JOURNAL OF VIROLOGY, May 1982, p. 389–394
0022-538X/82/050389-06$02.00/0

Vol. 42, No. 2

Guanidine-Resistant Mutants of Aphthovirus Induce the Synthesis of an Altered Nonstructural Polypeptide, P34

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Received 23 October 1981/Accepted 28 December 1981

Extracts of cells infected with guanidine-resistant mutants of aphthovirus were examined for differences in virus-induced polypeptides by using electrofocusing. Four of 10 independent spontaneous mutants induced the synthesis of an altered nonstructural polypeptide, P34. The precursor of P34, P52, and a previously unmapped polypeptide, P20c, also carried these charge-change mutations. No mutations in other regions of the genome were detected, and the remaining six guanidine-resistant mutants appeared entirely normal by electrofocusing. However, when the P34 of one of the latter mutants was examined by tryptic peptide fingerprinting, it too differed from that of the guanidine-sensitive parent. The frequency of P34 alterations among guanidine-resistant mutants suggests that P34 is functionally involved in the antiviral action of guanidine.

Guanidine inhibits specifically the mutiplication of some picornaviruses at concentrations that have little effect on cell growth (4, 22). This was first demonstrated for aphthovirus (foot-and-mouth disease virus) by Pringle (21). Evidence has accumulated that the primary action of guanidine is to inhibit replication of both aphthovirus (3) and poliovirus RNA, although the exact mode of inhibition is unknown (1, 7). Guanidine does not appear to inhibit elongation of RNA chains, since the RNA polymerase purified from aphthovirus-infected cells (3) or poliovirus-infected cells (2) is insensitive to guanidine.

Cells infected with either aphthovirus or poliovirus make a normal complement of virus polypeptides in the presence of guanidine (1; D. V. Sangar, unpublished observation). Hence the virus protease function is not susceptible to guanidine. The final viral maturation step, cleavage of VP0 to VP2 and VP4, is delayed in the presence of guanidine (11), but this could be an indirect result of an interrupted supply of RNA genomes for encapsidation.

Genetic and physicochemical studies on mutants of poliovirus able to grow in the presence of guanidine (*gr* mutants) implicated the capsid proteins as the site of action of guanidine. Cooper (9) found that *gr* mutations were linked genetically to temperature-sensitive (*ts*) mutations that were believed to be carried by capsid proteins. In some cases the capsid protein mutants themselves exhibited increased sensitivity to guanidine (10). Likewise Korant (16) observed capsid protein modifications to viruses made *gr* by multiple passage in the presence of guanidine. However, properties of deletion mutants of poliovirus seem to rule out the direct involvement of capsid proteins; these mutants make no detectable capsid protein, yet still retain the ability to synthesize RNA by a mechanism that is fully sensitive to guanidine (8).

In summary, none of the virus polypeptides of known function (RNA polymerase, structural, or processing) appears likely to be the primary target for guanidine. This paper describes a new approach to the problem of locating *gr* mutations in the picornavirus genome. Electrofocusing can be used to detect missense mutations that cause a change in the isoelectric point (pI) of a polypeptide. The technique has been used for the physical mapping of coat protein *ts* mutations in aphthovirus (13, 14). The study of polypeptides induced in virus-infected cells permitted the search for mutations to be extended to the nonstructural polypeptides (12), and this led to the identification of mutants with *ts* RNA polymerases (18). Using this approach, we show here that spontaneous *gr* mutations in aphthovirus are associated exclusively with alterations in P34, a stable nonstructural polypeptide of unknown function.

MATERIALS AND METHODS

Isolation of *gr* mutants. The origin of the guanidine-sensitive (*gs*) aphthovirus parental strain O Pacheco (*ts*$^+$*gs*) was described previously (17). Spontaneous *gr* mutants were isolated both from the *ts*$^+$*gs* parent and from *tsgs* mutants. Two methods of isolation were adopted. The first was used for *ts*$^+$*gr*1, *ts*34*gr*, and *ts*58*gr*. Guanidine-sensitive virus was titrated on monolayers of BHK-21 cells grown in 6-cm petri

