CCA	0	Q	CAA	2		CGA	0
	CTG	22		CCG	14		CAG	10		CGG	1
I	ATT	2	T	ACT	1	N	AAT	1	S	AGT	0
	ATC	24		ACC	21		AAC	16		AGC	2
	ATA	0		ACA	0	K	AAA	4	R	AGA	0
M	ATG	19		ACG	2		AAG	22		AGG	0
V	GTT	1	A	GCT	1	D	GAT	5	G	GGT	2
	GTC	11		GCC	27		GAC	20		GGC	27
	GTA	2		GCA	0	E	GAA	11		GGA	1
	GTG	10		GCG	10		GAG	13		GGG	2

[a] The above distribution includes the initiator methionine.
[b] Term, Terminator.

homogeneity with considerable NADH sensitivity. The method employed was identical to that used for purification of the same enzyme from *E. coli* (10) and *A. anitratum* (8), and the overall recovery of activity was better than 60% (Table 1). The only significant difference between the behavior of these other citrate synthases during the purification and that of the *Pseudomonas* enzyme is that the latter separated during DEAE-cellulose chromatography into three peaks of activity (Fig. 3). Each peak was pooled separately, concentrated, and purified further by gel filtration through Sephadex G200; all three citrate synthase fractions emerged from this column at the same elution volume, suggesting that they do not differ in size or degree of association of subunits. Peaks A and B each gave one major protein band, of apparent molecular weight about 48,000, upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. There were also traces of other bands, in particular a minor component of about 47,000 M_r. Peak C contained about equal amounts of two major bands of about 48,000 and 47,000 M_r and traces, totalling less than 10%, of at least nine other bands in the molecular weight range 60,000 to 100,000.

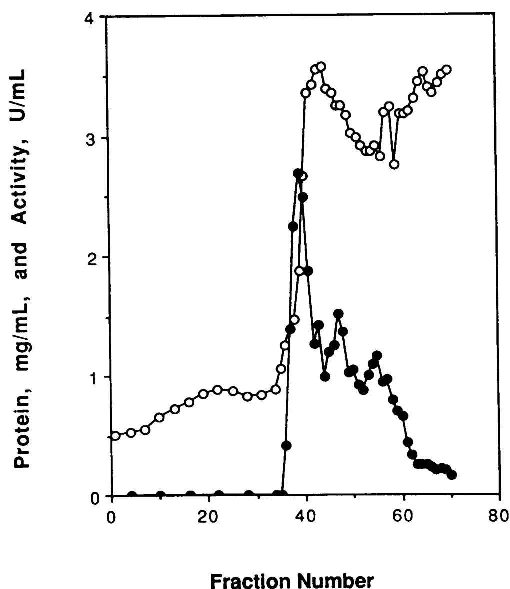

FIG. 3. Elution profile of citrate synthase from pPKB from DEAE-cellulose, showing protein (○) and citrate synthase activity (●). Fractions were pooled as follows: peak A, 36 to 43 inclusive; peak B, 45 to 50 inclusive; peak C, 53 to 60 inclusive.

None of the three citrate synthase peaks gave a single N-terminal amino acid sequence upon automatic Edman degradation. For all three peaks the sequence Ala-Asp-Lys-Lys-Ala-Gln-Leu-Ile... could be recognized, which is that predicted from the DNA sequence of the cloned gene in pPKB (Fig. 2) with the initiator methionine removed. Cycle 1 in all cases contained a significant amount of methionine, but since alanine was not especially prominent in cycle 2, we believe that this second sequence arose from a variety of contaminating proteins or peptides and not from the retention of the N-terminal methionine in a fraction of the citrate synthase molecules. Previous work on the (desensitized) citrate synthase purified directly from *P. aeruginosa* gave the N-terminal sequence expected from the DNA sequence, again with the N-terminal methionine removed (V. Bhayana, Ph.D. thesis). Thus, it appears that the initiator methionine is removed from the mature protein in both *P. aeruginosa* and *E. coli*. None of the contaminating sequences found in peaks A through C obviously arose through "fraying" of the N-terminus of *P. aeruginosa* citrate synthase, and their origins are unknown. In spite of the two major components detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in peak C, the N-terminal sequence found was similar to that of peaks A and B, which were almost pure. It is possible that both components in peak C have the same N-terminal sequence, or that one of the components was not degraded in the sequenator.

