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The base sequence of the *nifF* gene of *Klebsiella pneumoniae* and homology of the predicted amino acid sequence of its protein product to other flavodoxins

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The nucleotide sequence of a 629 base-pair segment of DNA spanning the *nifF* gene of *Klebsiella pneumoniae* is presented. The structural gene comprises 531 base-pairs (175 codons, excluding the translational initiator and terminator) encoding an acidic polypeptide of 18950 Da. The *nifF* product thus belongs to the long-chain class of flavodoxins. It shows some sequence homology to the short-chain flavodoxins from *Desulfovibrio vulgaris*, *Clostridium* MP and *Megasphaera elsdenii*, and much stronger homology to long-chain flavodoxins from *Azotobacter vinelandii* and *Anacystis nidulans*. The long chain flavodoxins thus seem to constitute a well-conserved sub-group. The homology with the *A. vinelandii* flavodoxin is particularly strong, which may reflect their common function in nitrogen fixation.

INTRODUCTION

The *nif* regulon of *Klebsiella pneumoniae* comprises at least 17 contiguous genes organised in seven or eight transcriptional units (MacNeil *et al.*, 1978; Merrick *et al.*, 1980; Pühler & Klipp, 1981; Shen *et al.*, 1983). These occupy 23 kb of the genome, which have been mapped physically and cloned on small plasmids (Riedel *et al.*, 1979; Pühler & Klipp, 1981; for review see Kennedy *et al.*, 1981).

Two *nif* genes code for proteins required for electron transport to nitrogenase. The *nifF* gene encodes a flavodoxin which is the immediate electron donor to component 2 of nitrogenase (Kp2), and *nifJ* a pyruvate:flavodoxin oxidoreductase which provides an efficient link to metabolic sources of reducing power (Nieva-Gomez *et al.*, 1980; Hill & Kavanagh, 1980; Shah *et al.*, 1980). Both proteins have been purified and shown to function *in vitro* in electron transport to nitrogenase (Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1980).

The *nifF* gene lies in a monocistronic transcriptional unit between *nifL* and *nifM* and is transcribed in the opposite direction to the flanking genes (Pühler & Klipp, 1981; Beynon *et al.*, 1983; Drummond *et al.*, 1983). Sequence analysis of the *nifF–nifL* intergenic region has been carried out and the regulatory sequences identified. The *nifF* promoter shows the unusual structure characteristic of other promoters within the *nif* cluster (Beynon *et al.*, 1983), and like them is activated by the *nifA* product when concentrations of oxygen and fixed nitrogen are sufficiently low (Drummond *et al.*, 1983). The *nifF* product is thus unique among flavodoxins hitherto described in that there is compelling genetic evidence for a specific function *in vivo*. This information is difficult to obtain using biochemical techniques owing to the functional interchangeability of flavodoxins *in vitro* (Mayhew & Ludwig, 1975).

MATERIALS AND METHODS

Restriction enzymes were bought from New England Biolabs and used following standard procedures (Maniatis *et al.*, 1982) in TARS buffer (33 mM-Tris/acetate, pH 7.9, 66 mM-potassium acetate, 10 mM-magnesium acetate, 0.5 mM-dithiothreitol, 4 mM-spermidine, 10 μg of pancreatic RNAase/ml). T4 DNA ligase was bought from Boehringer Corp., and used in TARS buffer supplemented with 1 mM-rATP. Nucleotide triphosphates used for sequencing were obtained from PL laboratories and a 17-nucleotide sequencing primer was obtained from Celltech.

The DNA fragments used for sequence determination were excised from pMD422 (M. H. Drummond, unpublished work), a pBR322 derivative (Bolivar *et al.*, 1977) carrying an *Eco*RI–*Sal*I fragment of 1447 bp, and pRD545 (R. A. Dixon, unpublished work), a pACYC177 derivative (Chang & Cohen, 1978), carrying a *Sma*I–*Xho*I fragment of about 1250 bp (see Fig. 1). These small plasmids were in turn derived from pGR112 and pCM1 respectively (Riedel *et al.*, 1979). Small fragments generated by *Bsp*RI or *Sau*3A digestion of the *Eco*RI–*Sal*I fragment isolated from pMD422 were cloned into M13 mp7 (Messing *et al.*, 1981). Other M13 clones were constructed by ligating specific fragments generated by double digestion of pMD422 or pRD545 into M13 mp8 or mp9 (Messing & Viera, 1982; see Fig. 1). The base sequence was determined by using the chain termination method (Sanger *et al.*, 1977), and the data compiled by computer analysis (Staden, 1979).

