

SIMPLE Approach for Isolating Mutants Expressing Fimbriae[∇]

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Genomes of members of the family *Enterobacteriaceae* contain large repertoires of putative fimbrial operons. Since many of these operons are poorly expressed in vitro, a convenient method for inducing elaboration of the encoded fimbriae would greatly facilitate their functional characterization. Here we describe a new technique for identifying fimbriated bacteria from a library of transposon mutants by screening with immunomagnetic particles for ligand expression (SIMPLE). The SIMPLE method was applied to identify the T-POP mutants of *Salmonella enterica* serotype Typhimurium carrying on their surfaces filaments composed of PefA, the major subunit product of a fimbrial operon (*pef*) that is not expressed during growth in Luria-Bertani broth. Four such mutants were identified from a library of 24,000 mutants, each of which carried a T-POP insertion within the *hns* gene, which encodes a global silencer of horizontally acquired genes. Our data suggest that the SIMPLE method is an effective approach for isolating fimbriated bacteria, which can be readily applied to fimbrial operons identified by whole-genome sequencing.

Comparative genomics reveals that members of the family *Enterobacteriaceae* possess large repertoires of fimbrial gene sequences, representing hypervariable regions within their genomes (16, 29). Many of the fimbrial operons identified by whole-genome sequencing are not expressed in vitro, which is one of the reasons why only a small fraction of the encoded surface structures has been characterized functionally.

For example, sequence analysis of the enterohemorrhagic *Escherichia coli* O157:H7 isolate reveals the presence of 14 fimbrial operons (11). Construction of *lacZ* transcriptional fusions to the genes encoding the major fimbrial subunit shows that 9 of these 14 operons are not expressed under in vitro growth conditions (16). The *Salmonella enterica* serotype Typhimurium genome contains 13 fimbrial operons, termed *csg* (*agf*), *fim*, *pef*, *lpf*, *bcf*, *stf*, *saf*, *stb*, *stc*, *std*, *sth*, *sti*, and *stj* (18). Laboratory-grown cultures of *Salmonella* serotype Typhimurium commonly elaborate only type 1 fimbriae encoded by the *fim* operon (6, 7) and thin curled fimbriae (also known as thin aggregative fimbriae or curli) encoded by the *csg* gene cluster (10, 23, 28). Expression of the remaining fimbrial operons cannot be detected readily by Western blotting (12) or flow cytometry (13) after growth of *Salmonella* serotype Typhimurium under standard laboratory conditions. Although expression of most fimbrial operons is suppressed in vitro, seroconversion to fimbrial antigens (12) and phenotypes of *Salmonella* serotype Typhimurium mutants (30) during infection of mice provide indirect evidence for in vivo expression of these surface structures. The considerable gap between the

number of fimbrial structures whose function can be studied using in vitro assays ($n = 2$) and the number of fimbrial operons present in the genome ($n = 13$) has been a major obstacle for mechanistic studies of the role of adherence during *Salmonella* serotype Typhimurium pathogenesis.

The histone-like nucleoid-structuring (H-NS) protein is a global silencer of gene expression. In *Salmonella* serotype Typhimurium, H-NS silences genes with GC contents lower than that of the genomic average (i.e., genes acquired by lateral gene transfer) by restricting the access of RNA polymerase to DNA (17, 20). Mutational analysis shows that inactivation of the *hns* gene increases transcription of the *pef* operon (21). Chromatin immunoprecipitation indicates the presence of H-NS binding sites upstream of the *bcfA*, *stfA*, *safA*, *fimA*, *stbA*, *lpfA*, and *sthA* genes (17, 20). These data suggest that H-NS functions as a transcriptional repressor of multiple fimbrial operons in *Salmonella* serotype Typhimurium.

The goal of this study was to devise a screening with immunomagnetic particles for ligand expression (SIMPLE) method for identifying fimbriated bacteria within mutant libraries. This method was developed using the *pef* operon located on the *Salmonella* serotype Typhimurium virulence plasmid (8). Northern blotting analysis shows that the *pef* operon is not expressed after growth of *Salmonella* serotype Typhimurium at neutral pH, but transcription is induced under acidic growth conditions (i.e., in LB broth at pH 5.1) (21). Despite transcription of the *pef* operon in LB broth at pH 5.1, previous studies were not able to detect expression of PefA by Western blotting in *Salmonella* serotype Typhimurium grown under this condition, presumably because elaboration of fimbriae is subject to posttranscriptional control mechanisms (12). Here we describe the isolation of *Salmonella* serotype Typhimurium mutants expressing fimbriae encoded by the *pef* operon, using the SIMPLE method.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used are listed in Table 1. Unless otherwise noted, cultures were grown from a single colony,

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TABLE 1. Bacterial strains used in this study

