Host Factor for Coliphage Q β RNA Replication as an Aid in Elucidating Phylogenetic Relationships: The Genus *Pseudomonas*

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Host Factor (HF) is a heat-stable RNA-binding protein required along with Q β replicase for *in vitro* transcription of coliphage Q β RNA. We have found that HF activity and antigenicity are conserved among certain Gram-negative bacterial species. We examined selected species within the genus *Pseudomonas* for the presence of the HF polypeptide's antigenicity and Q β RNA replication function. While we were unable to detect either of these in *Pseudomonas diminuta* or *Pseudomonas vesicularis*, the other eleven species tested contained cross-reacting material to *Escherichia coli* HF. Furthermore, in six of these eleven species we were able to detect HF activity. The detection of HF structure and function allowed the examined species to be grouped into three categories which we have called 'sets'. The results correlate well with those of previous studies on ribosomal RNA homology (Palleroni *et al.*, 1973).

INTRODUCTION

The elucidation of phylogenetic relationships among bacterial species is a complex and confusing problem. The lack of a fossil record has forced the study of bacterial evolution to rely on an examination of the degree of similarity among extant organisms. Taxonomic schemes based upon studies of morphology, nucleic acid homologies and intermediary metabolism have not always resulted in the unambiguous elucidation of phylogenetic relationships (Palleroni & Doudoroff, 1972). One such group whose phylogeny has not proven amenable to these approaches is the genus *Pseudomonas*. Palleroni *et al.* (1973) have, on the basis of ribosomal RNA homology studies, divided the genus into five groups some of which appear to be as related to *Escherichia coli* as they are to each other. Clearly, other approaches are desirable in order to unravel the taxonomic inconsistencies inherent in such a large, artificially created collection of species.

One approach is to examine the evolutionary conservation of the structure and function of proteins (see, for example, Patel & Ornston, 1976). We have found that Host Factor (HF) for coliphage Q β RNA replication appears to have the correct 'range' to assist in the determination of the phylogeny of the genus *Pseudomonas*.

Q β is one of the small, sex-specific RNA coliphages. It replicates its single-stranded RNA genome using the four-subunit enzyme, Q β replicase (for a review, see Zinder, 1975). Only the polypeptide called synthetase is specified by the Q β genome. The other three subunits are present in uninfected *E. coli* and normally perform roles in protein synthesis. In addition, a fourth protein present in uninfected *E. coli* is required for the initiation of transcription

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of Q β RNA (Shapiro, Franze de Fernandez & August, 1968; Franze de Fernandez, Eoyang & August, 1968). This protein, known as Host Factor (HF), is a 12 000 molecular weight polypeptide, usually found as a hexamer, that is stable at temperatures up to 90 °C for 5 min (Franze de Fernandez, Hayward & August, 1972). In uninfected *E. coli*, the great majority of HF is found associated with the 30S ribosomal subunit (Carmichael *et al.*, 1975; DuBow *et al.*, 1977). HF is an RNA-binding protein that binds tightly to and inhibits the *in vitro* translation of poly(A), but not poly(U) (Carmichael *et al.*, 1975). In addition, HF binds to R17 and Q β phage RNAs through the apparent recognition sequence AAUAAA (Senear & Steitz, 1976).

We have previously reported the presence in *Pseudomonas putida* of an 11 000 molecular weight polypeptide that cross-reacts serologically with *E. coli* HF and is equally active in allowing *in vitro* Q β RNA replication (DuBow & Blumenthal, 1975). In order to determine the evolutionary conservation of HF, we screened a number of different bacterial species for the presence of HF. We found that HF was present in some, but not all, Gram-negative species (DuBow *et al.*, 1977) but could not be detected in any Gram-positive species tested. It therefore seemed reasonable that the presence or absence of HF among certain Gram-negative bacterial species might be a useful tool in contributing toward an understanding of phylogenetic relationships.

The genus *Pseudomonas* was chosen for this study because it has been extensively studied and because it remains a heterogeneous collection of species whose evolutionary taxonomy is largely unresolved (Palleroni & Doudoroff, 1972). On the basis of HF activity and antigenicity we have found that the genus can be divided into three 'sets'.

METHODS

Materials. Bacteriological culture media were from Difco. Nucleoside triphosphates were from P-L Biochemicals (Milwaukee, Wisconsin, U.S.A.). Dithioerythritol was from Pierce Chemical (Rockford, Illinois, U.S.A.). Toluene-Omnifluor and [5,6-³H]UTP (36.84 Ci mmol⁻¹) were from New England Nuclear. Trizma base and phenylmethylsulphonylfluoride were from Sigma. DNAase (RNAase-free) was from Worthington. Q β RNA, made by the method of Yamamoto *et al.* (1970), was a gift of Stanley Brown of this laboratory. Q β replicase was prepared according to Kamen (1972) with modifications described previously (Blumenthal, Landers & Weber, 1972). *Escherichia coli* HF was purified from a high-salt ribosomal wash (Nishizuka, Lipmann & Lucas-Lenard, 1968) according to Kamen *et al.* (1972). *Escherichia coli* HF antiserum was a gift of Gordon Carmichael.