Antiserum to citrate synthase peak A was raised in rabbits, and this reacted with the protein from all three peaks, in a reaction characteristic of identity (Fig. 4). Where two precipitin lines were obtained with peaks A and B, however, peak C showed only one, which fused with the major line for the other two peaks. The antiserum used in this experiment precipitated purified peak A citrate synthase activity from solution completely and also precipitated at least 98% of all citrate synthase activity from a crude extract of *P. aerugi-*

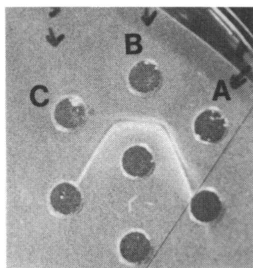

FIG. 4. Precipitation of protein from peaks A, B, and C by antiserum raised against peak A citrate synthase. The center well contained 10 μl of undiluted antiserum, and wells A, B, and C contained 15-μl portions of purified citrate synthase, 2.0 mg/ml in standard buffer, from peaks A, B, and C, respectively.

nosa UM74, from which the pPKB clone was ultimately derived (data not shown).

Kinetic properties of purified *P. aeruginosa* citrate synthase. In the absence of KCl, all three peaks of purified *Pseudomonas* citrate synthase gave hyperbolic kinetics which could be interpreted in terms of an ordered bisubstrate mechanism, with oxaloacetate binding first (1, 36). The calculated kinetic parameters for this mechanism are listed in Table 3, and little difference between the peaks was seen. KCl at 0.1 M activated the enzyme about fourfold in each case. Whereas in crude extracts *P. aeruginosa* citrate synthase was inhibited about 95% by NADH (data not shown), the three purified fractions were inhibited to a maximum of 54 to 75% (Table 3). Inhibition by 0.5 mM NADH could be reversed by 5'-AMP in all three cases, as has been described for partially purified enzyme (14, 35).

Amino acid sequence homology between citrate synthases of gram-negative bacteria. The *P. aeruginosa* citrate synthase is the third such enzyme to be sequenced from gram-negative sources, the other two being from *E. coli* (2, 24) and *A. anitratum* (8). The three sequences are aligned in Fig. 5 along with those of the enzymes from *Rickettsia prowazekii* (37) and from pig heart (3), the only citrate synthase for which a three-dimensional structure is known (27, 36). The percentages of identical residues for all possible pairs of sequences in Fig. 5, evaluated with the alignments in that figure, are listed in Table 4. As can be seen, the overall amino acid identities between the three pairs of allosteric gram-negative citrate synthases were about 70% (the highest identity was between the enzymes from *Acinetobacter* and *Pseudomonas*), the similarity extending throughout the lengths of the sequences. Active-site residues, identified in the pig heart sequence by direct crystallographic study of interaction of the enzyme with substrates and substrate analogs, were all represented by identical or similar residues in the gram-negative bacterial sequences, with the exception of pig heart Arg-46, which had no obvious equivalent among the bacteria.