dishes. Guanidine-HCl at a concentration of 650 μg/ml was incorporated into an overlay containing Eagle medium supplemented with 2% (vol/vol) bovine serum and 0.6% (wt/vol) agarose. Large plaques were isolated and recloned in the presence of guanidine. In the other method, which was used to isolate ts^+gr2 and *ts*107*gr*, *gs* virus was cloned by using agar-cell suspension culture. Bottles (2 oz., ca. 60 ml) containing BHK cell monolayers were infected with virus from separate *gs* plaques and incubated in the presence of 800 μg of guanidine-HCl per ml for 4 to 6 h until the appearance of cytopathic effect, when the bottles were frozen at −20°C. Virus was cloned twice in agar-cell suspension culture in the absence of guanidine, and the clones were assayed in monolayers under normal and guanidine overlays at 37°C to screen for resistance to guanidine.

The guanidine resistance of the presumptive mutants was checked by assaying the yield of virus produced after a single growth cycle in the presence of guanidine as follows. Duplicate test tubes containing 2×10^5 cells were infected with virus at a multiplicity of infection of about 100 and incubated in the presence of guanidine-HCl at different concentrations (0, 200, 300, and 400 μg/ml), at 37°C for 4 to 6 h. The yield of each tube was then assayed on cell monolayers in the absence of guanidine. Viruses that exhibited a significant reduction in sensitivity to guanidine (10- to 100-fold at the highest inhibitor concentration) were designated *gr* mutants.

Electrofocusing. Preparation of cytoplasmic extracts of virus-infected cells, electrofocusing, and two-dimensional gel electrophoresis were carried out as described previously (18). High-resolution isoelectric focusing was performed as for electrofocusing, with the following modifications. Gels (260 by 2.5 mm) contained pH 7 to 9 LKB Ampholine ampholytes. Electrophoresis was for 2 h at 400 V followed by 24 h at 1,100 V and 2 h at 1,600 V.

Tryptic peptide fingerprinting. Labeled cytoplasmic extracts of infected cells were mixed with a [^{3}H]leucine/[^{14}C]leucine ratio of 5:1. These extracts were then subjected to two-dimensional electrophoresis. After autoradiography, P34 was excised from the gel. The polypeptide was eluted into 1 ml of 0.1 M Tris-hydrochloride (pH 7.9)–1% (vol/vol) sodium dodecyl sulfate–0.1% (vol/vol) 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride for 1 to 2 days at 37°C. Carboxymethylation and trypsin digestion were carried out as described previously (23). The digest was lyophilized and dissolved in 0.2 ml of sample buffer containing 0.04 M pyridine acetate (pH 2.7). After clarification by centrifugation for 5 min in an Eppendorf microfuge the digest was loaded onto a 0.6- by 48-cm column of Technicon Chromobeads type P cation-exchange resin. The chromatogram was developed at 54°C with a 0 to 2 M pyridine acetate gradient in sample buffer containing 0.6% thiodiglycol and 1% Brig 35 (BDH); 200 0.25-ml fractions were collected in scintillation vials containing glass fiber disks, dried, and assayed for radioactivity.

RESULTS

Two-dimensional electrophoresis of virus-induced polypeptides. Ten independent *gr* mutants were examined for charge changes in virus-induced polypeptides. Initially, the mutants