RESULTS AND DISCUSSION

Sequence of *nifF*

The sequence shown in Fig. 2 was determined on both strands except between positions 443 and 457 which

Abbreviations used: kb, kilobases; bp, base pairs.

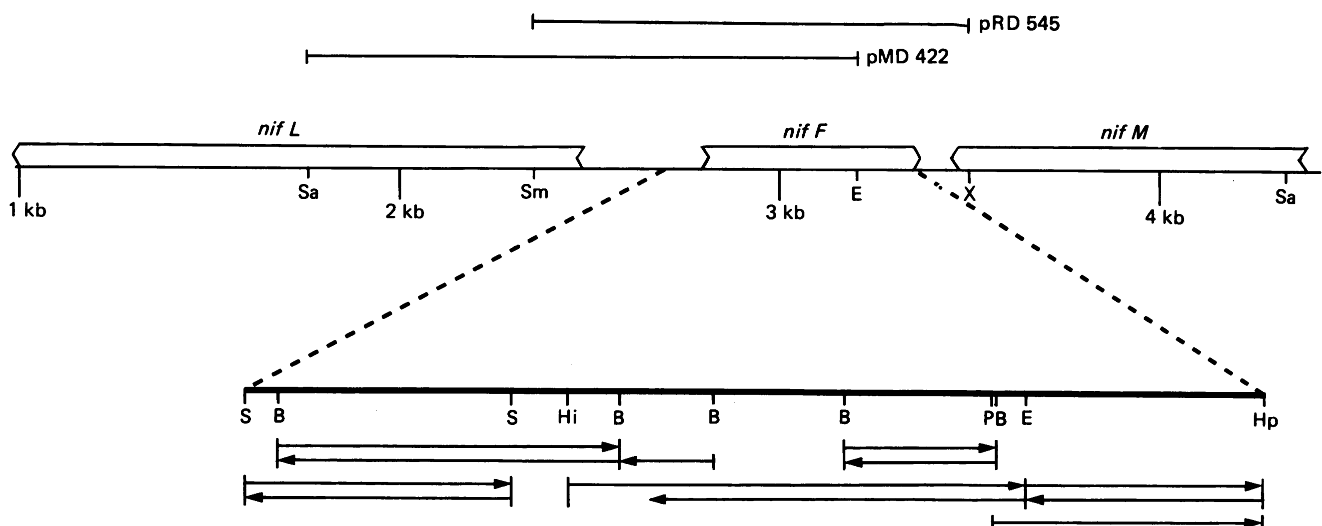

Fig. 1. Physical and genetic map of the region surrounding the *nifF* gene of *K. pneumoniae* and summary of DNA sequence data obtained from M13 clones

Positions of restriction sites have been determined by sequence analysis to the left of the *Hpa*I site (the present paper; M. H. Drummond, unpublished work) and by restriction analysis to the right of the *Hpa*I site (Riedel *et al.*, 1979). B, *Bsp*RI; E, *Eco*RI; Hi, *Hinc*II, Hp, *Hpa*I; P, *Pvu*II; S, *Sau*3A; Sa, *Sal*I, Sm, *Sma*I. The segments of DNA cloned into the plasmids pMD422 and pRD545 are shown at the top of the Figure. The positions and translational polarities of the structural genes are indicated by the open boxes. The arrows below the expanded section of the restriction map show the positions, directions and extents of the sequences obtained from M13 clones.

