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Effect of Type 1 Piliation on In Vitro Killing of *Escherichia coli* by Mouse Peritoneal Macrophages

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***Escherichia coli* K-12 mutants possessing defined lesions affecting type 1 pilus production, receptor binding, or length were examined for their ability to resist killing by mouse peritoneal macrophages in vitro. Mutants were mixed pairwise at known ratios in wells containing macrophages, and after incubation, the ratio of the survivors was assayed. The difference in phagocytic killing between type 1 piliated cells and isogenic nonpiliated cells was significant, the piliated cells being approximately threefold more resistant. Pilus length had little effect upon survival, as the long-piliated mutants were no more resistant to killing than the normal-length parents. Interestingly, the receptor-binding function of type 1 pili was most important in effecting resistance, as mutants lacking the ability to bind receptor were killed as effectively as nonpiliated mutants. These data are consistent with the notion that pili actually impede killing by macrophages rather than serve as passive physical barriers to uptake.**

Type 1 pili (fimbriae) of *Escherichia coli* are organelles that are involved in effecting colonization by binding to host epithelial cells through a mannose-sensitive interaction with a receptor on the host cell (reviewed in reference 23). Considerable evidence suggests that the binding is carried out by a minor component of the pilus, the product of the *pilE* (also called *fimH*) gene (1, 11, 14, 15).

Although the role of type 1 piliation as a virulence factor has been questioned because of the ubiquity of the organelles (7), epidemiological evidence associates these pili more frequently with *E. coli* isolates from extraintestinal infections than with commensal isolates (10). Experimental evidence also associates the organelles with an increased ability of *E. coli* to colonize extraintestinal sites (5, 9, 14).

A paradigm that has emerged for the role of type 1 piliation as a virulence factor is that the pili, while being an advantage to the bacterium in colonizing mucosal surfaces, are a disadvantage once the bacterium is in the blood or interstitial fluids. This is because it is imagined that the pili, in effect, tether the bacteria to phagocytic cells, resulting in ingestion (3, 8). This process is nonopsonic and has been described as a type of lectinophagocytosis (19).

Our initial purpose in undertaking the experiments herein described was to confirm and extend the role of type 1 pili in the phagocytic process by taking advantage of the recently acquired ability to construct mutants with different pilus morphologies and receptor-binding capabilities (15, 17). Such mutants had the potential for permitting a more precise probing of the pilus-phagocyte interaction. To our surprise, we found that type 1 piliation provided an advantage to the bacterium in avoiding killing by unelicited mouse peritoneal macrophages. This ability was dependent upon the pili having receptor-binding capabilities and was independent of the length of the pilus.

The bacterial mutants employed here are all *E. coli* K-12 derivatives that have specific lesions in genes involved in type 1 piliation (Table 1). The mutants exhibit three basic piliation phenotypes, each of which is associated with a defined lesion in a single gene: (i) the nonpiliated mutant contains the neomycin phosphotransferase gene inserted in the *pilA* (also called *fimA*) gene which encodes the pilin monomer that makes up the pilus shaft, (ii) the receptor-binding-defective mutant contains a Tn*5* insertion in the *pilE* gene (the length of the pilus is also increased somewhat by this lesion), (iii) the long-piliated mutant contains a defined deletion in the *pilD* (also called *fimG*) gene. This gene is adjacent to the *pilE* gene. All lesions are in the chromosome, and thus, the phenotypes exhibited are what a normal *E. coli* mutant would present to a phagocyte. In addition to the *E. coli* K-12 strains, a K1 capsular strain (5) was employed as a positive control in devising the phagocytosis assay.

The relevant properties of the mutants used in the assay are shown in Table 2. Since the strains were mixed in the assays, each strain bearing a given piliation lesion had two versions: one which could utilize maltose and one that could not. Table 2 also shows pictograms of each of the mutant phenotypes. Electron micrographs showing the actual piliation phenotypes of the parental and mutant strains are presented in Fig. 1.