Assays. HF assays and Ouchterlony immunodiffusion analyses were performed as described previously (DuBow & Blumenthal, 1975). Crude extracts (S-30) and heat-treated crude extracts were prepared according to DuBow *et al.* (1977).

Organisms and growth. Bacterial strains and their sources are listed in Table 1. All species (with the exception of *Pseudomonas acidovorans*) were grown on 2 $\times$ broth (16 g nutrient broth, 4 g NaCl and 1 g D-glucose per litre H₂O). *Pseudomonas acidovorans* was grown on L-broth (10 g tryptone, 10 g NaCl and 5 g nutrient broth per litre H₂O). All organisms were harvested at the late-exponential phase of growth by centrifuging in a Sorvall GSA rotor at 9000 rev. min⁻¹ for 20 min at 4 °C.

RESULTS

Detection of HF antigen

Purified *P. putida* HF forms a band of partial identity in an Ouchterlony immunodiffusion plate when tested against *E. coli* HF using *E. coli* HF antiserum (DuBow & Blumenthal, 1975). A band of partial identity is also formed if S-30 fractions are used instead of the purified proteins (DuBow *et al.*, 1977). The S-30 fractions from the *Pseudomonas* species listed in Table 1 were tested for the presence of antigenic material which would cross-react with antiserum prepared against purified *E. coli* HF. We found that S-30 fractions from all but two of the species tested contained cross-reacting material (Fig. 1). The S-30 fractions from *P. vesicularis* and *P. diminuta* did not contain detectable *E. coli* HF cross-reacting

Table 1. *Host factor (HF) activity and antigenicity in pseudomonads*

HF activity and antigenicity were determined as described by DuBow & Blumenthal (1975).

Set	Species	Strain no.	ATCC no.	Source*	HF antigen†	HF activity‡
A	<i>Pseudomonas aeruginosa</i>	131	17503	Indiana	+	+
	<i>Pseudomonas fluorescens</i>	E38	13985	Berkeley	+	+
	<i>Pseudomonas putida</i>	A90	12633	Indiana	+	+
	<i>Pseudomonas stutzeri</i>	221	17588	Berkeley	+	+
	<i>Pseudomonas maltophilia</i>	67	13637	Berkeley	+	+
	<i>Xanthomonas vesicatoria</i>	1127		Purdue	+	+
B	<i>Pseudomonas delafieldii</i>	134	17506	Berkeley	+	ND
	<i>Pseudomonas facilis</i>	332	17695	Berkeley	+	ND
	<i>Pseudomonas acidovorans</i>	14	15668	Berkeley	+	ND
	<i>Pseudomonas cepacia</i>	382	17759	Berkeley	+	ND
	<i>Pseudomonas solanacearum</i>	1047		Purdue	+	ND
C	<i>Pseudomonas diminuta</i>	501	11568	Berkeley	—	—
	<i>Pseudomonas vesicularis</i>	500	11426	ATCC	—	—

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† +, Present; — absent.

‡ +, At least an eightfold increase in incorporation into acid-precipitable material over background; —, no detectable increase in incorporation over background; ND, not determinable (see text).

material (Fig. 2). To investigate the possibility of the presence of an inhibitor in these two S-30 fractions, purified *E. coli* HF was added to each fraction to yield a final concentration equivalent to that present in an *E. coli* S-30 fraction. The S-30 fractions from these two species containing exogenous *E. coli* HF formed bands of complete identity in an Ouchterlony immunodiffusion plate when tested against an S-30 fraction from *E. coli* (Fig. 2).

HF activity in heat-treated crude extracts

We have previously shown that the ability of a heat-treated crude extract from *P. putida* to stimulate Q β RNA replication *in vitro* by Q β replicase is due to the presence of the HF polypeptide (DuBow & Blumenthal, 1975). RNA synthesis is totally dependent on the presence of both Q β RNA and Q β replicase. We have now tested heat-treated crude extracts from other *Pseudomonas* species for functional activity in this assay (Table 1).

The S-30 fractions from *P. diminuta* and *P. vesicularis* (set C) did not contain detectable HF antigen and their heat-treated crude extracts failed to stimulate Q β RNA replication *in vitro*. However, when saturating amounts of purified *E. coli* HF were added to the reaction, Q β RNA replication was almost fully stimulated. Thus, no inhibitor is present.