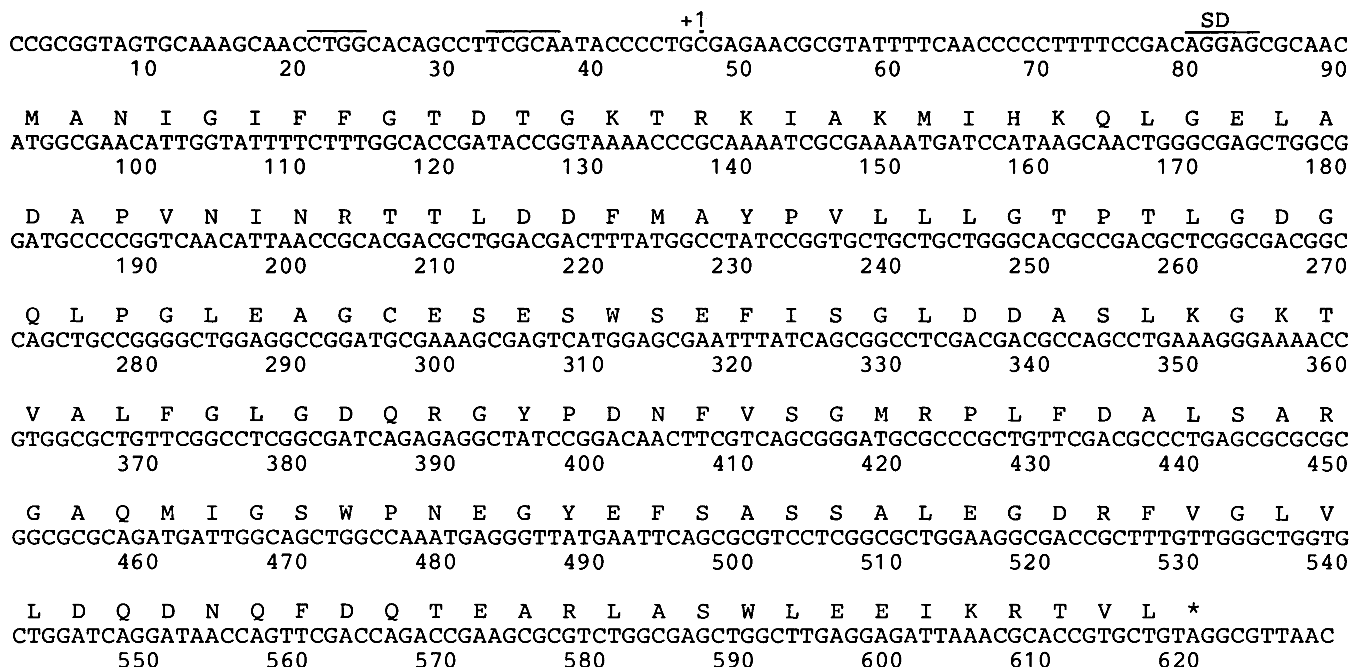

Fig. 2. Sequence of the *K. pneumoniae nifF* gene with predicted translation product

The base sequence from the midpoint of the *Bsp*RI site upstream from the promoter to the *Hpa*I site downstream from the stop codon is shown. The *nif* promoter consensus sequence between positions 21 and 37 is overlined, as is the Shine–Dalgarno sequence at position 80–84. The transcription start point is designated +1 (Beynon *et al.*, 1983). The primary structure of the *nifF* product is shown directly above the nucleotide sequence in one-letter code.

includes G+C-rich inverted repeat, the distal portion of which yielded poor sequence data in both directions. This could be alleviated but not entirely corrected by substituting dITP for dGTP (Mills & Kramer, 1979) and running very hot gels. The sequence spans *nifF* from the midpoint of the *Bsp*RI site upstream from the promoter to the *Hpa*I site downstream from the stop codon. The *nif* promoter consensus sequence and *nifF* transcription

Table 1. Amino acid compositions of short-chain and long-chain flavodoxins

The amino acid composition predicted for the *K. pneumoniae nifF* product is compared with that of flavodoxins whose amino acid sequence has been determined (for references, see text). The initiating formylmethionine residues are not included.

