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Isolation and characterization of the positively acting regulatory gene *QUTA* from *Aspergillus nidulans*

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ABSTRACT

The positively acting regulator gene QUTA from Aspergillus nidulans has been identified and located within a cluster of quinic acid utilisation (QUT) genes isolated within a recombinant phage lambda (λ Q1). The DNA sequence of the QUTA gene reveals a single uninterrupted reading frame coding for a protein of mw 90.416 Kd. The QUTA protein sequence has a protein motif in the form of a putative "DNA finger" that shows strong homology to other such motifs in the GAL4, PPR1, ARGRII, LAC9 and QA1F regulatory gene products of S. cerevisae, K. lactis and N. crassa. The data presented confirm the view deduced by genetical analysis that the QUTA gene of A. nidulans encodes a protein capable of interacting with QUT specific DNA sequences.

INTRODUCTION

The interrelationship between quinate degradation to protocatechuate and polyaromatic amino acid (AROM) biosynthesis in Aspergillus nidulans is the subject of intense study. The pathways share two intermediates, 3-dehydroquinate and dehydroshikimate and have two isoenzymes that interconvert these two substrates (1,2). The AROM pathway is constitutively expressed at a low level whereas the quinic acid utilization (QUT) pathway is inducible by quinate, some of its breakdown products and is subject to catabolite repression (1,2). The presence of quinate induces the production of quinate dehydrogenase, 3-dehydroquinase and dehydratase, the enzymes required for catabolism to protocatechuate. The three genes encoding these enzymes QUTB, QUTE and QUTC map in a cluster on chromosome VIII together with three other loci, QUTD a permease (3), QUTA a positively acting regulatory gene and QUTR a gene with a negative role in regulation (4). Five of the six enzymatic steps leading to chorismate in AROM biosynthesis are encoded by the complex AROM locus which specifies a mosaic pentafunctional polypeptide (5,6). It is believed that this locus arose by multiple gene fusion as the DNA sequence shows strong homology with the five

equivalent separate aro loci of Escherichia coli (7). The 3-dehydroquinase activity of the pentafunctional AROM polypeptide shows no homology at the DNA or protein level with the 3-dehydroquinase encoded by the QUTE gene strongly indicating convergent evolution (5,8). The relationship between two pathways leads to two fundamental questions:- (1) How are the pools of common intermediates kept functionally separated under normal physiological conditions, to prevent internal induction of the quinate pathway in wild type strains? (2) What is the precise mechanism by which quinate induces the enzymes required for catabolism to protocatechuate? As part of a programme of research designed to answer these questions, we report in this communication the isolation and characterization of the positively acting regulatory QUTA gene from A. nidulans. We show that the derived protein sequence shares significant homologies with other eukaryotic DNA regulatory proteins.

MATERIALS AND METHODS

Organisms and Materials

The origins of the phage lambda, Escherichia coli and Aspergillus nidulans strains have been detailed previously (8,9).

Materials were obtained as described in Charles et al, (5) and media for the growth of A. nidulans, E. coli or phage lambda were those reported previously (10,11,12,13).

Transformation of A. nidulans

A. nidulans was transformed by the method of Ballance et al, (14), with each transformation mixture using a suspension of 10^6 regenerable sphaeroplasts mixed with 15μgof the appropriate DNA. Transformation mixtures were plated in selective minimal medium containing quinate as sole carbon source.

Recombinant DNA Techniques

Recombinant DNA techniques were as previously described (7,9,15). Southern blot analysis was carried out by the method of Jeffreys et al (16), DNA restriction fragments were recovered by the method of Tautz and Renz (17). Di-deoxy chain terminator sequencing using ^{35}SdATP and buffer gradient gels was as described by Sanger et al (18) and Biggin et al (19). Specific oligonucleotides were synthesized by the method of Matthes et al (20) with modifications to the wash cycle as described by Sproat and Gait (21).