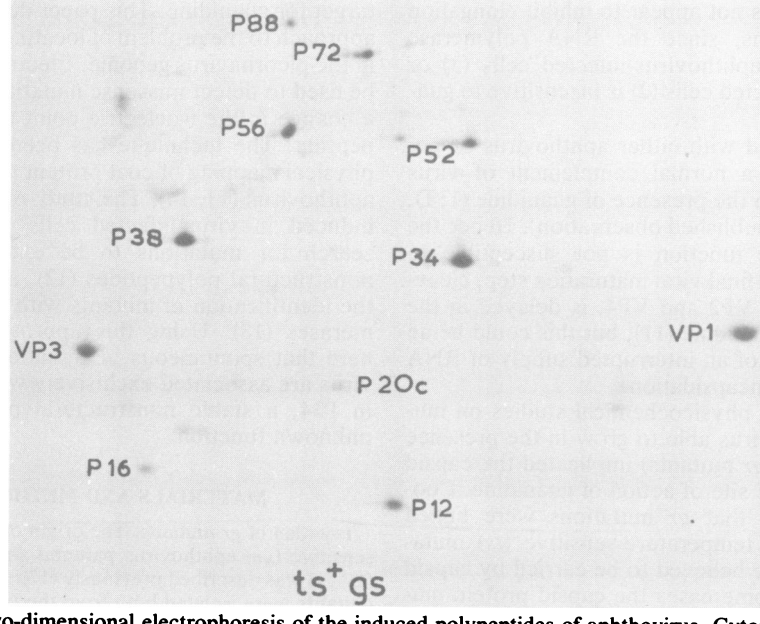

FIG. 1. Two-dimensional electrophoresis of the induced polypeptides of aphthovirus. Cytoplasmic extracts were prepared from cells infected with ts^+gs virus and pulse-labeled for 10 min with [^{35}S]methionine. Electrophoresis and autoradiography were as described previously (18). Electrofocusing, in the horizontal dimension, was with the origin (anode) on the left.

were screened by one-dimensional electrofocusing of cytoplasmic extracts prepared from infected cells pulse-labeled for 10 min with [^{35}S]methionine. Three of the mutant viruses were found to induce the synthesis of altered nonstructural polypeptides P34 and P52; the remaining seven mutants were indistinguishable from their *gs* parents (data not shown).

Polypeptide extracts of these three *gr* variants were then analyzed by two-dimensional electrophoresis. Figure 1 illustrates a complete two-dimensional electropherogram of polypeptides induced by one of the *gs* parents, *ts*$^+$*gs*. The identification of the polypeptides has been described previously (12). Figure 2 shows the polypeptides of each of the *gr* variants corun with those of *ts*$^+$*gs*; only the central portion of each autoradiograph is shown in Fig. 2. An extract of uninfected BHK cells was also analyzed (Fig. 2D), and the resulting pattern provided reference points to assist in the interpretation of the mutational shifts. In all three *gr* mutants the shifts in the pI of P34 were found to be different from each other (Fig. 2). Thus, the P34 of *ts*$^+$*gr* (Fig. 2A) was more basic than that of *ts*$^+$*gs*, whereas acidic shifts of differing magnitude were detected in the P34 of *ts*34*gr* (Fig. 2B) and *ts*$^+$*gr*2 (Fig. 2C). One of the mutants, *ts*34*gr*, was a double mutant, but its charge change was attributed to the *gr* rather than to the *ts* mutation, since the polypeptide pattern produced by *ts*34*gs*, the parent of *ts*34*gr*, was identical to that of *ts*$^+$*gs* (data not shown).

As expected, the pI of P34 varied in exactly the same manner as that of its precursor polypeptide, P52 (Fig. 2). Close examination of the