The assay used to assess phagocytosis employed unelicited mouse peritoneal macrophages. Bacterial cells were grown overnight in L broth (18) and diluted in tissue culture medium to produce a bacterium-to-macrophage ratio of ca. 1 to 10. Each well contained ca. 10^6 mouse peritoneal macrophages that had been harvested by peritoneal lavage of five to seven CD-1 female mice (Charles River) and which had been allowed to attach to microtiter plates overnight in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum. Prior to the assay, the medium was removed and replaced by identical medium containing the bacteriostatic antibiotic spectinomycin (3 μg/ml) to limit growth. Bacteria were then added, and a sample was immediately removed, diluted, and plated on maltose tetrazolium agar (24) to obtain preincubation CFU. The microtiter plates were then subjected to a brief centrifugation (15 min, 400 × *g*) to concentrate the bacteria and incubated for 5 h at 37°C.

A summary of incubation conditions, scoring, representative results, and the mathematical handling of the data

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TABLE 1. Bacterial strains

Strain	Description	Source or reference
ORN115	*thr-1 leuB thi-1* Δ(*argF-lac*)*U169 malA xyl-7 ara-13 mtl-1 gal-6 rpsL fhuA2 supE44 pilG* Pil$^+$ (does not exhibit phase variation of piliation)	22
CAB1	018ac:K1:H7, requires nicotinamate for growth	5
ORN105	*thr leu proA2* Δ(*argF-lac*)*U169 galK his argE rpsL supE mtl xyl recBC sbcB* Pil$^+$ Tet^r (has Tn*10* between *hsd* and *serB*)	22
ORN175	same as ORN115, except Mal$^+$	P1 transduction from ORN105
TST1	*araD139* Δ(*argF-lac*)*205 flbB5301 ptsF25 relA1 rpsL150 malE52*::Tn*10 deoC1* λ$^-$	B. Bachmann
ORN176	Same as CAB1, except *malE52*::Tn*10*	P1 transduction from TST1
ORN147	Same as ORN115, except *pilA'-kan*	17
ORN177	Same as ORN147, except Mal$^+$	P1 transduction from ORN105
ORN178[a]	Same as ORN115, except *tetR* gene from Tn*10* adjacent to *pilE*	P. W. Russell and P. E. Orndorff, unpublished data
ORN179	Same as ORN178, except Mal$^+$	P1 transduction from ORN178 to ORN177 (selected for Tet^r, scored for Kan^s)
ORN180	Same as ORN178, except with a 462-base-pair deletion of *pilD*. Produces long pili	P. W. Russell and P. E. Orndorff, unpublished data
ORN181	Same as ORN180, except Mal$^+$	P1 transduction from ORN180 to ORN177 (selected for Tet^r score Kan^s)
ORN140	Same as ORN115, except *pilE104*::Tn*5* (formerly called *pilF104*::Tn*5*)	17
ORN182	Same as ORN140, except Mal$^+$	P1 transduction from ORN105

[a] ORN178 was constructed from ORN174 (5) by P1 transduction.

is illustrated in Fig. 2. Potential synergistic effects of mixing mutants were taken into account by (i) including separate control wells with individual mutants and (ii) employing a high macrophage-to-bacterium ratio in the assays. In all cases, the results obtained with the pairwise mixtures were statistically the same as the results obtained with separate wells for each individual mutant. Possible nonspecific killing by macrophages (i.e., killing by the products of macrophage metabolism) was assessed by prelysing the macrophages with 0.1% Triton X-100 (at this concentration, Triton X-100 has no effect upon the bacteria) and incubating the bacteria with the lysed macrophages. In all cases, no effect of the lysed macrophage products could be detected. Furthermore electron microscopy revealed that there was an active uptake of all bacterial strains under the conditions employed (Fig. 3).