RESULTS AND DISCUSSION

Identification and location of the QUTA gene

The biological activity of three (QUTD, QUTB and QUTE) of four tightly linked QUT genes isolated in recombinant phage λ-Q1 (9) has been demonstrated by the transformation of appropriate qut mutant strains (3,7,8) and their integrity confirmed by DNA sequence analysis (7). To determine if the QUTA gene is also contained in phage λ-Q1 two recessive non-inducible qutA mutant strains (qutA361 and qutA444) were subject to transformation with λ-Q1 DNA, and in both cases QUT transformants were recovered on quinic acid media (Table 1). In order to localise the QUTA gene within the λQ1 A. nidulans DNA, three plasmids, pAL3.7, pAL6.1 and pAL7 were constructed by ligating BamH1 single and partial digests of λQ1 DNA into the plasmid vector pBR322. Figure 1 summarises the position and extent of A. nidulans DNA in each of these plasmids.

Mutant strains qutA361 and qutA444 were subject to transformation with all three plasmids and the regenerable sphaeroplasts plated in selective medium with quinate as sole carbon source. Table 1 summarises the results of these transformations, and inspection of the data shows the following points:- (a) pAL3.7 fails to transform either mutant strain; (b) pAL6.1 and pAL7 transform both mutant strains; (c) therefore the bulk or complete QUTA gene must be contained within the 3.4Kb BamH1 fragment common to both pAL6.1 and pAL7, and is therefore located to the right of the QUTE gene (see Figure 1).

Table 1

Transforming DNA	Transformation frequency $x10^{-6}$ $\mu mole^{-1}$ (μg^{-1}) for mutant			
	R153	qutA361	R1553	qutA444
λQ1	31	(1)	22	(0.7)
pAL 6.1	3.35	(0.5)	8.97	(1.3)
pAL 7	6.0	(0.8)	19.5	(2.6)
pAL 3.7	0		0	
NO DNA	0		0	

Transformation of qutA361 and qutA444 mutants of A. nidulans. Sphaeroplasts prepared from mutant strains were transformed with samples of DNA of λQ1 or of the various plasmids indicated (see Figure 1). The number of transformants per μmole (and per μg) transforming DNA are given.

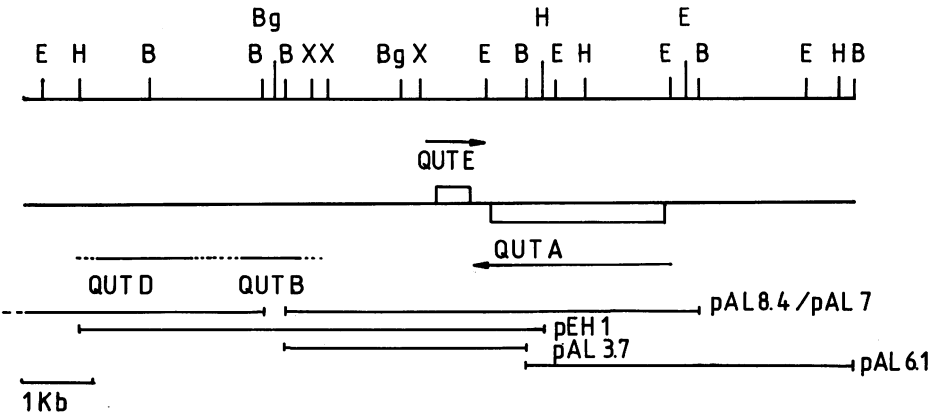


Figure 1

A restriction map of the A. nidulans DNA contained within phage λ Q1: B = BamH1; Bg = Bg1II; E = EcoR1; H = HindIII. The positions of the QUTA and QUTE genes are derived from DNA sequence analysis, the boxes and large arrows denoting the coding strand and direction of transcription. The position of the QUTD gene is located by transformation of qutD mutants using plasmids pAL8.4 and pEH1, and the QUTB gene by DNA cross hybridization to the equivalent QA-3 gene from N. crassa.

To confirm that the presumed transformants were the result of DNA integration into the genome, DNA from twelve λQ1 and twelve pAL6.1 treated strains in the R153 qutA361 background was prepared, digested with HindIII or EcoR1 and immobilised on nitrocellulose. After probing with $^{\alpha 32}$P labelled λ DB286 or pBR325 DNA analysis of the autoradiograph (data not shown) showed that vector sequences were present in all of the λQ1 transformed strains and over half the pAL6.1 transformed strains, confirming that colonies growing on selective plates were genuine transformants.