Similarity of *P. aeruginosa* citrate synthase to citrate synthases of other pseudomonads. To investigate the degree of homology between the *P. aeruginosa* citrate synthase which we have cloned in pPKB and those of other pseudomonads, we prepared Southern blots of genomic DNA from a second isolate of *P. aeruginosa* and from isolates of *P. putida*, *P. stutzeri*, and *P. alcaligenes* as specified in Materials and Methods and probed them with the nick-translated 942-bp *Sal*I fragment, first at 65°C and then at 55°C. With all species tested, most restriction digests gave a single hybridizing band, whose size varied with the organism; the 942-bp *Sal*I band appeared to be present in DNA from *P. alcaligenes* and *P. stutzeri*. Examples of the hybridization patterns obtained at 65°C are shown in Fig. 6; no additional band appeared when the hybridization was repeated at 55°C. To test the antigenic relationship between the citrate synthases of these other pseudomonads and that cloned in pPKB, we prepared crude extracts from all of them and tested the ability of the antiserum to pool A *P. aeruginosa* citrate synthase to precipitate citrate synthase from them. All the extracts contained significant citrate synthase activity, but in only one case (*P. alcaligenes*) was partial precipitation obtained (data not shown). By this criterion, it therefore appears that *P. aeruginosa* UM211 contains a citrate synthase which is more distantly related to that of strain UM74 than would have been expected for bacteria classified in the same species.

DISCUSSION

Scattered information of the literature suggests that the citrate synthase of *P. aeruginosa*, while typical of a gram-negative bacterium in that it shows allosteric inhibition by NADH, has functional properties which place it in a position intermediate between the extreme types of *E. coli* and *A. anitratum* citrate synthase. The citrate synthase of *E. coli* is very sensitive to NADH, showing a hyperbolic saturation by that nucleotide, with a K_d of about 2 μM at pH 7.8; the enzyme from *A. anitratum*, on the other hand, is saturated by NADH in a sigmoid fashion, with about 150 μM being needed for half-maximal inhibition (23, and references therein). As shown in Table 3, the *P. aeruginosa* citrate synthase is intermediate between the enzymes from *E. coli* and *A. anitratum* in a number of kinetic constants: affinity for substrates, sensitivity to NADH, and ability to be activated by KCl. The degree of sequence homology found between the *P. aeruginosa* enzyme and those of the other two gram-negative bacteria is so high that a very small number of critical differences in particular amino acid side chains must account for these functional differences.

The citrate synthases of *E. coli* (5, 32) and *P. aeruginosa* (14) are both desensitized to NADH by treatment with the sulfhydryl group reagent 5,5'-dithiobis-(2-nitrobenzoic acid); *Acinetobacter* citrate synthases are unaffected by this treatment (23, 35). Recently we reported that the reactive sulf-

TABLE 3. Kinetic properties of purified *P. aeruginosa* citrate synthase[a]

Peak	K_{OAA}(μM)	K_{iOAA}(μM)	K_{AcCoA}(μM)	V_{max} (μmol/min per mg)	Activation by 0.1 M KCl (fold)	NADH inhibition		K_{act} for 5'-AMP (μM)
						K_i(μM)	Maximum (%)	
A	15 ± 3	13 ± 4	37 ± 3	10.4 ± 0.5	4.6	22	65	160
B	15 ± 3	13 ± 4	47 ± 3	14.1 ± 0.4	3.6	29	75	200
C	18 ± 5	22 ± 4	93 ± 10	18.2 ± 1.3	3.6	12	54	680

[a] All values were determined in 20 mM Tris chloride, pH 7.8, containing 1 mM EDTA. For values of the kinetic parameters K_{OAA}, K_{iOAA}, K_{AcCoA}, and V_{max}, with their errors, see reference 1. Where no experimental error is given, parameters were calculated by hand; the estimated uncertainties are no greater than 10%. Abbreviations: OAA, oxaloacetic acid; AcCoA, acetyl coenzyme A; K_{act}, $K_{activation}$ (tested with 0.5 mM NADH).