	K. pneumoniae		*A. vinelandii*		*Clostridium* MP		*M. elsdenii*		*D. vulgaris*	
Residue	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%
A	15	8.57	14	7.82	6	4.35	18	13.14	17	11.49
C	1	0.57	1	0.56	3	2.17	2	1.46	4	2.7
D	14	8.00	15	8.38	9	6.52	10	7.30	9–16	6.08–10.81
N	6	3.43	5	2.79	8	5.80	5	3.65	2–9	1.35–6.08
E	11	6.29	16	8.94	19	13.77	16	11.70	9–16	6.08–10.81
Q	7	4.00	5	2.79	2	1.45	0	0	0–7	0–4.73
F	10	5.7	13	7.26	5	3.62	4	2.92	6	4.05
G	20	11.43	20	11.17	14	10.14	15	10.95	18	12.16
H	1	0.57	0	0	0	0	0	0	1	0.68
I	8	4.57	6	3.35	15	10.87	5	3.65	9	6.08
K	7	4.00	12	6.70	10	7.25	9	6.57	3	2.03
L	21	12.00	20	11.17	8	5.80	7	5.11	13	8.78
M	4	2.29	1	0.56	5	3.62	5	3.65	1	0.68
P	7	4.00	5	2.79	3	2.17	5	3.65	3	2.03
R	8	4.571	5	2.79	2	1.45	2	1.46	7	4.73
S	12	6.86	15	8.38	8	5.80	7	5.11	8	5.41
T	10	5.71	8	4.47	5	3.62	8	5.84	7	4.73
V	7	4.00	10	5.59	10	7.25	13	9.49	9	6.08
W	3	1.71	3	1.68	3	2.17	4	2.92	1	0.68
Y	3	1.71	5	2.79	3	2.17	2	1.46	6	4.05
Total	175		179		138		137		148	
M_r	18950		19480		14280		14430		15730	
Polarity	43%		45%		46%		42%		32%	

start point have been determined by Beynon *et al.* (1983). The coding region is open in one frame only and is preceded by a Shine and Dalgarno sequence of five nucleotides which fulfils the most stringent critria described by Stormo *et al.* (1982) for identifying translational initiation sites.

The *nifF* product has been shown to possess an *N*-terminal alanine and the sequence Arg-Thr-Val-Leu at its *C*-terminus (Deistung *et al.*, 1985). This confirms the position of the translation stop and is consistent with docking of the initial methionine. The *nifF* polypeptide is thus predicted to comprise 175 residues having a molecular mass of 18950 Da. This is close to measurements of 22000 Da (Nieva-Gomez *et al.*, 1980) and 19000 ± 500 Da (Deistung *et al.*, 1985) obtained using SDS/polyacrylamide-gel electrophoresis, and estimates of 20000 Da (Nieva-Gomez *et al.*, 1980) and 21000 Da (Yoch *et al.*, 1974) based on gel filtration.

The predicted amino acid composition of the *K. pneumoniae* flavodoxin corresponds closely to that determined by analysis of the purified protein (Deistung *et al.*, 1985) and is compared with that of four other flavodoxins in Table 1. The *nifF* product has 14 aspartic and 11 glutamic acid residues as against 8 arginines and 7 lysines, making it an acidic protein like the other flavodoxins.

The G + C content of the *nifF* structural gene is 60.0%, marginally higher than the 58.4% G+C of the *nifH* and *nifD* genes (Sundaresan & Ausubel, 1981; Scott *et al.*, 1981), which encode subunits of nitrogenase. In view of its size, this is not sufficiently different from the 55–57.6% G+C content estimated for entire *K. pneumoniae* genomes (Hill, 1966; Star & Mandel, 1969) to suggest an evolutionary origin outside the species (Postgate, 1982).

The codon usage shown by *nifF* is similar to that of *nifK* and *nifD*. There is a marked preference for G or C in third positions, 75% of codons showing this pattern, which has been reported in other prokaryotes (Dunn *et al.*, 1981; Winter *et al.*, 1983; Kagawa *et al.*, 1984). Nif polypeptides are major constitutents of nitrogen-fixing cell and one would expect the codon usage of *nif* genes in *K. pneumoniae* to resemble that of strongly expressed genes in *E. coli* (Grosjean & Fiers, 1982; Kalnins *et al.*, 1983; Ikemura, 1981). However this is not true for all amino acids. In particular, alanine and valine show the codon usage typical of weakly expressed genes. Since the relative abundance of synonymous tRNAs is strongly correlated to codon preference (Ikemura, 1981), the codon usage observed for these *nif* genes could reflect differences between *E. coli* and *K. pneumoniae* in the size of tRNA pools.