Bacterial strain(s)	Genotype	Source or reference
<i>E. coli</i>		
DH5 α MCR	F' <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i>	Gibco BRL
ORN172 Nal ^r	<i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i> Nal ^r <i>thr-1 leuB thi-1</i> Δ (<i>argF-lac</i>)U169 <i>malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44</i> Δ <i>fimBEACDFGH::Km</i>	31
<i>Salmonella</i> serotype Typhimurium		
IR715	Wild-type isolate ATCC 14028, Nal ^r	27
EHW2	IR715 Δ <i>fimAICDHF::Km</i>	30
LT2	Laboratory strain	15
TH3923	LT2 pJS28(Carb ^r P22-9 ⁺)/F'114ts Lac ⁺ <i>zzf-20::Tn10[tetA::MudP](Tc^s)</i> <i>zzf-3823::Tn10dTc[del-25](T-POP)/leuA414 hsdSB Fels2-</i>	K. Hughes
TH4881	LT2 pNK2880(Carb ^R , Tn10 <i>tnpA</i> [<i>ats-1 ats-2</i>])/f <i>liC5050::MudJ fjb5001::MudCm</i>	22
SL1344(T-POP)	Wild-type isolate SL1344 <i>fimZ2::T-POP</i>	5
SR11	Xylose-fermenting mutant of wild-type isolate BA ₂	24
AJB3	SR11 Nal ^r	3
ADH19	SR11 Nal ^r Δ <i>pefBACD::Km</i>	13
SPN22	SR11 Nal ^r Δ <i>fimAICDHF::Km</i>	This study
SPN23	LT2 Δ <i>fimAICDHF::Km</i>	This study
SPN66 and SPN67	SR11 Nal ^r Δ <i>fimAICDHF::Km hns(-195)::T-POP</i>	This study
SPN68	SR11 Nal ^r Δ <i>fimAICDHF::Km hns(+281)::T-POP</i>	This study
SPN69	SR11 Nal ^r Δ <i>fimAICDHF::Km hns(-224)::T-POP</i>	This study
SPN71	SR11 <i>fimZ2::T-POP</i>	This study
SPN74	SR11 Nal ^r Δ <i>fimAICDHF::Km fimZ2::T-POP</i>	This study
SPN81	SR11 <i>hns(-195)::T-POP</i>	This study
SPN84	SR11 <i>hns(+281)::T-POP</i>	This study

statically, at 37°C in 0.22- μ m filter-sterilized Luria-Bertani (LB) broth buffered to pH 7.0 with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) hydrate for 48 h or on Miller LB agar plates (1.5% Difco agar) overnight. Antibiotics and other chemicals were added as needed: tetracycline (Tc), 20 mg/liter; kanamycin (Km), 100 mg/liter; carbenicillin (Carb), 100 mg/liter; naladixic acid (Nal), 50 mg/liter; 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), 40 mg/liter.

Transduction. P22 HT *int-105* and KB1 *int* were used to generate generalized transducing lysates of *Salmonella* serotype Typhimurium LT2 as previously described (19). *Salmonella* serotype Typhimurium strain SR11 can serve as a recipient for P22-mediated transduction, but this strain is resistant to P22-mediated lysis. To use SR11 derivatives as donors for transduction, we used phage KB1 *int*, which forms plaques on this *Salmonella* serotype Typhimurium strain. Transductants were struck for single colonies on Evans blue uridine agar (4), and light green colonies were cross-struck against P22 H5 (for P22 HT *int-105*) or KB1 *int* (for KB1) to confirm phage sensitivity.

Strain construction. Strains SPN22 (SR11 Δ *fimAICDHF::Km*) and SPN23 (LT2 Δ *fimAICDHF::Km*) were generated by transducing SR11 and LT2, respectively, to kanamycin resistance using a P22 lysate of EHW2 (14028 Nal^r Δ *fimAICDHF::Km*). SPN71 (SR11 *fimZ2::T-POP*) and SPN74 (SR11 *fimZ2::T-POP* Δ *fimAICDHF::Km*) were generated by transducing the SR11 wild type and SR11 Δ *fimAICDHF::Km* (SPN22), respectively, to tetracycline resistance with a P22 lysate of SL1344(T-POP) (*fimZ2::T-POP*). Plasmid pNK2880 was introduced into SPN22 (Δ *fimAICDHF::Km*) by P22 transduction to carbenicillin resistance from a lysate of TH4881. The *hns(-195)::T-POP* and *hns(+281)::T-POP* insertions were introduced into the SR11 wild type by transduction with a KB1 lysate of SPN66 [Δ *fimAICDHF::Km hns(-195)::T-POP*] and SPN68 [Δ *fimAICDHF::Km hns(+281)::T-POP*] to generate SPN81 [SR11 *hns(-195)::T-POP*] and SPN84 [SR11 *hns(+281)::T-POP*], respectively, by selecting for tetracycline resistance.

T-POP mutant pools. Sixteen pools of random Tn10dTc[del-25] (T-POP) (22) insertion mutants were generated using a P22 lysate grown on TH3923 to transduce into *Salmonella* serotype Typhimurium strain SPN22 carrying plasmid pNK2880 (22). Transductants were selected on LB plus Tc agar (approximately 1,500 mutants per plate) and pooled by flooding with 5 ml of LB broth at pH 7 and resuspending colonies.

Western blotting analysis. Polyclonal rabbit anti-PefA serum has been previously described (13). The serum was diluted 1:5 in phosphate-buffered saline (PBS) at pH 7.4 plus 0.2% sodium azide and preabsorbed eight times (9) with

ORN172 carrying plasmid pGEX-4T-2 (25) and four times with ADH19 (SR11 Δ *pefBACDI::Km*). Unless otherwise noted, cultures were resuspended, by measuring optical density at 600 nm (OD₆₀₀), to a concentration of approximately 2×10^8 CFU/10 μ l in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and boiled for 5 min to generate cell lysates. Cell lysates were then separated by 15% SDS-PAGE, transferred to Immobilon-P (Millipore) membranes using a Trans-Blot semidry transfer cell (Bio-Rad), incubated with anti-PefA serum diluted to a 1:500 final dilution, detected with goat anti-rabbit alkaline phosphatase and an Immun-Star chemiluminescent substrate (Bio-Rad), and visualized with X-Omat Blue film (Kodak).