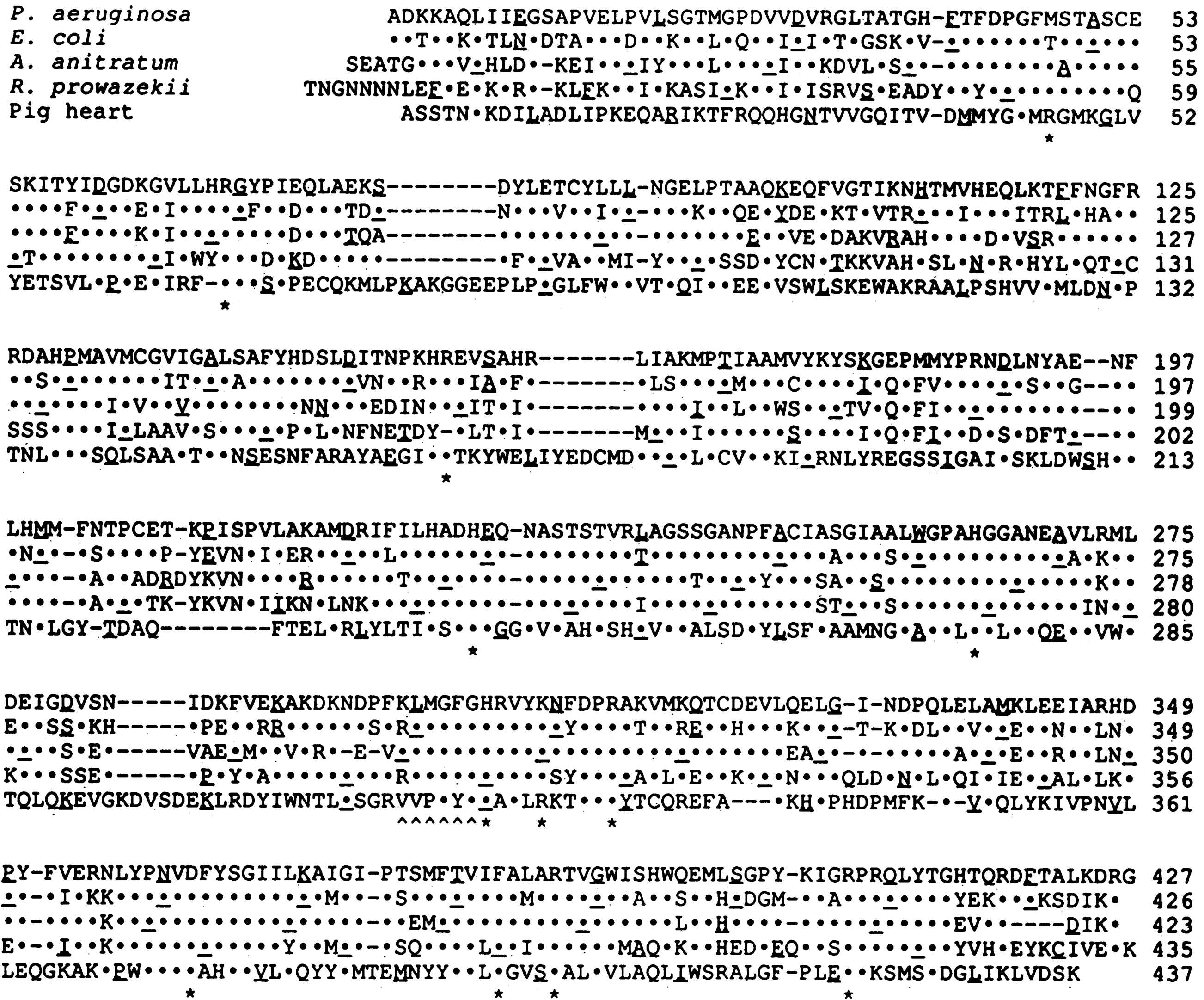

FIG. 5. Alignment of amino acid sequences for one eucaryotic and four bacterial citrate synthases. The standard one-letter abbreviations are used. Sources of the sequence data: *Pseudomonas aeruginosa* (this paper); *E. coli* (2, 24) (this sequence should be corrected by changing F-288 to V, for a reason explained in reference 1); *A. anitratum* (8); *R. prowazekii* (37); and pig heart (3). Dots indicate residues which are identical to those in the *Pseudomonas* sequence, and dashes mark deletions introduced to improve the alignment. Below the pig heart citrate synthase sequence, symbols mark residues involved in substrate binding (36), both side-chain (*) and main-chain (^) interactions. Every 10th residue is underlined, and the number of the last residue in each line is given on the far right.