Homology of *nifF* product to other flavodoxins

Two classes of flavodoxin are distinguished on the basis of size (Mayhew & Ludwig, 1975), short-chain flavodoxins having molecular masses in the range 14500–16000 Da, and long-chain flavodoxins in the range 18500–23000 Da. The *nifF* product clearly belongs to the latter class. The complete amino acid sequence of a long-chain flavodoxin from *Azotobacter vinelandii* is known (Tanaka *et al.*, 1977) as well as that of short-chain flavodoxins from *Megasphaera elsdenii* (Tanaka *et al.*, 1973, 1974*a*), *Clostridium* MP (Tanaka *et al.*, 1974*b*) and *Desulfovibrio vulgaris* (Dubourdieu *et al.*, 1973). These have been shown to possess significant sequence homology, especially at the *N*-terminus (Mayhew & Ludwig, 1975).

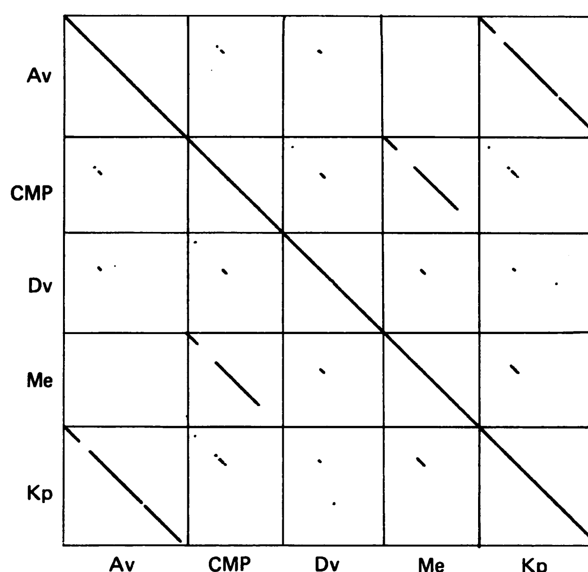

Fig. 3. Amino acid sequence comparison of all flavodoxins of completely characterized primary structure

The graphics program DIAGON was used with a span of 18 and minimum score for proportional matching of 228 giving a double matching probability of 1.32×10^{-5}. The flavodoxins are isolated from *Azotobacter vinelandii* (Av), *Clostridium* MP (CMP), *Desulfovibrio vulgaris* (Dv), *Megasphaera elsdenii* (Me), and *Klebsiella pneumoniae* (Kp) (for references see the text).

Fig. 3 shows a comparison of these published sequences with each other and with the predicted *K. pneumoniae* sequence, as generated by the graphics program DIAGON (Staden, 1982). This program assigns a score to each amino acid match based on the relatedness odds matrix MDM_{78} of Dayhoff (1969), which reflects observed substitutions of amino acids in related proteins. Scores are summed over a defined span and plotted if in excess of a specified value, which can be raised or lowered to increase or decrease the stringency of the comparison. Fig. 3 has been plotted at a rather high stringency and it can be seen that the homology between the *Azotobacter vinelandii* flavodoxin and the *K. pneumoniae nifF* product is particularly strong and extends for the entire length of the proteins, rather than tailing off towards the *C*-terminus as the other matches tend to do. In Fig. 4 the two amino acid sequences are aligned, and a padding character has been inserted at position 27 in the *K. pneumoniae* sequence to optimize the match.

The *A. vinelandii* flavodoxin is widely supposed to transfer electrons in nitrogenase *in vivo* (Benemann *et al.*, 1969), but this has been questioned (Nieva-Gomez *et al.*, 1980), because the molecule is synthesized in the presence of ammonium, which represses the synthesis of other *nif* genes. Its very close resemblance to the *K. pneumoniae nifF* product suggests that this doubt is unjustified. It is interesting to note that the *nifF* and *nifJ* promoters of *K. pneumoniae* retain a low level of expression in the presence of ammonium (Drummond *et al.*, 1983; Dixon *et al.*, 1980). The homology also suggests that the entity which donates electrons to the nitrogenase flavodoxin in *A. vinelandii* will be found to resemble the *nifJ* product of *K. pneumoniae*, at least with respect to the domain of