SIMPLE protocol. BioMag protein G particles (QIAGEN) were resuspended by vortexing and then added in 100- μ l aliquots to 1.5-ml Eppendorf tubes and magnetically separated out of solution using a magnetic particle concentrator for Eppendorf tubes (DynaL MPC-S). The storage solution was aspirated, and then the particles were washed by adding 750 μ l of TN buffer (0.1 M Tris-HCl, 0.15 M NaCl [pH 7.5 final], sterilized with a 0.22- μ m filter) and vortexing. This wash step was repeated two more times. The particles were then resuspended in 100 μ l of preabsorbed anti-PefA serum and incubated for 1 h at room temperature, with vortexing every 5 min. The particles were then magnetically separated, the serum was aspirated, and the particles were washed three times as described before with 750 μ l of TN buffer. The particles were resuspended in 650 μ l of particle-blocking (PB) buffer (TN buffer plus 1% casein, added aseptically and prepared fresh 2 h prior to use) in Eppendorf tubes and incubated at room temperature for half an hour with constant inversion on an automatic roller. Culture (100 μ l; mixed by gentle swirling and at no time vortexed) containing 1 to 5×10^8 CFU (by OD₆₀₀) of bacteria (either a mutant pool or mixtures of two bacterial strains) was added, and the tubes were incubated for 1 h at room temperature with constant inversion. The particles were washed three times with PB buffer, and the tubes were gently inverted 10 times to resuspend the particles. Particles were either resuspended in 1 ml of PBS (to generate serial 10-fold dilutions of the resuspended particles that were spread on agar plates containing the appropriate antibiotics) or in the appropriate growth medium (to incubate bacteria statically at 37°C for 48 h; and the resulting culture was used to repeat the above protocol). In competition experiments using mixtures of two bacterial strains, the SIMPLE protocol was performed with each mixture in triplicate.

Enrichment (*n*-fold) was calculated by dividing the output ratio (i.e., the mutant/wild-type ratio recovered after enrichment) by the input ratio (i.e., the

mutant/wild-type ratio present in the inoculum). For statistical analysis, data were transformed logarithmically and analyzed by Student's *t* test.

Inverse PCR. DNA regions adjacent to the T-POP insertion site were amplified by inverse PCR (1). In brief, genomic DNA from the mutant of interest was digested with either AluI or TaqI and ligated with T4 DNA ligase, and the divergent primers TPOP-OUT (CGCTTTTCCCGAGATCATATG [bp 2168 to 2188 of GenBank accession number AY150213]) and TPOP-AluTaq (GCACTGTCTCCTGTTACTCC [bp c1977 to c1956 of GenBank accession number AY150213]) were used with PCR SuperMix HiFi (Invitrogen) to amplify the region of interest from the resultant circular DNA. The reactions were then run on 1% agarose gels, and the amplified fragment was purified with a QIAEX II gel purification kit (QIAGEN), cloned into pCR2.1 with a TOPO TA cloning kit (Invitrogen), transformed into chemically competent *E. coli* DH5 α MCR, and plated on LB plus Carb plus X-Gal. Plasmid minipreps (QIAGEN) of white colonies were digested with EcoRI to confirm insertion, and the insert was sequenced by SeqWright (Houston, TX) using the universal M13-Forward and M13-Reverse primers.

Electron microscopy. For immune electron microscopy, bacteria were grown in a static culture, washed twice in PBS, and resuspended in electron microscopy (EM)-grade water (EM Science) at a titer of approximately 1×10^9 CFU/ml. Bacteria were allowed to adhere to a formvar/carbon-coated grid (EM Science) for 2 min, and grids were incubated for 20 min in a primary antiserum (rabbit) diluted 1:250 in PBS containing 1% bovine serum albumen (BSA). Grids were washed five times for 1 min each in PBS containing 1% BSA. Grids were incubated for 20 min in goat anti-rabbit antibody-10-nm-gold conjugate (EM Science) diluted 1:20 in PBS containing 1% BSA. Grids were washed three times for 1 min each in PBS containing 1% BSA and three times for 1 min each in EM-grade water (EM Science) before they were analyzed by electron microscopy.

RESULTS

Enrichment for fimbriated bacteria by immunomagnetic separation. The goal of this study was to establish a genetic screen suitable for detecting mutants that elaborate fimbriae. The most direct approach to accomplishing this goal was detection of the major fimbrial subunit on the surfaces of live bacterial cells using specific antiserum. We reasoned that by incubating a bacterial culture with magnetic particles coated with antiserum raised against a fimbrial protein, it would be possible to enrich fimbriated bacteria using immunomagnetic separation. To provide a proof of concept for this approach, we chose to study expression of the *pef* operon because its major fimbrial subunit, PefA, can be readily detected by Western blotting in static LB broth cultures of a non-type 1-fimbriated *E. coli* strain (ORN172) carrying a cosmid (pFB11) bearing the *Salmonella* serotype Typhimurium *pef* operon (2). We determined whether fimbriated bacteria could be enriched by immunomagnetic separation after incubating a 30,000:1 mixture of ORN172 and ORN172(pFB11) with a slurry of anti-PefA-coated magnetic beads. Immunomagnetic separation resulted in a 2,100-fold enrichment for fimbriated bacteria [ORN172(pFB11)] (Fig. 1A). This enrichment was antibody mediated, because no fimbriated bacteria [ORN172(pFB11)] could be recovered from beads that were not coated with antiserum. Expression of PefA could be detected on the surface of ORN172(pFB11) but not on the surface of ORN172 by using immunogold labeling with rabbit anti-PefA serum (Fig. 1B), which demonstrated that the antiserum used for immunomagnetic separation specifically bound PefA assembled into fimbrial filaments. Collectively, our results show that immunomagnetic separation can be used to enrich fimbriated bacteria.