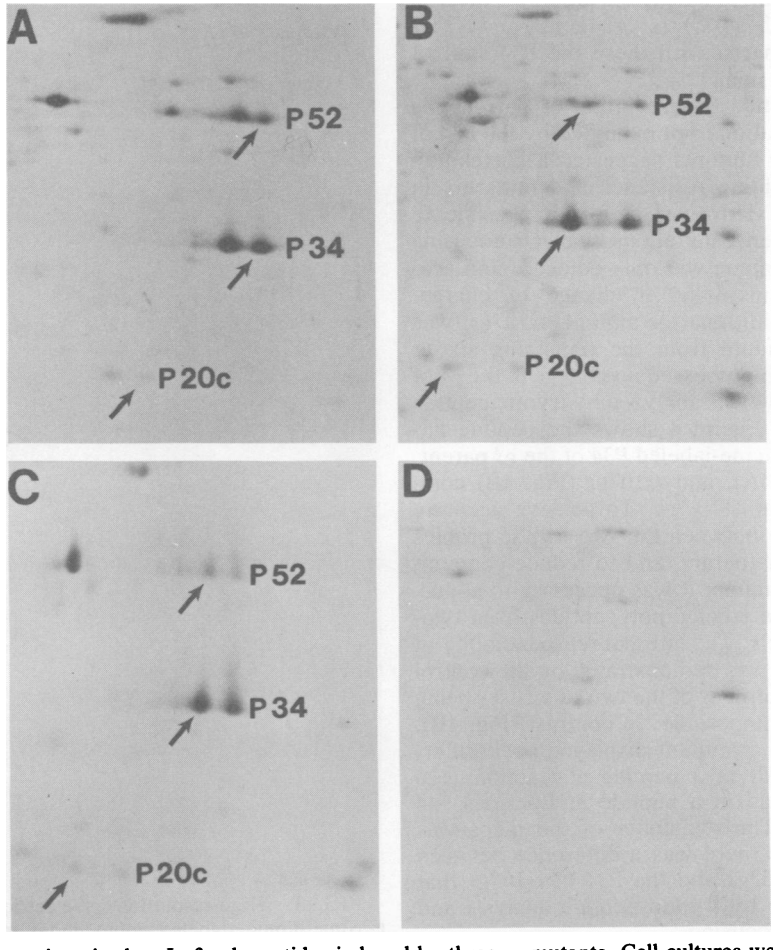

FIG. 2. Alterations in the pI of polypeptides induced by three *gr* mutants. Cell cultures were infected with individual viruses, and their cytoplasmic extracts were mixed and subjected to two-dimensional electrophoresis as in Fig. 1. Panels: A, *ts*$^+$*gs* and *ts*$^+$*gr*1; B, *ts*$^+$*gs* and *ts*34*gr*; C, *ts*$^+$*gs* and *ts*$^+$*gr*2; D, uninfected BHK cells. The figure shows the middle portion of each autoradiograph, and arrows indicate polypeptides not present in *ts*$^+$ *gs* extracts only.

two-dimensional gels revealed that the previously unmapped polypeptide P20c also carried these charge-change mutations. This shows that P20c and P34 are derived from the same region of P52. The relative intensity of labeling of P20c declined during a 30-min chase with unlabeled methionine (data not shown). Unlike P34, P20c thus appeared to be an unstable polypeptide.

High-resolution isoelectric focusing of P34. High-resolution isoelectric focusing was used to screen the *gr* mutants for very slight deviations in the pI of P34. Polypeptide extracts were analyzed by electrophoresis in long gels containing a narrow pH range of Ampholine ampholytes (pH 7 to 9). Under these conditions P34 focused approximately in the middle of the gel. Mutant and parental viruses were compared by analyzing mixtures in one dimension. By this means one additional *gr* mutant (*ts*58*gr*) was found to induce the synthesis of an altered P34 (Fig. 3, gel E). For comparison Fig. 3 also shows the P34 shifts of ts^+gr1 (gel C), ts^+gr2 (gel B) and *ts*34*gr* (gel D). Compared with these the P34 shift of *ts*58*gr* is very small.

Tryptic peptide fingerprinting of polypeptide P34. The alteration in pI exhibited by the P34 of four of the *gr* mutants suggested a correlation between guanidine resistance and mutation in the P34 gene. However, it is not possible to locate all missense mutations by electrofocusing since the technique will only detect amino acid substitutions involving a change of charge. Hence, one additional *gr* mutant, *ts*107*gr*, was chosen at random from the remaining six *gr* mutants, which possessed no change in the pI of P34, and its P34 was analyzed by tryptic peptide fingerprinting. Figure 4 shows the peptide fingerprints of leucine-labeled P34 of the *gs* parent, *ts*107*gs* (Fig. 4A), and *ts*107*gr* (Fig. 4B) compared with that of ts^+gs. To achieve accurate reproducibility between the two tryptic profiles of each chromatogram, and to reduce contaminants to a minimum, it was necessary to copurify each pair of labeled polypeptides from two-dimensional gels. The internal reproducibility of this technique was demonstrated by the control (Fig. 4A), the profiles of the two *gs* viruses being virtually superimposable. In contrast (Fig. 4B), the P34 of the *gr* mutant displayed peak differences (arrowed) in a peptide at fractions 120 through 126 and in a peptide at fractions 144 through 157. The remainder of the maps was similar. Hence there was a difference between the P34 of *ts*107*gs* and the P34 of *ts*107*gr* that was detectable by tryptic peptide analysis and not by electrofocusing.