DNA sequence of the QUTA gene

The 1.0, 1.7 and 1.9Kb EcoR1, 0.6Kb HindIII and 3.4Kb BamH1 fragments from λQ1 (see Figure 1), covering the entire QUTA gene, were subcloned into suitably digested M13 vectors in both relative orientations and sequenced with universal primer. Gaps in the sequence were filled by extension using specific oligonucleotides as primers to complete the sequence on both strands. The nucleotide sequence determined is shown in Figure 2 with the inferred amino acid sequence of the QUTA specified protein. The presumed ATG start codon is the first to follow an in phase translational stop codon strongly suggesting that it is the in vivo translation initiator methionine. The molecular weight of the deduced

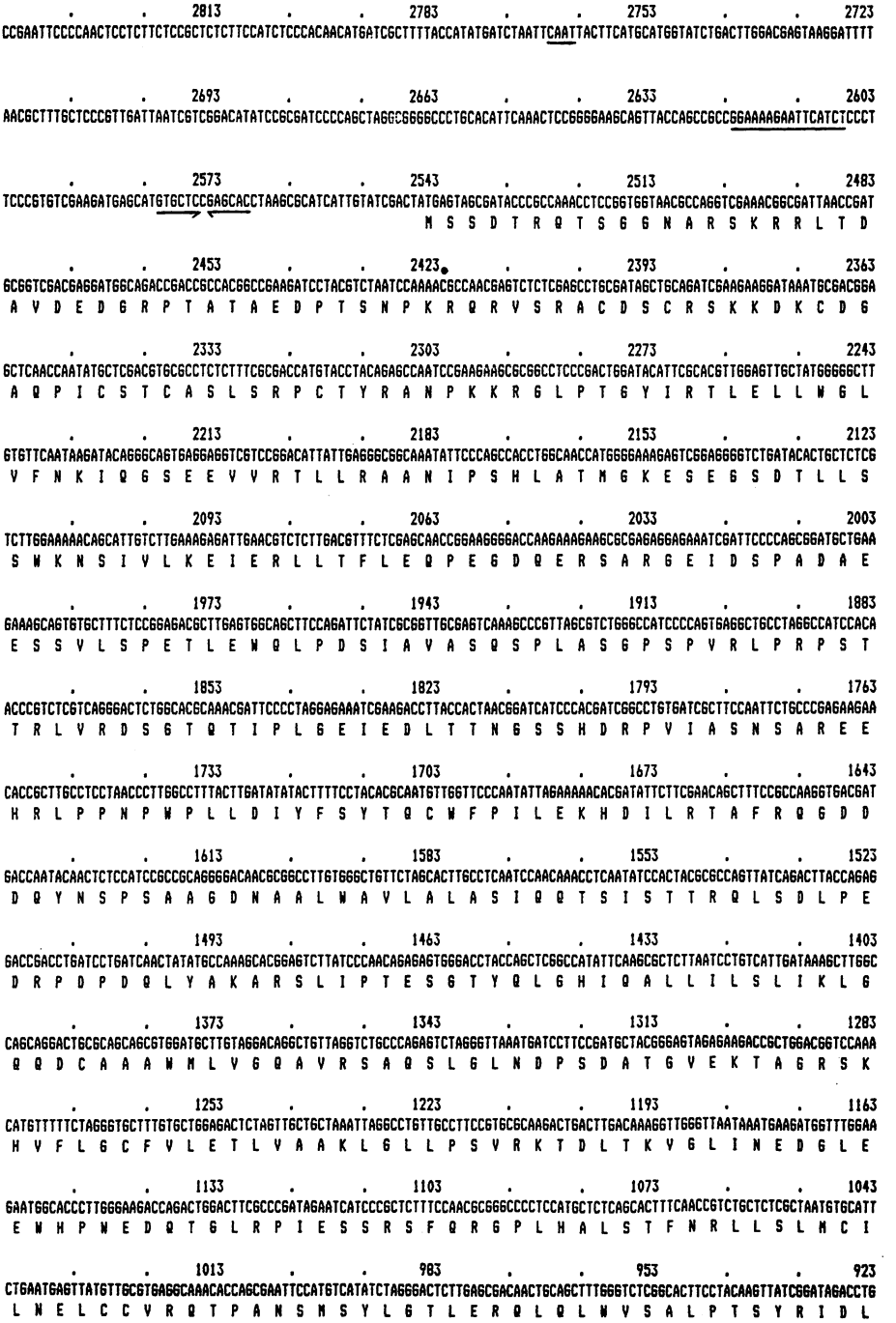
polypeptide is 90.432 Kd, and the codon usage (data not shown) does not show any consistent or major bias.