Type 1 fimbriae and growth conditions modulate PefA expression in *Salmonella* serotype Typhimurium. Although the cloned *pef* operon is well expressed in *E. coli*, PefA is not

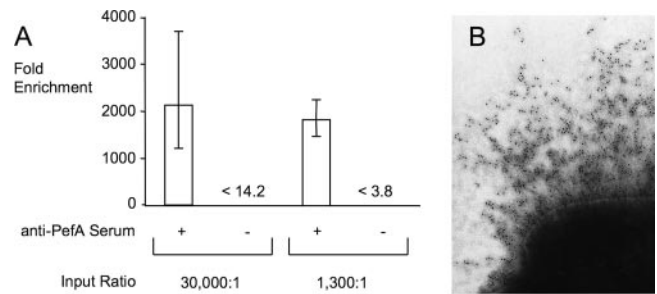


FIG. 1. Enrichment by immunomagnetic separation of *E. coli* cells expressing PefA on their surface. (A) *n*-fold enrichment of ORN172 (pFB11) was accomplished by a single round of immunomagnetic separation by particles coated with anti-PefA serum (+) or not coated with serum (-). Magnetic particles were incubated with mixtures (30,000:1 or 1,300:1) of strains ORN172 (nonfimbriated) and ORN172(pFB11) (expressing fimbriae encoded by the *pef* operon). Data are shown as geometric means \pm standard deviations of three independent experiments. In case no colonies of ORN172(pFB11) were recovered from particles, the limit of detection is indicated (<14.2 and <3.8, respectively). (B) Detection of fimbrial filaments composed of PefA on the surfaces of *E. coli* ORN172(pFB11) cells by using immunogold labeling.

expressed after growth of *Salmonella* serotype Typhimurium in static LB broth culture (12). To test whether immunomagnetic separation could be used to isolate mutants expressing the *pef* operon in LB broth, we used a *Salmonella* serotype Typhimurium *fimAICDHF* deletion mutant (SPN22) to prevent the possibility that the elaboration of type 1 fimbriae would interfere with binding of bacteria to magnetic particles. PefA was not detected by Western blotting in cultures of the *Salmonella* serotype Typhimurium wild type (SR11) or in the *fimAICDHF* deletion mutant (SPN22) grown statically in LB broth at neutral pH. After growth of the *Salmonella* serotype Typhimurium wild type (SR11) at pH 5.1, a faint band could be detected by Western blotting after overnight exposure, suggesting poor expression of PefA under this condition. Interestingly, deletion of the biosynthesis genes for type 1 fimbriae (*fimAICDHF*) resulted in markedly increased PefA expression after static growth of *Salmonella* serotype Typhimurium in LB broth at pH 5.1 (Fig. 2A). The *fimZ*::T-POP insertion, which places the FimZ transcriptional activator of the *fimAICDHF* operon under the control of a tetracycline-inducible promoter, was introduced into the *Salmonella* serotype Typhimurium wild type (SR11) and the *fimAICDHF* deletion mutant (SPN22). PefA was expressed in the *Salmonella* serotype Typhimurium *fimZ*::T-POP mutant (SPN71) grown in LB broth at pH 5.1 only when tetracycline was absent. In contrast, the *Salmonella* serotype Typhimurium Δ *fimAICDHF*::Km *fimZ*::T-POP mutant (SPN74) grown in LB broth at pH 5.1 expressed PefA in both the presence and the absence of tetracycline (Fig. 2B). These data suggested that the presence of type 1 fimbrial biosynthesis genes interfered with expression of fimbriae encoded by the *pef* operon in *Salmonella* serotype Typhimurium by an unknown mechanism. Similar observations were made recently for *E. coli*, where expression of type 1 fimbriae affects expression of P-antigen-recognizing (P) fimbriae negatively (26).

In the course of these studies, we noted that inoculation of LB broth cultures directly from -80°C freezer stocks pre-