DISCUSSION

Examination of polypeptides induced by *gr* mutants indicates a predominance of alterations in the nonstructural polypeptide P34. Since each *gr* mutation arose spontaneously during only one or two passages in the presence of guanidine, it is likely that a single mutation was responsible for both guanidine resistance and the alteration to P34. Guanidine-sensitive revertants of the *gr* mutants could not be studied owing to the lack of any selection procedure for isolating them. Therefore, it was not possible to confirm the physical location of *gr* mutations by the covariant reversion test as was done previously for *ts* mutations (12, 18). Nevertheless there are several reasons for believing that the P34 gene is a frequent, and possibly the only, site of mutation to guanidine resistance. (i) No change in any other polypeptide was observed except in polypeptides P20c and P52, which are

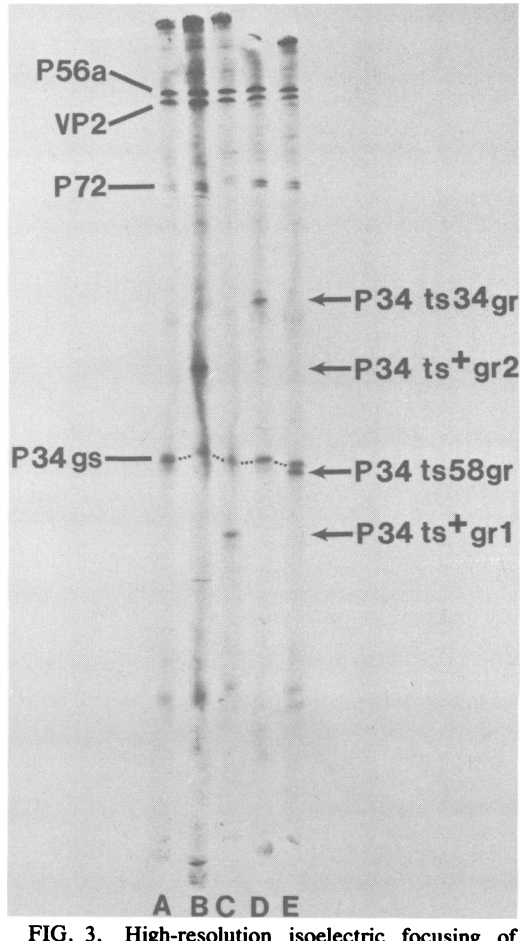

FIG. 3. High-resolution isoelectric focusing of [^{35}S]methionine-labeled polypeptides induced by (A) ts^+gs, (B) ts^+gs and ts^+gr2, (C) ts^+gs and ts^+gr1, (D) ts^+gs and *ts*34*gr*, and (E) ts^+gs and *ts*58*gr*. Labeling was for 30 min followed by a chase for 30 min with unlabeled methionine. Electrophoresis was as described in the text, with the origin (anode) at the top.

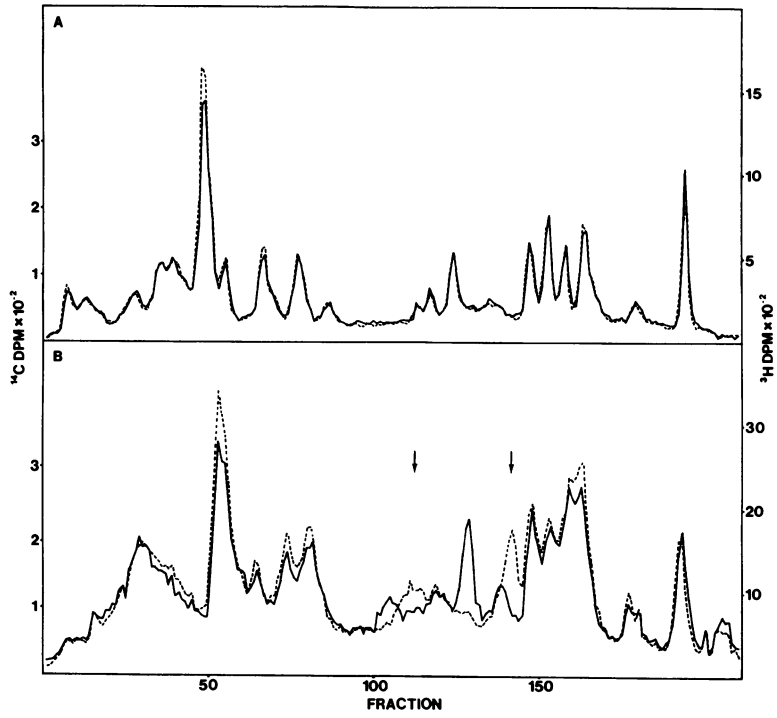

FIG. 4. Comparison by tryptic peptide fingerprinting of [^{14}C]leucine-labeled P34 of ts^+gs (----------) with [^{3}H]leucine-labeled P34 (———) of (A) *ts*107*gs* and (B) *ts*107*gr*.