Heat-treated crude extracts from the other eleven species, all of whose S-30 fractions contained detectable HF antigen, were also tested for their ability to stimulate Q β RNA replication *in vitro*. We were able to detect HF activity in six of these eleven species (set A). The heat-treated crude extracts from the other five species failed to stimulate Q β RNA replication *in vitro*. However, we were also unable to detect *in vitro* Q β RNA replication above the background level with these heat-treated crude extracts when saturating amounts of purified *E. coli* HF were added to the reactions. Thus, it seems that the S-30 fractions from these five species contain a heat-stable inhibitor of the Q β RNA replication reaction (set B).

Fig. 1. HF antigenicity in sets A and B. Ouchterlony double diffusion analysis was performed as described previously (DuBow & Blumenthal, 1975). The centre wells each contained $20\ \mu\text{l}$ of antiserum (A) prepared against purified *E. coli* HF. The outer wells contained $50\ \mu\text{l}$ of S-30 fractions from the following organisms: 1, *E. coli*; 2, *P. cepacia*; 3, *P. solanacearum*; 4, *P. aeruginosa*; 5, *P. stutzeri*; 6, *P. fluorescens*; 7, *Xanthomonas vesicatoria*; 8, *P. maltophilia*; 9, *P. acidovorans*; 10, *P. facilis*; 11, *P. delafieldii*.

Fig. 2. HF antigenicity in *P. vesicularis* and *P. diminuta* (set C). Ouchterlony double diffusion analysis was performed as described previously (DuBow & Blumenthal, 1975). The centre well contained $10\ \mu\text{l}$ of antiserum (A) prepared against purified *E. coli* HF. The outer wells contained: 1 and 4, $20\ \mu\text{l}$ S-30 fraction from *E. coli*; 2, $20\ \mu\text{l}$ S-30 fraction from *P. diminuta*; 3, $20\ \mu\text{l}$ S-30 fraction from *P. diminuta* containing $0.1\ \mu\text{g}$ purified *E. coli* HF; 5, $20\ \mu\text{l}$ S-30 fraction from *P. vesicularis* containing $0.1\ \mu\text{g}$ purified *E. coli* HF; 6, $20\ \mu\text{l}$ S-30 fraction from *P. vesicularis*.

DISCUSSION

We have previously shown that the presence of Host Factor (HF) for coliphage Q β RNA replication appears to be a useful criterion in the elucidation of phylogenetic relationships among some Gram-negative bacterial species (DuBow & Blumenthal, 1975; DuBow *et al.*, 1977). On the basis of our detection of HF antigen and Q β RNA replication activity within certain species of the genus *Pseudomonas*, we are able to distinguish three categories which we refer to as 'sets'.

By rRNA homology studies, Palleroni *et al.* (1973) divided the genus *Pseudomonas* into five 'groups'. They included in their study representative species of the genus *Xanthomonas* which, by some criteria, appear closely related to the pseudomonads. Palleroni *et al.* (1973) also found that some of the 'groups' within the genus *Pseudomonas* are, by rRNA homology, as closely related to *E. coli* as they are to each other, a finding that supports the contention that this genus is an artificial and heterogeneous grouping. It is apparent that our sets A and B correspond to their closely related groups (I and V) and (II and III) respectively. In all of these species, we were able to detect antigenic determinants that would cross-react with *E. coli* HF antiserum.

Palleroni *et al.* (1973) suggested that the five rRNA homology groups exhibit a sufficient degree of unrelatedness to warrant the creation of separate genera. The existence of enough known phenotypic characters to distinguish all the species from one group from those of another is perhaps only present for *P. diminuta* and *P. vesicularis* (our set C; Palleroni *et al.*, 1973, group IV) which have some unique properties. For example, *P. diminuta* and *P. vesicularis* are two of only three pseudomonads that require growth factors (Palleroni & Doudoroff, 1972). Perhaps the inclusion of these two species within the genus *Pseudomonas* does not reflect true phylogenetic relationships but, rather, may be due to convergent evolution.

HF is an RNA-binding protein that is found associated with ribosomes in *E. coli* (Carmichael *et al.*, 1975) and *P. putida* (DuBow & Blumenthal, 1975). In *E. coli* we have found that HF is associated with the 30S ribosomal subunit (DuBow *et al.*, 1977). It is interesting to note that the conservation of HF parallels that of the 3'-terminal sequence of prokaryotic 16S rRNA (Shine & Dalgarno, 1975) and translational specificity of ribosomes (Stallcup, Sharrock & Rabinowitz, 1974), which has been shown to be determined by the 30S ribosomal subunit (Leffler & Szer, 1973). It is thus not surprising that our results on the conservation of the HF polypeptide correlate well with the rRNA homology studies of Palleroni *et al.* (1973).

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