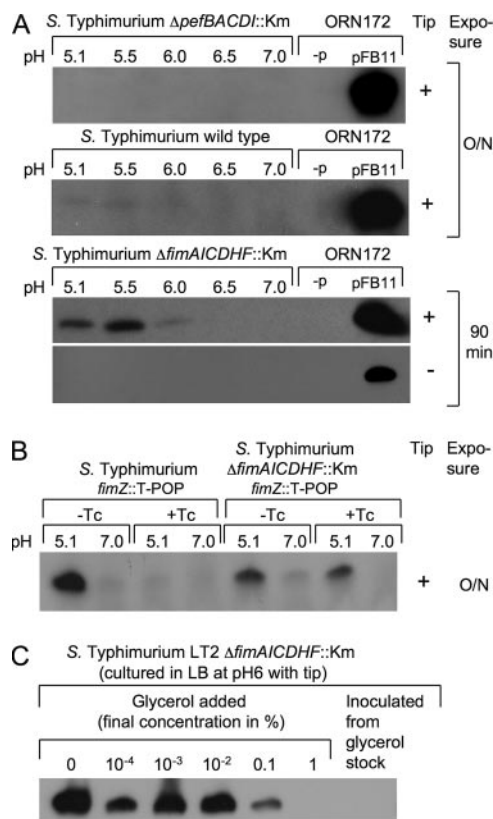


FIG. 2. Expression of PefA detected in *Salmonella* serotype Typhimurium strains by Western blotting. (A) Expression of PefA in *Salmonella* serotype Typhimurium strains ADH19 ($\Delta pefBACD::Km$), SR11 (wild type), and SPN22 ($\Delta fimAICDHF::Km$) grown statically in the presence (+) or absence (-) of a yellow pipette tip in LB broth buffered to pH 5.1, 5.5, 6.0, 6.5, or 7.0. Expression of PefA by *E. coli* strain ORN172 carrying a cosmid bearing the *pef* operon (pFB11) in LB broth at pH 7 is shown in the far right lane of each blot as a positive control. *E. coli* strain ORN172 without cosmid (-p) is shown as a negative control in the second lane from the right. Film was exposed for 90 min or overnight (O/N) as indicated on the right. (B) Expression of PefA in *Salmonella* serotype Typhimurium strains SPN71 (*fimZ::T-POP*) and SPN74 ($\Delta fimAICDHF::Km$ *fimZ::T-POP*) grown in LB broth buffered to pH 5.1 or pH 7 in the presence (+Tc) or absence (-Tc) of tetracycline. (C) Expression of PefA in *Salmonella* serotype Typhimurium strain SPN23 ($\Delta fimAICDHF::Km$) grown in LB broth (pH 6) supplemented with the indicated concentrations of glycerol. Cultures were inoculated from a single colony (left lanes) or directly from glycerol stock (far right lane) and incubated overnight in the presence of a yellow pipette tip.

vented PefA expression in *Salmonella* serotype Typhimurium. A follow-up experiment suggested that this effect was due to an inhibition of PefA expression by the presence of small amounts of glycerol in the culture medium (Fig. 2C). Furthermore, expression of PefA at pH 5.1 was increased considerably in *Salmonella* serotype Typhimurium if the 200- μ l yellow pipette tips used to inoculate static LB broth cultures were left in the culture tubes during overnight incubation (Fig. 2A). For the screen described below, bacteria were grown without inoculation with yellow tips.

Screening with immunomagnetic particles for ligand expression (SIMPLE). The *Salmonella* serotype Typhimurium *fimAICDHF* (SPN22) deletion mutant was used to generate a

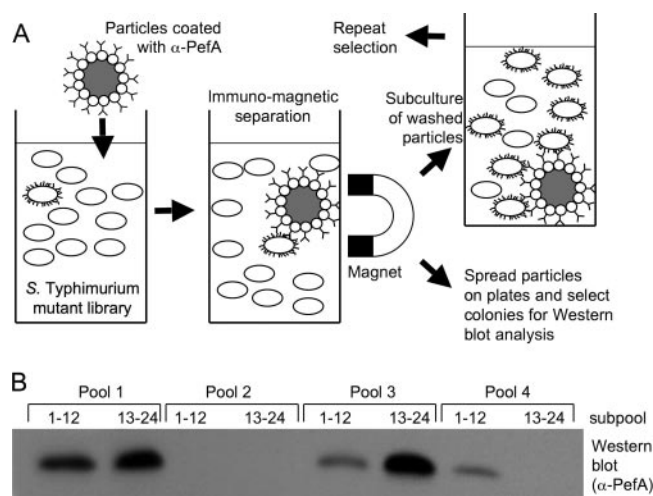


FIG. 3. Enrichment for *Salmonella* serotype Typhimurium mutants expressing PefA by using the SIMPLE method. (A) Schematic illustrates the principle of the SIMPLE method. (B) Western blotting analysis of four pools, each originally consisting of approximately 1,500 independent mutants, after two rounds of enrichment by the SIMPLE method. From each pool, 24 colonies were randomly selected and grown statically in LB broth at pH 7, and a mixture of 12 mutants was loaded per lane.

library of random T-POP insertion mutants (22). To this end, a plasmid encoding a Tn10 transposase with broad target specificity (pNK2880) was introduced into the *Salmonella* serotype Typhimurium *fimAICDHF* (SPN22) deletion mutant. Bacteriophage P22 was grown on a serotype Typhimurium strain (TH3923) carrying the T-POP transposon on an *E. coli* F' plasmid, and the resulting phage lysate was used to deliver the T-POP transposon into SPN22(pNK2880). Transductants were selected on LB agar plates containing tetracycline (the resistance conferred by T-POP). Approximately 24,000 mutants generated by this method were grouped into 16 pools of approximately 1,500 mutants each. Each pool was used to inoculate a static LB broth culture buffered to pH 7, a growth condition under which PefA is not expressed in the SPN22 parental strain. Each pool was incubated with magnetic particles coated with anti-PefA serum, and particle-bound bacteria were resuspended in LB broth (pH 7) and used to inoculate a second static LB broth (pH 7) culture to repeat the enrichment procedure. After two enrichment cycles, 24 randomly selected colonies from each pool were grown statically in LB broth at pH 7, and expression of PefA was assessed for subpools of 12 mutants by Western blotting (Fig. 3). Seven of the 16 pools were positive for PefA expression. Individual bacterial colonies from each positive pool were grown statically in LB broth at pH 7 to identify T-POP mutants expressing PefA. One PefA-expressing mutant from each pool was selected, and the T-POP insertion was transduced into the *Salmonella* serotype Typhimurium *fimAICDHF* deletion mutant (SPN22) by KB1 *int*-mediated transduction to remove the transposase encoded by plasmid pNK2880. For three pools, we were not able to obtain transductants expressing PefA (even after repeated attempts with different colonies from each pool). In these cases, T-POP may have undergone secondary transposition events due to the continued presence of pNK2880 during the screening procedure.