Analysis of the QUTA protein

The current model for the action of the QUTA gene proposes that it encodes a positively acting DNA regulatory protein (4). If this is true, homology (in the form of conserved protein motifs) with other known eukaryotic regulatory proteins may be expected. Study of positively acting DNA regulatory proteins in eukaryotes and both positively and negatively acting proteins in prokaryotes has identified at least two conserved protein structural motifs. The first of these, the helix-turn-helix generalised, model for the interaction of DNA binding proteins with their target is proposed to be present in most and possibly all such proteins (23). The second, the so called DNA finger or "chromatin plough" is proposed to be a common motif in many eukaryotic DNA regulatory proteins (24). This motif is characterised by several cysteine residues that help to maintain a compact structure in a moveable amino terminal portion of the protein that is rich in basic amino acids. This structure is proposed to probe and disrupt the structure of the chromatin and allow access to the DNA to facilitate binding. Cysteine rich sequences have been identified in five other generalised categories; retrovial low molecular weight nucleic acid binding proteins, adenovirus E1A gene products, aminoacyl tRNA synthetases, large T antigens, bacteriophage proteins, as well as eukaryotic DNA regulatory proteins (25).

A number of these proteins have been demonstrated to bind metal ions such as zinc (of which the Xenopus laevis transcription factor IIIA is the best characterised), and it has been suggested that many of the cysteine rich motifs may represent metal binding domains that would be important in DNA binding and gene regulation (25,26,27).

Both visual and computer aided searches of the QUTA protein sequence have revealed possible candidates for the helix-turn-helix and "chromatin plough" protein motifs. Pabo & Sauer (23) have compiled a table of helix-turn-helix motifis, some of the sequences have been proved to form the motif whereas others are inferred from homology and emprical folding predictions (28). At each position in the motif a subset of allowed amino acids is conserved, and we note that amino acid residues 446-465 in the QUTA protein have in 17/20 cases one of the conserved subset of amino acids, possibly identifying a helix-turn-helix motif in a linear position closely similar to another putative motif in the ARGRII gene product of S. cerevisiae (29). Empirical protein folding predictions on the QUTA motif show that the



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      .     .   893     .     .   863     .     .   833     .     .   803
CAGACAGTCTCGACCAAGCCGGCATCACCCCACATATTCGGCCTAGAGATGATGTACGAGGCCGTTGCAACCGCTATAAGCTTACAGCTGGCAGTCCAGAAAAACGAAAGAAACGGATTA
 Q  T  V  S  T  K  P  A  S  P  H  I  F  G  L  E  M  M  Y  E  A  V  A  T  A  I  S  L  Q  L  A  V  Q  K  N  E  R  N  G  L

      .     .   773     .     .   743     .     .   713     .     .   683
CGACGTGCTTCGGAAGGCTCCAAGCGGTTGGTCTCACTACTTCAAGCCTACATGGAGACCTATAGCCTTTCTGCTACCTGCCCGACTTTCGGTATAGCTCTGACTTTAGGCCTTCCACTT
 R  R  A  S  E  G  S  K  R  L  V  S  L  L  Q  A  Y  M  E  T  Y  S  L  S  A  T  C  P  T  F  G  I  A  L  T  L  G  L  P  L

      .     .   653     .     .   623     .     .   593     .     .   563
CCCTGCATGAAGAAAACTGCCCCATGGCCCTTTGAGGCTTCTCATGGCATCAACCACAAACTCCAATCGTTTTCTGCCCATCTCGCAACGGTTTGGATCCATAATACTGGCTCGCAGAGA
 P  C  M  K  K  T  A  P  W  P  F  E  A  S  H  G  I  N  H  K  L  Q  S  F  S  A  H  L  A  T  V  W  I  H  N  T  G  S  Q  R

      .     .   533     .     .   503     .     .   473     .     .   443
AGACCGAGACACGCTACACAGCAAGGTACCCATCCAAGGAGTCCGATGGCCATAAGCCTGCCCGGAAACAATATGCGTTTGACGAACTTAATCGAAGAATCTCGAACTGGGAATCTTTCA
 R  P  R  H  A  T  Q  Q  G  T  H  P  R  S  P  M  A  I  S  L  P  G  N  N  M  R  L  T  N  L  I  E  E  S  R  T  G  N  L  S