TABLE 4. Percentages of amino acid identities between citrate synthases[a]

Citrate synthase source	% Identical amino acids with citrate synthase from:				
	Pig	*E. coli*	*A. anitratum*	*P. aeruginosa*	*R. prowazekii*
Pig	(100)	24.4	23.8	22.3	21.6
E. coli		(100)	68.0	70.0	61.0
A. anitratum			(100)	75.9	60.5
P. aeruginosa				(100)	59.7
R. prowazekii					(100)

[a] Listed are percentages of those residues which are placed opposite one another in the alignment in Fig. 5 which are identical. Higher percentages of identity could be reached if individual pairs of sequences were aligned optimally. No penalty has been assigned where gaps were introduced.

hydryl group in the *E. coli* enzyme is that of Cys-206 (9). As can be seen from Fig. 6, Cys-206 in the *P. aeruginosa* citrate synthase corresponds to this and is presumably the reactive cysteine, while the *A. anitratum* sequence has alanine at the equivalent position.

Weitzman and co-workers have suggested that a number of pseudomonads may contain two citrate synthases, one NADH sensitive and one not. They found that two peaks of citrate synthase may be separated from several pseudomonad strains by various chromatographic methods and that the two enzymes are of different sizes and have different sensitivities to NADH (22, 31). In one case, the NADH-insensitive activity accumulated in stationary-phase cells (31). The two peaks of activity did not interconvert, so that they could not be different forms of a single enzyme, as had been found by Cazzulo and co-workers for the citrate synthase of a marine pseudomonad (15, 20).

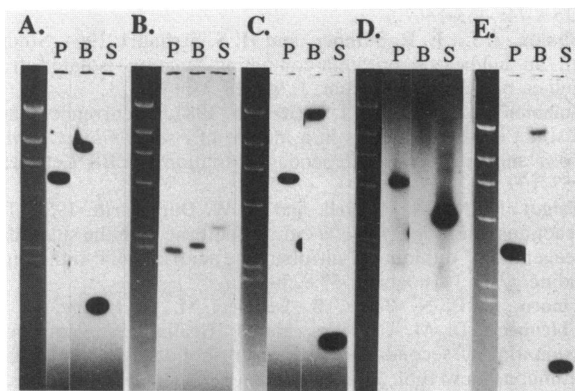

FIG. 6. Southern blots of genomic DNA from various pseudomonads, probed with DNA from the structural gene for *P. aeruginosa* citrate synthase. In each panel, the first track shows DNA size markers derived by digesting λ DNA with HindIII, and the other three are the results of hybridization with pseudomonad genomic DNA digested with *Pst*I (P), *Bam*HI (B), and *Sal*I (S). (A) *P. aeruginosa* UM 74; (B) *P. aeruginosa* UM 211; (C) *P. alcaligenes*; (D) *P. putida*; (E) *P. stutzeri* (for strain designations, see Materials and Methods).

Our results with the cloned *P. aeruginosa* gene, and with the enzyme produced from it in *E. coli*, reemphasize the point that pseudomonad citrate synthases often give more than one peak on chromatography columns. In our case, there is no question that all three peaks arise from a single gene product, since the plasmid pPKB contains only one open reading frame long enough to code for a protein of molecular weight near 48,000. Why the three peaks separate on DEAE-cellulose is still unknown; all three, however, are of the same molecular size and are sensitive to NADH. The enzyme from peak C gave a different pattern of precipitin lines on Ouchterlony plates than the other two (Fig. 4) and contained much more of the 47,000-M_r contaminant, which may be a partly proteolyzed form of the enzyme. The Michaelis constant for acetyl coenzyme A and the amount of 5'-AMP needed to reactivate enzyme inhibited by 0.5 mM NADH were also different for peak C enzyme than for the other two (Table 3). Since almost all the citrate synthase activity present in a crude extract of *P. aeruginosa* UM74 could be precipitated with antiserum raised against our purified peak A enzyme, it is unlikely that more than one activity exists in this strain.