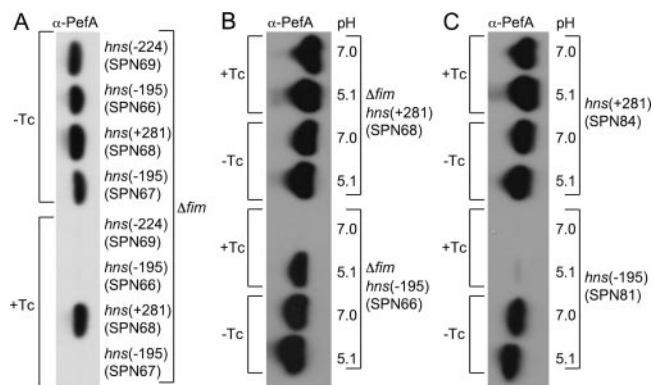


FIG. 4. Expression of PefA detected by Western blotting in *Salmonella* serotype Typhimurium *hns* mutants. Bacteria were cultured statically in LB broth at 37°C in the presence of a yellow pipette tip. (A) *Salmonella* serotype Typhimurium SR11 Δ *fimAICDHF*::Km carrying T-POP insertions in *hns* at position -224 (SPN69), -195 (SPN66, SPN67), or +281 (SPN68) was grown at pH 7 in the presence (+Tc) or absence (-Tc) of tetracycline. (B) *Salmonella* serotype Typhimurium SR11 Δ *fimAICDHF*::Km carrying T-POP insertions in *hns* at position -195 (SPN66) or +281 (SPN68) was grown at pH 7 or pH 5.1 in the presence (+Tc) or absence (-Tc) of tetracycline. (C) *Salmonella* serotype Typhimurium SR11 carrying T-POP insertions in *hns* at position -195 (SPN81) or +281 (SPN84) was grown at pH 7 or pH 5.1 in the presence (+Tc) or absence (-Tc) of tetracycline. α , anti.

For the remaining four pools, transduction did yield T-POP mutants (SPN66, SPN67, SPN68, and SPN69) expressing PefA in LB broth at pH 5.1 and pH 7. Interestingly, three of the four mutants (SPN66, SPN67, and SPN69) did not express PefA after growth in LB broth at pH 7 in the presence of tetracycline (Fig. 4), suggesting that the *tetA* promoter of T-POP may drive expression of a negative regulator of PefA expression in these strains.

Inactivation of *hns* relieves PefA suppression in *Salmonella* serotype Typhimurium. DNA regions flanking the T-POP transposon insertion sites in strains SPN66, SPN67, SPN68, and SPN69 were cloned by inverse PCR, and the respective nucleotide sequences were determined. The T-POP insertions were located either within or upstream of the *hns* open reading frame at base pair position -224 (SPN69), -195 (SPN66 and SPN67), or +281 (SPN68), relative to that of the start codon (Fig. 5). The fact that PefA was detected in a mutant in which the T-POP insertion had disrupted the *hns* open reading frame (SPN68) suggested that the expression of PefA is negatively regulated by the H-NS protein. Mutants SPN66, SPN67, and SPN69 carried T-POP insertions in an orientation in which the *hns* gene was located downstream of the *tetA* promoter. Our data suggested that the T-POP insertions in strains SPN66, SPN67, and SPN69 disrupted the *hns* promoter, resulting in the expression of PefA in the absence of tetracycline. Sequence analysis further suggested that growth of strains SPN66, SPN67, and SPN69 in the presence of tetracycline would result in the expression of *hns* from the *tetA* promoter, resulting in PefA repression. Predictions from the sequence analysis of T-POP insertion sites (Fig. 5) were thus in good agreement with the expression of PefA observed for the respective mutants (Fig. 4).

SIMPLE method validation. Our data suggested that the *hns* mutants were identified using the SIMPLE method because

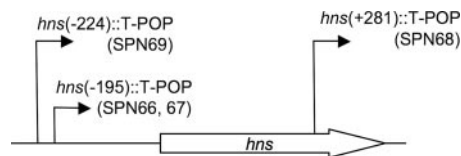


FIG. 5. Positions of T-POP insertions identified by the SIMPLE method for *Salmonella* serotype Typhimurium mutants expressing PefA. The open arrow indicates the position and orientation of the *hns* open reading frame. The T-POP insertion sites in mutants SPN66, SPN67, SPN68, and SPN69 are indicated. Arrows indicate the orientation of the tetracycline-inducible *tetA* promoter of T-POP.

PefA expressed on their surfaces bound to particles coated with anti-PefA serum. This assumption was tested experimentally by detecting the expression of PefA on the surface of strain SPN68 [Δ *fimAICDHF*::Km *hns*(+281)::T-POP] cells by immunogold labeling after growth at pH 7. Expression of PefA could be detected by this method in *Salmonella* serotype Typhimurium Δ *fimAICDHF*::Km *hns*(+281)::T-POP (SPN68) but not in *Salmonella* serotype Typhimurium Δ *fimAICDHF*::Km (SPN22) cells (Fig. 6A).