      .     .   413     .     .   383     .     .   353     .     .   323
ACCACTGACTCATATCTTTCGCCAACTTGGATGCGGACTTCGAACGATGAAAACGCTGTATTATCTCTACCGACGCCAGCATCGTCTCTTAATATCGCGTCTGGAGTTGAAACGAACCCT
 T  T  D  S  Y  L  S  P  T  W  M  R  T  S  N  D  E  N  A  V  L  S  L  P  T  P  A  S  S  L  N  I  A  S  G  V  E  T  N  P

      .     .   293     .     .   263     .     .   233     .     .   203
ACTTCGCAGCAAATCACCCATCAACATCGCTCTTCGGTCGTTGGAAAGCCTAACTCAGCACTGATAATGTCCGACCTGACGACGCCTTTTCCTGCGTCCGGTCATCACTATCAACAAACG
 T  S  Q  Q  I  T  H  Q  H  R  S  S  V  V  G  K  P  N  S  A  L  I  M  S  D  L  T  T  P  F  P  A  S  G  H  H  Y  Q  Q  T

      .     .   173     .     .   143     .     .   113     .     .    83
TATGATGATGCCAGTTTGCACTTCAATTCTTTAGCCGACATCCAGAGCACTAGCTCAGCACAACGACCAAGGATTGCTCCTGACCTCGATGCCTTGTTTGACGAGTTGGCCTCGCTTGAT
 Y  D  D  A  S  L  H  F  N  S  L  A  D  I  Q  S  T  S  S  A  Q  R  P  R  I  A  P  D  L  D  A  L  F  D  E  L  A  S  L  D

      .     .    53     .     .    23     .     .
GGGACAGATAGGTAACCAAGCTCTCTTCTGCACGCAAGTTATAAAACTAACTAGCACTAGGATGGACAACCAGCCCGAATTC
 G  T  D  R  U
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Figure 2

The nucleotide sequence of the QUTA gene. The sequence shown has the same orientation as the mRNA and the inferred protein sequence of the single uninterrupted reading frame is shown with the single letter code. Nucleotide 1 in the DNA sequence corresponds to nucleotide 895 in the 3' non translated region of the QUTE gene which has been reported previously (8).

sequence predicts a weak helical structure followed by a turn and a strong second helical prediction. The corresponding QA1-F gene of N. crassa has been sequenced and shown to encode a protein of mw 88.96 Kd (30,31) and shares significant homologies with QUTA. This homology is not linearly distributed but falls into three distinct regions in a manner similar to the two related regulatory genes LAC9 (from Kluveromyces lactis) and GAL-4 (from S. cerevisiae) active in galactose metabolism, reported by Salmeron & Johnston (24). Residues 41 to 117 in the amino terminal of the QUTA and QA-1F protein products show 58% homology, residues 264 to 542 show 36% homology, and residues 809 to 824 in the carboxy terminal show 56% homology. The homology in the amino terminal protein sequence (Region 1), comprising the DNA finger is also shared by the LAC9; GAL4; PPR1 and ARGRII gene products (see Table 2A). Salmeron & Johnston (24) have proposed that

Table 2

Table 2A

(A)	49.	C	D	S	C	R	S	K	K	D	K	C	D	G	A	Q	P	I	C	S	T	C	A	S	L	S	R	P	C			
(B)	95.	C	D	A	C	R	K	K	K	W	K	C	S	K	T	V	P	T	C	T	N	C	L	K	Y	N	L	D	C			
(C)	11.	C	D	I	C	R	L	K	K	L	K	C	S	K	E	K	P	K	C	A	K	C	L	K	N	N	W	E	C			
(D)	34.	C	K	R	C	R	L	K	K	I	K	C	D	Q	E	F	P	S	C	K	R	C	A	K	L	E	V	P	C			
(E)	21.	C	W	T	C	R	G	R	K	V	K	C	D	L	R	H	P	H	C	Q	R	C	E	K	S	N	L	P	C			
(F)	76.	C	D	Q	C	R	A	A	R	E	K	C	D	G	I	Q	P	A	C	F	P	C	V	S	Q	G	R	S	C			