The Southern blotting experiments with genomic DNA from other pseudomonads (Fig. 6) indicate the presence of a single citrate synthase gene in each genome, closely enough related to the *P. aeruginosa* citrate synthase gene to permit strong hybridization under stringent conditions. Other citrate synthase genes, much less homologous, may also exist in these organisms. Since antiserum raised against peak A from *P. aeruginosa* UM74 did not precipitate significant citrate synthase activity from extracts of any of these strains, however, the homology detected by hybridization at the level of the gene does not extend to the major antigenic determinants on the proteins specified by those genes.

ACKNOWLEDGMENTS

We thank Mary Lynn Duckworth for instruction in λEMBL3 cloning; James C. Jamieson for preparing the antiserum to peak A *P. aeruginosa* citrate synthase; Neil Holliday for reading this manuscript; and the Microbiology Department, University of Manitoba, for unrestricted access to the Sharples continuous-flow centrifuge, the French press, and other equipment. The original λEMBL3 library of *P. aeruginosa* genomic DNA was constructed by Aimee Ryan, and the plasmid subclone from λP4-2, pSS2, was made by Shelley Stopera.

This work was supported by the Natural Sciences and Engineering Research Council, Canada, and by the University of Manitoba Research Board.

LITERATURE CITED

1. **Anderson, D. H., and H. W. Duckworth.** 1988. *In vitro* mutagenesis of *Escherichia coli* citrate synthase to clarify the locations of ligand binding sites. J. Biol. Chem. **263:**2163–2169.
2. **Bhayana, V., and H. W. Duckworth.** 1984. Amino acid sequence of *Escherichia coli* citrate synthase. Biochemistry **23:**2900–2905.
3. **Bloxham, D. P., D. C. Parmelee, S. Kumar, K. A. Walsh, and K. Titani.** 1982. Complete amino acid sequence of porcine heart citrate synthase. Biochemistry **21:**2028–2036.
4. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27:**151–160.
5. **Danson, M. J., and P. D. J. Weitzman.** 1973. Functional groups in the activity and regulation of *Escherichia coli* citrate synthase. Biochem. J. **135:**513–524.
6. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Donald, L. J., and H. W. Duckworth.** 1986. Molecular cloning of the structural gene for *Acinetobacter* citrate synthase. Biochem. Biophys. Res. Commun. **141:**797–803.
8. **Donald, L. J., and H. W. Duckworth.** 1987. Expression and base sequence of the citrate synthase gene of *Acinetobacter anitratum*. Biochem. Cell Biol. **65:**930–938.
9. **Duckworth, H. W., D. H. Anderson, A. W. Bell, L. J. Donald, A. L. Chu, and G. D. Brayer.** 1987. Structural basis for regulation in Gram-negative bacterial citrate synthases. Biochem. Soc. Symp. **54:**83–92.
10. **Duckworth, H. W., and A. W. Bell.** 1982. Large-scale production of citrate synthase from a cloned gene. Can. J. Biochem. **60:**1143–1147.
11. **Flechtner, V. R., and R. S. Hanson.** 1970. Regulation of the tricarboxylic acid cycle in bacteria. A comparison of citrate synthase from different bacteria. Biochim. Biophys. Acta **222:**253–264.
12. **Grantham, R., C. Gautier, and M. Guoy.** 1980. Codon frequencies in 119 individual genes confirm consistent choices of degenerate bases according to genome type. Nucleic Acids Res. **8:**4309–4323.
13. **Grosjean, H., and W. Fiers.** 1982. Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. Gene **18:**199–209.
14. **Harford, S., and P. D. J. Weitzman.** 1975. Evidence for isosteric and allosteric nucleotide inhibition of citrate synthase from multiple-inhibition studies. Biochem. J. **151:**455–458.
15. **Higa, A. I., E. Massarini, and J. J. Cazzulo.** 1978. Purification and some properties of the citrate synthase from a marine *Pseudomonas*. Can. J. Microbiol. **24:**215–221.
16. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Constructing and screening cDNA libraries in λgt10 and λgt11, p. 49–78. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
17. **Inouye, S., Y. Asai, A. Nakazawa, and T. Nakazawa.** 1986. Nucleotide sequence of a DNA segment promoting transcription in *Pseudomonas putida*. J. Bacteriol. **166:**739–745.
18. **Kaiser, K., and N. E. Murray.** 1985. The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries, p. 1–47. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Massarini, E., and J. J. Cazzulo.** 1975. Two forms of citrate synthase in a marine pseudomonad. FEBS Lett. **57:**134–138.