The enrichment factor for the *hns* mutants, by the SIMPLE method, was quantified in a competition experiment in which magnetic beads were incubated with an 8,300:1 or a 430:1 mixture of strains SPN22 (Δ *fimAICDHF*::Km) and SPN68 [Δ *fimAICDHF*::Km *hns*(+281)::T-POP] grown at pH 7. Immunomagnetic separation resulted in a 16-fold or a 36-fold enrichment, respectively, for strain SPN68 [Δ *fimAICDHF*::Km *hns*(+281)::T-POP] when the experiment was performed with magnetic particles coated with anti-PefA serum (Fig. 6C). In contrast, the same experiment performed with magnetic particles that had not been coated with serum did not result in a marked enrichment for strain SPN68.

To determine whether the presence of an intact *fim* operon might interfere with the SIMPLE method, the *hns*(+281)::T-POP insertion in strain SPN68 was transduced into the *Salmonella* serotype Typhimurium wild type (SR11). Mixtures of strains SR11 (wild type) and SPN84 [*hns*(+281)::T-POP] were subjected to enrichment using magnetic particles as described above. Bacteria expressing PefA were enriched approximately 10-fold with magnetic particles coated with anti-PefA serum, while no significant enrichment was observed in the absence of anti-PefA serum. In the presence of type 1 fimbriae, enrichment for PefA-expressing bacteria by the SIMPLE method was reduced to approximately 10-fold compared to the approximately 16- to 36-fold enrichment observed in the absence of type 1 fimbrial biosynthesis genes (Fig. 6C). The degree of enrichment correlated positively with the fraction of cells expressing PefA on their cell surfaces as assessed by immunogold labeling, which was approximately 65% of cells for strain SPN68 [Δ *fimAICDHF*::Km *hns*(+281)::T-POP] and approximately 5% of cells for strain SPN84 [*hns*(+281)::T-POP]. These data suggested that type 1 fimbriae did not interfere directly with bacterial binding to magnetic beads but rather with PefA expression on the bacterial surface. Interestingly, similar levels of PefA expression were detected for cultures of SPN68 [Δ *fimAICDHF*::Km *hns*(+281)::T-POP] and SPN84 [*hns*(+281)::T-POP] by Western blotting (Fig. 4B and C), suggesting that rather than decreasing the total amount of PefA, the presence of type 1 fimbrial biosyn-

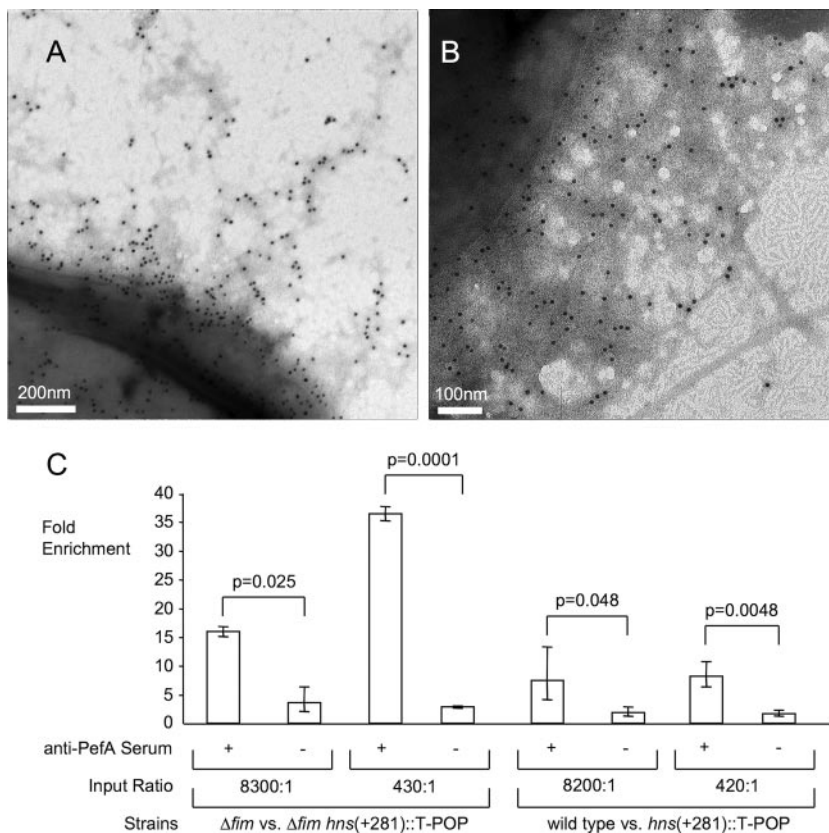


FIG. 6. Enrichment by immunomagnetic separation for *Salmonella* serotype Typhimurium $hns(+281)::T-POP$ mutants expressing PefA on their surfaces. Detection of fimbrial filaments composed of PefA on the surface of (A) *Salmonella* serotype Typhimurium SR11 $\Delta fimAICDHF::Km hns(+281)::T-POP$ (SPN68) or (B) *Salmonella* serotype Typhimurium SR11 $hns(+281)::T-POP$ (SPN84) by immunogold labeling. Bacteria were grown statically in LB broth at pH 7 without a yellow pipette tip. (C) n -fold enrichment is shown for SPN68 [$\Delta fim::Km hns(+281)::T-POP$; left panel] or SPN84 [$hns(+281)::T-POP$; right panel] accomplished by a single round of immunomagnetic separation with particles coated with anti-PefA serum (+) or not coated with serum (-). Magnetic particles were incubated with the indicated mixtures of strains SPN22 and SPN68 (left panel) or SR11 and SPN84 (right panel). Data are shown as geometric means \pm standard deviations of three independent experiments. Statistical significance (P value) of differences between treatment groups (shown in brackets) is indicated above the bars.

thesis genes may interfere with the assembly of PefA on the cell surface.