(A)= QUTA; (B)= LAC9; (C)= GAL4; (D)= PPR1; (E)= ARGR11; (F)= QA-1F

Table 2B

QUTA	150.	K	E	I	E	R	L	L	T	F	L	E					810.	L	D	A	L	F	D	E	L							
PPR1	205.	K	E	I	S	A	L	G	T	M	R	E					810.	L	D	E	F	F	T	E	L							
QUTA	446.	K	T	D	L	T	K	V	G	L							313.	N	A	A	L	W	A	V	L	A						
ARGR11	384.	K	T	D	L	T	V	H	G	L							444.	N	A	L	L	N	A	L	L	A						

Homologies between the protein sequence of QUTA and other eukaryotic DNA regulatory proteins. 2(a) compares the sequences of the cysteine rich region which are believed to make up the "DNA finger". 2(b) summarises homologies between the QUTA protein sequence and the protein sequence of the yeast proteins encoded by the PPR1 and ARGRII genes. Boxes in 2(a) identify identical amino acids, and in 2(b) identify identical or closely related amino acids. The number preceding each protein sequence is the position of the first amino acid within the entire sequence.

region 1 can be split into two domains, regions IA and IB, IA comprising the DNA finger or "chromatin plough" and IB comprising sequences essential for gene/pathway specific regulation. Region IB of QUTA (residues 41 to 77) shows highly limited (< 15%) homology with the IB region of the LAC9 and GAL4 gene protein products, but shows 65% homology with the IB region of QA-1F (30,32), strongly suggesting that this region is important for pathway specific regulation (24). Table 2B further shows regions of homology between QUTA and two other positively acting eukaryotic DNA regulatory proteins, PPR1, involved in the pyrimidine pathway and regulation, and ARGRII involved in controlling the anabolism and catabolism of arginine (29,33).

5' and 3' non translated DNA sequence of QUTA

The 5' nontranslated sequence of the QUTA gene was searched for inverted repeat structures, and sequence motifs typically associated with a

role in transcription regulation. The results of the search show the following points:- (a) There is a 12nt perfect inverted repeat from nt 2569-2581, (b) There is a CAAT sequence from nt 2764-2767, this sequence is typically found 70-80 nt upstream from the transcription initiation start point and is important for the binding of RNA polymerase II to the DNA (34). (c) There is a sequence of 16 bases from nt 2607-2622 that shows 67% homology with the concensus N. crassa upstream activator sequence (GGATAANNNNTTATCC) which has been shown to bind the QA-1F regulatory gene protein (35).

Similarly, inspection of the 3' non translated DNA sequence reveals the following points:- (a) There is an AUAAA sequence starting at 38nt downstream of the translational TAA stop codon (underlined in Figure 2). The AAUAAA sequence is required for polyadenylation of mRNA's transcribed by RNA polymerase II (36,37), and is found at variable distances upstream of the polyadenylation site. (b) The consensus sequence YGTGTTYY (where Y = pyrimidine) is found downstream from the AAUAAA sequence and polyadenylation site and is required for efficient formation of 3' termini in many (67%) mammalian mRNA's (38). This sequence is not present in the 3' flanking region of QUTA but is present 54 nt downstream of the second of two major polyadenylation sites in the A. nidulans AROM locus (7), and 149 nt downstream of the translational stop codon in the QUTE gene (8). The possible in vivo significance of the putative conserved motifs is the subject of continuing investigation using oligonucleotide directed site specific mutagenesis. Previous genetic analysis of the QUTA gene has shown that the most common class of mutants are recessive and are unable to induce the three enzyme structural genes QUTB, QUTC and QUTE in haploid strains. Rare dominant qutA mutants non inducible for all three enzyme structural genes do occur but no constitutive mutants have been recovered (4). These observations strongly suggest that the QUTA gene encodes a positively acting DNA regulatory protein, although the absence of constitutive mutants formally allows the possibility that the QUTA protein may act in concert with other protein(s) to positively control transcription of the QUT gene cluster (4).

In conclusion, we can say that the data presented in this communication concur strongly with the view deduced by classical genetical analysis that the QUTA gene of A. nidulans is a positively acting DNA regulatory gene.

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