TABLE 2. Relevant phenotypic characteristics of bacterial strains tested

Strain	Capsule Type	Pili	Receptor Binding[a]	Pilius Length[b]	pil Gene Affected	Use of Maltose[c]	Pictogram
ORN115	K12	+	+	N	(wt)	−	
ORN175	K12	+	+	N	(wt)	+	
ORN178	K12	+	+	N	(wt)[d]	−	
ORN179	K12	+	+	N	(wt)	+	
ORN147	K12	−	−	−	pilA	−	
ORN177	K12	−	−	−	pilA	+	
ORN180	K12	+	+	L	pilD[e]	−	
ORN181	K12	+	+	L	pilD	+	
ORN140	K12	+	−	L	pilE	−	
ORN182	K12	+	−	L	pilE	+	
ORN176	K1	+	+	N	(wt)	−	
CAB1	K1	+	+	N	(wt)	+	

[a] Ability to agglutinate guinea pig erythrocytes.
[b] N, Normal length; −, no pili; L, long pili.
[c] Ability to utilize maltose.
[d] Strains ORN178 and ORN179 contain the tetracycline resistance gene (*tetR*) adjacent to *pilE*. This insertion does not affect piliation, and thus the wild-type designation (wt) is given. The *tetR* gene was used as a marker to construct strains ORN180 and ORN181, which also contain the *tetR* gene in the same location.
[e] Strains ORN180 and ORN181 contain a defined deletion of the *pilD* gene (also called the *fimG* gene [15]). Details of this construction are to be described elsewhere (P. W. Russell and P. E. Orndorff, unpublished data).