In summary, our data demonstrate that SIMPLE is a powerful method for identifying mutants expressing fimbriae from a complex mutant library.

DISCUSSION

At the dawn of the genomic era, *Escherichia coli* and *Salmonella* serotypes were among the organisms best characterized genetically. Despite a large body of knowledge about these organisms' biology, whole-genome sequencing revealed the presence of much larger repertoires of fimbrial operons than predicted by the number of fimbrial structures observed on the surfaces of these bacteria. The fact that the majority of fimbrial operons were identified only by sequence analysis reflects the tight control of their expression in vitro, a property that has prevented a thorough characterization of the encoded adhesins.

Efforts to characterize the growing number of putative fimbrial operons identified by whole-genome sequencing would greatly benefit from the development of reliable approaches

for isolating fimbriated bacteria from a mutant library. Currently, such screens commonly employ transposon mutagenesis of a strain carrying a fusion between the gene of interest (in this case a fimbrial operon) and a reporter gene that, when expressed, alters the colony phenotype. Two shortcomings limit the usefulness of this approach for analysis of fimbrial operons. First, a screen for colony phenotypes requires that fimbriae be expressed on agar plates, an assumption that may not always be correct. For example, type 1 fimbriae of *Salmonella* serotype Typhimurium are expressed in LB broth but not on LB agar plates (13). Second, the use of transcriptional fusions cannot detect posttranscriptional control mechanisms that may affect elaboration of fimbriae on the bacterial surface. The SIMPLE method developed in this study overcomes these limitations because it allows identification of bacteria expressing a fimbrial operon by directly targeting the encoded fimbrial filaments expressed in broth culture.

The *Salmonella* serotype Typhimurium genome contains 4,597 open reading frames (18), of which 490 genes are essential during growth in rich medium (14). By applying the formula $P = 1 - e^{-a/b}$, where P is the probability that a gene is mutated, a is the number of mutants, and b is the number of

nonessential genes, it can be estimated that our library of approximately 24,000 transposon mutants was large enough to contain insertions within 99.7% of *Salmonella* serotype Typhimurium nonessential genes. Assuming an equal probability for insertions in each gene, a bank of 24,000 transposon mutants should, on average, contain approximately six insertions in *hns*. Our screen identified seven mutants expressing PefA, although three of them could not be analyzed further, presumably because T-POP insertions in these strains had undergone secondary transposition events. The remaining four mutants contained insertions in the *hns* gene, a putative negative regulator. The number of *hns* insertions identified in our study was, thus, in good agreement with theoretical predictions. Expression of H-NS-repressed genes is thought to be induced when this repressor becomes displaced by a positive regulatory element from the promoter region (17, 20). No such positive regulatory elements for *pef* expression were identified in this study. To identify genes encoding positive regulatory elements, the T-POP transposon has to be inserted in the correct orientation (i.e., downstream of the single tetracycline-inducible promoter present on Tn10dTc[del-25]) and in close proximity (i.e., within 200 bp or less) of a start codon, to ensure activation of its open reading frame in the presence of tetracycline. A library of approximately 24,000 T-POP mutants was expected to contain insertions in the correct orientation and in close proximity to start codons of only 38% of *Salmonella* serotype Typhimurium genes. The mutant bank screened in this study was thus not of sufficient size for identifying positive regulatory elements with high probability.

The presence of type 1 fimbrial biosynthesis genes reduced the fraction of *Salmonella* serotype Typhimurium cells expressing PefA on their surfaces. Similar observations were made recently for *E. coli*, where the expression of type 1 fimbriae affects the expression of P fimbriae negatively (26). The fact that our screen was performed with a strain background carrying a deletion of type 1 fimbrial biosynthesis genes resulted in increased sensitivity of the SIMPLE method (Fig. 6C). H-NS binding sites are present in promoter regions of multiple *Salmonella* serotype Typhimurium fimbrial operons (17, 20). The use of a *hns fim* mutant background may further improve analysis of *Salmonella* serotype Typhimurium fimbrial operons by the SIMPLE method.

Our results demonstrate that the SIMPLE method can be used to isolate mutants elaborating a fimbrial protein, PefA, whose expression is not observed in *Salmonella* serotype Typhimurium grown under standard laboratory conditions. One potential drawback of the SIMPLE approach is that it requires the availability of antibodies directed against fimbrial proteins. However, the fact that the majority of *Salmonella* serotype Typhimurium fimbrial operons are not expressed in vitro has not been a major obstacle for generating specific antibodies against major fimbrial subunits (13). The SIMPLE method should be tractable with any mutagenesis strategy and readily applicable to other bacteria and possibly to any surface structure to which antiserum can be raised.

In summation, we have demonstrated that the SIMPLE method is readily applicable to the isolation of a mutant elaborating a fimbrial structure on its surface from a complex library.

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