related to P34. (ii) Alterations in P34 are extremely rare among other types of mutant. Thus none of the 70 *ts* mutations of the aphthovirus genetic map exhibits an alteration in P34 (unpublished observations), and the pI of P34 is highly conserved in evolution (15). (iii) Four of 10 *gr* mutations were associated with changes in the pI of P34. This is consistent with the fact that only one-third of all possible missense mutations causes a change in amino acid charge (24). (iv) The inference that the remaining *gr* mutations may also have been carried by P34 was confirmed, for one of them, by tryptic peptide fingerprinting.

Genetic data are also consistent with the location of *gr* in the P34 gene. Studies of genetic recombination between mutants of aphthovirus led to the construction of a genetic map in which *gr* was located near the center (17, 20). The *gr* mutants used in these recombination studies were derived from ts^+gr1 (Fig. 2) and possessed the same alteration in P34 (data not shown). A possible alignment of the genetic and biochemical maps has recently been proposed, based on the genetic loci of *ts* mutations carried by the capsid proteins (14) and the virus-induced RNA polymerase (18; for review, see reference 19). According to this alignment, the genetic locus of *gr* agrees well with its physical location in the P34-coding region of the genome. This location is also consistent with the reported properties of *ts*16 which lies close to *gr* on the genetic map. This chemically induced *ts* mutant exhibits a slightly reduced sensitivity to guanidine (20), which as we have shown covaries with temperature sensitivity in a high proportion of ts^+ revertants (data not shown). It was shown recently by biochemical analysis of genetic recombinants that *ts*16 is located on the 3′ side of the VP1-coding region (12), suggesting that the altered sensitivities of this mutant are conferred by a mutation in one of the nonstructural polypeptide genes. This approach is currently being used to map physically the six spontaneous *gr* mutations studied here which did not produce a change in the pI of any polypeptide.

Evidence reviewed earlier that *gr* mutations of poliovirus are carried by capsid proteins (9, 10, 16) conflicts with the results we obtained with aphthovirus. It is possible that guanidine-resistance is conferred by changes in different gene products in these two virus genera. However, the biochemical evidence suggests that the mode of action of guanidine on poliovirus replication is the same as in aphthovirus. If this analogy is correct *gr* mutations of poliovirus would be expected to be carried by polypeptide NCVPX. It is not difficult to reconcile the genetic informa-

tion on the locus of *gr* with this hypothesis since the genetic maps of poliovirus and aphthovirus are quite similar to each other. Alterations to NCVPX do not appear to have been sought among *gr* mutants of poliovirus; reported structural modifications in *gr* mutants (16) could have been due to second-site mutations, which frequently arise in multiply passaged stocks of virus.

In both poliovirus (10) and aphthovirus (D. McCahon and W. R. Slade, unpublished results) capsid protein mutants with altered guanidine sensitivity have been found, and this sensitivity covaries with the *ts* mutation. These mutants provide the strongest evidence of a link between capsid protein and the action of guanidine. However, it should be noted that, unlike the *gr* mutations studied here, structural mutations only increase the inhibitory effect of guanidine. The properties of deletion mutants which lack capsid proteins (8) suggest that structural defects have only a secondary effect on the response of picornaviruses to guanidine.

The function of the major protein encoded in the middle of the picornavirus genome (P34 in aphthovirus and NCVPX in poliovirus) is unknown. Evidence presented in this paper implicates P34 as the primary site of action of guanidine, and this suggests that continued RNA synthesis is dependent on the function of P34. In poliovirus-infected cells NCVPX is found associated with replication complexes bound to smooth membranes (5, 6). In the presence of guanidine, replicating structures are lost from smooth membranes (25). This suggests that the function of NCVPX may include binding RNA polymerase to the membrane, and that guanidine blocks in vivo replication by disrupting this bond.

ACKNOWLEDGMENTS

We thank D. McCahon, D. V. Sangar and T. Doel for constructive discussions, J. W. I. Newman for unpublished data, and W. R. Slade and J. R. Lake for isolation of some of the mutants.

K.S. is the holder of an Agricultural Research Council (Great Britain) studentship.

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