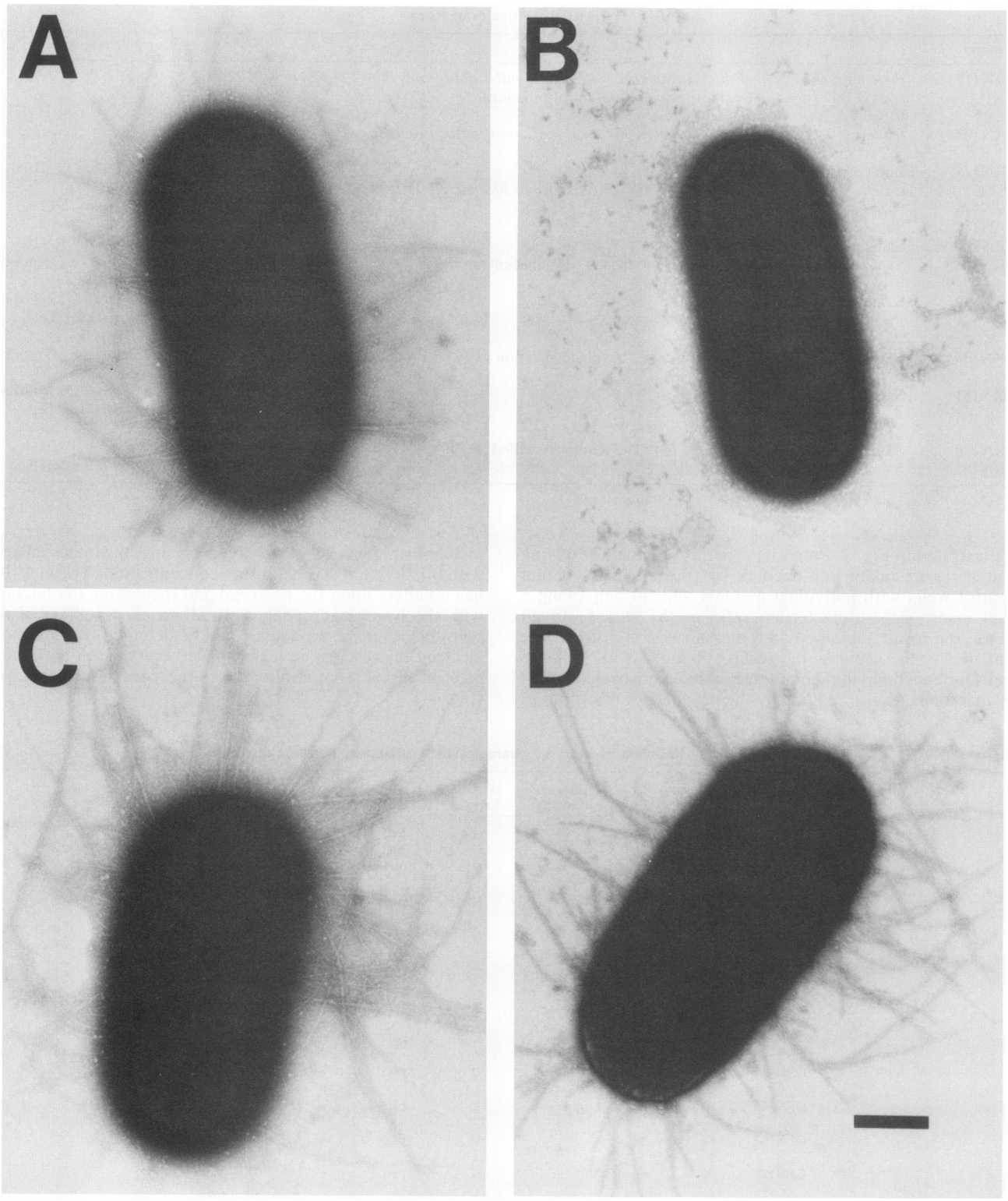
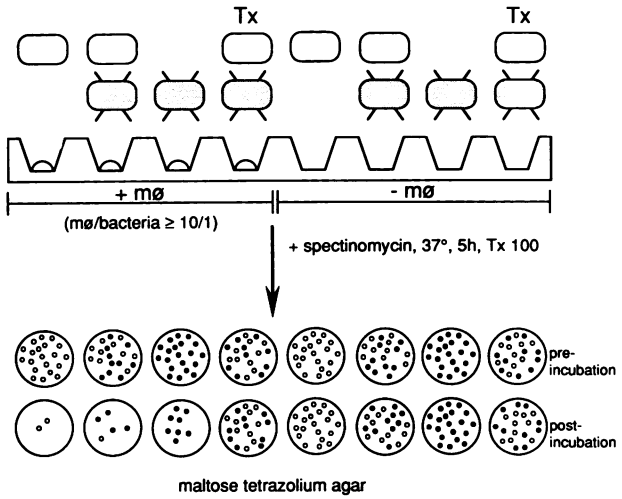


FIG. 1. Electron micrographs showing piliation phenotypes of *E. coli* K-12 strains used. (A) ORN115 (parental piliation); (B) ORN147 (*pilA*); (C) ORN180 (*pilD*); (D) ORN140 (*pilE*). Bar, 0.5 μm.

Depending upon the particular experiment, between 60 and 99% of the bacteria were killed during the incubation, the length of which was maximized to give the highest degree of killing. Although the degree of killing was noted in each experiment, the change in ratio observed after incubation remained similar regardless of the degree of killing—at least in the 60 to 99% range that we noted.

The results of assays using specific bacterial mutants are



$$\frac{\sum_{i=1}^{n}\left(\frac{\#\,mal^-\,post}{\#\,mal^-\,pre}\Big/\frac{\#\,mal^+\,post}{\#\,mal^+\,pre}\right)_i}{n}\ (+m\phi) \quad \times \quad \frac{\sum_{i=1}^{n}\left(\frac{\#\,mal^-\,pre}{\#\,mal^-\,post}\Big/\frac{\#\,mal^+\,pre}{\#\,mal^+\,post}\right)_i}{n}\ (-m\phi)$$