21. **Mermod, N., P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1984. Transcription of the TOL plasmid catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. EMBO J. 3:2461–2466.
22. **Mitchell, C. G., and P. D. J. Weitzman.** 1986. Molecular size diversity of citrate synthases from *Pseudomonas* species. J. Gen. Microbiol. **132**:737–742.
23. **Morse, D., and H. W. Duckworth.** 1980. A comparison of the citrate synthases of *Escherichia coli* and *Acinetobacter anitratum*. Can. J. Biochem. **58**:696–706.
24. **Ner, S. S., V. Bhayana, A. W. Bell, I. G. Giles, H. W. Duckworth, and D. P. Bloxham.** 1983. Complete sequence of the *gltA* gene encoding citrate synthase in *Escherichia coli*. Biochemistry **22**:5243–5249.
25. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26**:101–106.
26. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1894, 237[AL] (nom. cons. Opin. 5, Jud. Comm. 1952, 237) p. 141–199. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol 1. The Williams & Wilkins Co., Baltimore.
27. **Remington, S., G. Wiegand, and R. Huber.** 1982. Crystallographic refinement and atomic models of two different forms of citrate synthase at 2.7 and 1.7Å resolution. J. Mol. Biol. **158**:111–152.
28. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Simkin, J. L., E. R. Skinner, and H. S. Seshadri.** 1964. Studies on an acidic glycoprotein-containing fraction isolated from guinea-pig serum. Biochem. J. **90**:316–330.
31. **Solomon, M., and P. D. J. Weitzman.** 1983. Occurrence of two distinct citrate synthases in a mutant of *Pseudomonas aeruginosa* and their growth-dependent variation. FEBS Lett. **155**: 157–160.
32. **Talgoy, M. M., A. W. Bell, and H. W. Duckworth.** 1979. The reactions of *Escherichia coli* citrate synthase with the sulfhydryl reagents 5,5′-dithiobis-(2-nitrobenzoic acid) and 4,4′-dithiodipyridine. Can. J. Biochem. **57**:822–833.
33. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246**:40–41.
34. **Viebrock, A., and W. G. Zumft.** 1988. Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. J. Bacteriol. **170**:4658–4668.
35. **Weitzman, P. D. J., and M. W. Danson.** 1976. Citrate synthase. Curr. Top. Cell. Regul. **10**:161–204.
36. **Wiegand, G., and S. J. Remington.** 1986. Citrate synthase: structure, control and mechanism. Annu. Rev. Biophys. Biophys. Chem. **15**:97–117.
37. **Wood, D. O., L. R. Williamson, H. H. Winkler, and D. C. Krause.** 1987. Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. J. Bacteriol. **169**:3564–3572.
38. **Wozniak, D. J., D. C. Cram, C. J. Daniels, and D. R. Galloway.** 1987. Nucleotide sequence and characterization of *toxR*: a gene involved in exotoxin A regulation of *Pseudomonas aeruginosa*. Nucleic Acids Res. **15**:2123–2135.