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    1         11        21        31        41        51        61        71        81
Av. AKIGLFFGSNTGKTRKVAKSIKKRFDDETMSDALNVNRVSAEDFAQYQFLILGTPTLGEGELPGLSSDCENESWEEFLPKIEGLDFS
    * ** ***  ****** ** * *    *      * **    **  *  * ******* * ****   ** *** **
Kp. ANIGIFFGTDTGKTRKIAKMIHKQLG-ELADAPVNINRTTLDDFMAYPVLLLGTPTLGDGQLPGLEAGCESESWSEFISGLDDASLK
    * ** * ** ** *  **  *    * *       *      *  **  *  * *    * *           *      **
An. AKIGLFYGTQTGVTQTIAESIQQEFGGESIVDLNDIANADASDLNAYDYLIIGSPXWNVGEL--------QSDWEGIYDDLDSVNGQ

91        101       111       121       131       141       151       161       171
GKTVALFGLGDQVGYPEDYLDALGELYSFFKDRGAKIVGSWSTDGYEFESSEAVVDGKFVGLALDLDNQSGKTDERVAAWLAQIAPEFGLSL
************ ***         *      ***   ***   ****  * *     **** ** ***   *  * * **  *
GKTVALFGLGDQRGYPDNFVSGMRPLFDALSARGAQMIGSWPNEGYEFSASSALEGDRFVGLVLDQDNQFDQTEARLASWLEEIKRTVL
 *                    *  *    *  * *  * ** *** *  * *     ****  *              *    *
AKXXXXXXXXXXXXXXXXXXXXXMGILEEKISSLGSQTVGYWPIEGYDFNESKAVRNNQFVGLAIDXXXXXXXXXXXXXXWVSQLKSEFGL
```

Fig. 4. Alignment of long-chain flavodoxin sequences from *A. vinelandii* (Av), *K. pneumoniae* (Kp) and *A. nidulans* (An)

Asterisks denote exact matches. Hyphens are packing characters introduced to align the sequences, not to be confused with unidentified residues which are represented by X. The numbering of the longest sequence, Av, is also used to refer to the other two sequences.

this 120000 Da protein which is responsible for flavodoxin recognition.

Although the sequence of the long-chain flavodoxin from the cyanobacterium *Anacystis nidulans* is not yet complete (Tarr, 1983; Ludwig *et al.*, 1984) it clearly resembles the *K. pneumoniae* and *A. vinelandii* sequences and has been aligned with them in Fig. 4. At its *N*- and *C*-terminal extremities, it matches the *A. vinelandii* sequence more exactly than does the *K. pneumoniae* sequence, and no padding character is required around position 27 to align the sequences. However, the *K. pneumoniae* and *A. vinelandii* sequences match much more exactly between residues 52 and 78, and have eight additional residues in a central region relative to the *A. nidulans* sequence. Because the *A. nidulans* sequence is incomplete and the homology relatively weak in this central region, it is difficult to judge the correct position of the padding characters necessary to align the sequences, but they almost certainly correspond to residues somewhere between positions 55–120 in the *A. vinelandii* sequence, since there is clear homology flanking this region. In Fig. 4 they have been placed so as to align the tryptophan at position 74, a match found by Dayhoff (1969) to be particularly significant.

The matrix MDM_{78} may be used to assess the quality of the matches in Fig. 4. The average score of the matches of known amino acids of the *A. nidulans* sequence with the *K. pneumoniae* sequence is 1.93, and with the *A. vinelandii* sequence 2.4, whereas for *K. pneumoniae* and *A. vinelandii* the average score for the equivalent amino acids is 2.87, allowing a score of -10 for the 'missing' residue in the *K. pneumoniae* sequence. Thus, even before the 'missing' residues in the *A. nidulans* sequence are taken into account, the *K. pneumoniae* and the *A. vinelandii* sequences match each other more exactly than either does the *A. nidulans* sequence. This probably reflects their common function in nitrogen fixation, since *A. nidulans* is not diazotrophic (Stewart, 1980). However all three long-chain flavodoxins show greater mutual homology than any of them do to any short-chain flavodoxin so far described. The long-chain flavodoxins thus seem to comprise a well-conserved sub-group, despite the taxonomic diversity of the organisms in which they are found, the nitrogenase flavodoxins being particularly well conserved, as is nitrogenase itself (Sundaresan & Ausubel, 1981; Scott *et al.*, 1981; Ruvkun & Ausubel, 1980).

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