FIG. 2. Summary of the macrophage killing assay, representative results, and mathematical treatment of the data. Bacterial cells alone and in pairwise mixtures were added to 1-cm microtiter wells with (+) and without (−) macrophages (mϕ). In two of the wells, Triton X-100 (Tx) was added (final concentration, 0.1%) to kill the macrophages prior to the beginning of the assay. In the example shown, the piliated parental strain and a nonpiliated mutant were tested; white cells represent a Mal^+ strain, and gray cells represent a Mal^- strain. After incubation, Triton X-100 was added to each well to a final concentration of 0.1%, the contents were mixed, and dilutions were plated. Results pre- and postincubation are shown as being representative, although up to one generation of growth was observed in the absence of macrophages. The mathematical treatment of the data is shown at the bottom; it consists of a comparison of the numbers (#) of Mal^- and Mal^+ microorganisms surviving the incubation with macrophages, followed by a normalization of these results by multiplication to the identical, but inverse, comparison without macrophages. Thus, if both strains in the mixture were equally affected by the macrophages, the final numerical score should be 1. In most cases, $n = 4$ (i.e., the assays were performed in quadruplicate).

shown in Table 3. For convenience, we have used the pictograms as well as strain numbers to illustrate the mixtures. In set 1 (Table 3), mixtures having identical piliation phenotypes were used as a basis for comparison with mixtures comprised of cells of different piliation phenotypes (sets 2 through 8). Results deviating from unity represent an effect of the macrophages (see formula in Fig. 2). The only correction applied to the results was one to adjust for a slight and unexplained bias in the survival of Mal^- bacteria. This correction value was obtained by assaying pairwise mixtures of Mal^+ and Mal^- bacteria, identical for their piliation phenotype, noting the deviation from unity, and using this value to correct the remaining samples.

It can be seen (Table 3) that when paired with a nonpiliated mutant, parental piliation enhances resistance to macrophage killing significantly (sets 1 and 2). This effect holds if the pili are longer, but increased length does not increase

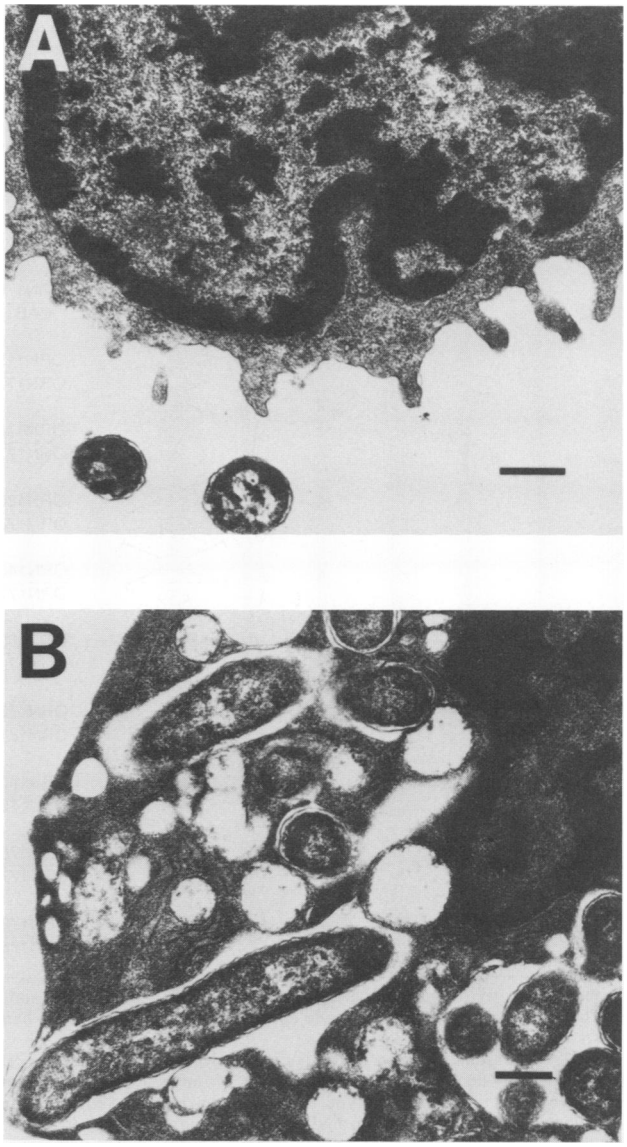
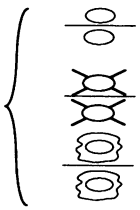
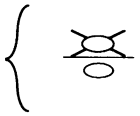
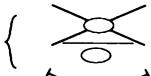
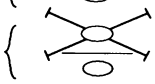
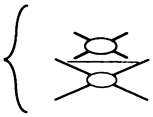
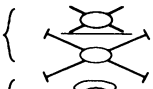


FIG. 3. Electron micrographs demonstrating the uptake of bacteria by macrophages during the assay. In this case, the macrophages and bacteria were incubated for 3.5 h at a ratio of 100 bacteria to 1 macrophage. Pre- and postincubation micrographs were obtained by first fixing the macrophages in Trump fixative and imbedding them in spurr resin. Sections were then stained in uranyl acetate (5%) and Reynold lead citrate. (A) Two cells of strain ORN147 (*pilA*) (lower left corner) outside a macrophage prior to incubation. (B) ORN147 bacteria within a macrophage at 3.5 h. Bar, 1.5 μm.

the resistance to killing over normally piliated bacteria (sets 2 and 3). In contrast, pili that lack the ability to bind receptor—and are also longer—are ineffective in resisting killing (set 4).

The above conclusions, derived from sets 1 to 4 (Table 3), hold when aberrantly piliated mutants are paired with parentally piliated cells (sets 5 and 6). That is, the long-piliated mutants were not statistically better or worse in avoiding killing than the parental cells (set 5), whereas mutants lacking receptor-binding ability were statistically worse than parentally piliated cells in avoiding killing (set 6). Thus, all sets support the notion that piliation effects resistance to

TABLE 3. Results of macrophage killing assay

Set Number	Pictograph[a]	Strain Combinations Tested[b]	Average post Incubation Ratio[c]	Standard Deviation	Significance[d]
1		ORN147/ORN177; ORN115/ORN175 vs ORN178/ORN175; ORN176/CAB1	1.0	0.27	
2		ORN115/ORN177 vs ORN147/ORN175; ORN178/ORN177 vs ORN147/ORN179	3.0	1.48	p=0.004
3		ORN180/ORN177 vs ORN147/ORN181	2.0	0.93	p=0.025
4		ORN140/ORN177 vs ORN147/ORN182	1.3	0.88	p=0.447
5		ORN180/ORN179; ORN178/ORN181 vs ORN180/ORN175	1.3	0.29	p=0.086
6		ORN140/ORN175 vs ORN115/ORN182	2.4	1.25	p=0.015
7		ORN176/ORN177 vs ORN147/CAB1	33.1	14.60	$p<10^{-4}$
8		ORN176/ORN175 vs ORN115/CAB1	20.4	0.21	$p<10^{-6}$

[a] Pictographs represent the phenotypes of mutants that were mixed and compared.

[b] The strain comparisons were made as Mal^- over Mal^+ (Fig. 2). When necessary, reciprocal values were calculated to match the pictograph designations shown in this table.

[c] Values represent the ratio of the strains shown in the pictographs. Deviation from unity represents an effect of macrophage killing, since all pre- and post-incubation values were normalized to values from incubation without macrophages, as shown in the formula in Fig. 2. In set 1, in which the strains compared were of identical piliation phenotype, a composite average is shown for convenience.

[d] Values represent the comparison of the set 1 value (average, 1.0) to the average value of each of the other sets. Values were calculated by the Student t test. Significantly different results are defined to have a value of $P < 0.05$.

killing and that receptor binding is crucial to that resistance. In support of the latter notion, we found that addition of 25 mM α-methyl-mannopyranoside (an inhibitor of pilus binding) to wells containing parentally piliated and nonpiliated cells reduced the survival-enhancing effects of normal piliation. However, the addition did not negate the advantage of piliation altogether. This could either mean that the concentration of α-methyl-mannopyranoside was not optimal or indicate a more complicated picture of the process by which pili enhance survival.

Results obtained with a K1 capsular strain of *E. coli* (5) revealed that piliation was, comparatively, a modest factor in preventing macrophage killing, as the K1 capsular variants were almost an order of magnitude more resistant to killing than even the most resistant *E. coli* K-12 (sets 7 and 8, Table 3).

The results presented herein indicate that piliation is correlated with an increased resistance to killing by unelicited mouse peritoneal macrophages. This resistance was dependent upon the receptor-binding function of the pili as *pilE* (adhesin-deficient) mutants were no more resistant to killing than nonpiliated strains. Pilus length did not appear to be a factor affecting resistance, as the long-piliated mutant strain was no more resistant than the normal-length parental strain.

Although pili may have any number of properties that could affect killing after uptake, we suspect that because of the ability of pili to effect binding to leukocytes (7), the resistance to macrophage killing is at least initiated at the uptake stage. Prevention of killing by type 1 pili could be most easily visualized as the pili physically preventing the macrophage from detecting bacterial surface components needed to signal uptake (28). However, if this were true, then we would expect the adhesin-deficient mutants to be as efficient (or more so) than the parentally piliated cells at resisting killing. Since this was not observed, we have come to the tentative conclusion that the receptor-binding function of type 1 pili actually impedes a course leading to killing. Perhaps this could be done by the adhesin stimulating a nonproductive-uptake pathway (i.e., uptake without killing)

in the macrophage (8) or a pathway preventing uptake entirely.

Our results run counter to a wealth of reports indicating that type 1 piliation is a disadvantage in a phagocyte-*E. coli* interaction in vitro (4, 6, 20, 25, 27; reviewed in reference 19). There could be a number of technical reasons for this discrepancy. For example, our assay measured ratios of bacterial populations after a comparatively long incubation with unelicited mouse peritoneal macrophages and looked strictly at killing by these cells. Measurements of absolute numbers of bacteria, shorter incubations, the use of different phagocytic populations, and other measurements of bacterium-phagocyte interaction (e.g., binding, uptake, killing, or combinations thereof) all could affect results.

With regard to the effect of piliation on *E. coli* in vivo, reports indicate that in clinical surveys (3, 19) and in experimental infections (2, 9), nonpiliated variants of *E. coli* tend to be isolated from host compartments that have active phagocytic cells. However, we wish to point out that there could be a number of reasons for this other than the often-suggested disadvantage of type 1 piliation in encounters with phagocytic cells (3, 19). In fact, nonpiliated variants predominate under most laboratory conditions that do not enrich specifically for piliated individuals (21). Consequently, arguments that intimate a disadvantage to piliated cells in certain host compartments ignore the advantage that nonpiliated cells normally have when adherence is not a factor (12).

To conclude, it is our feeling that there is no real consensus of opinion concerning the role of type 1 pili in bacterium-phagocyte interactions, since both early (16) and recent reports (8, 26, 28) question the role of type 1 pili in facilitating phagocytic killing. The assay we have developed for measuring phagocytosis employs isogenic mutants, mixed in a single sample well, at a low bacterium-to-macrophage ratio. In using the isogenic sets, we have attempted to standardize the bacterial population genetically. By using mixtures, we have provided an internal control and removed the need for absolute numbers to determine relative susceptibility to killing. Finally, by using a low bacterium-to-macrophage ratio, we have hoped to create a situation more in line with that found in vivo. In any case, the need for high bacterium-to-phagocyte ratios for cytological and other common measurements of phagocytosis (e.g., chemiluminescense, lysosome release, and protein iodination), some of which may not be indicative of uptake and killing (13, 20), is removed in this assay.

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